Dynamics of sperm-oviduct interaction that regulates maternal immune response in cattle

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General Introduction

According to United Nations, by year 2050, it is projected that we will reach almost 10 billion in terms of world population. With the addition of almost 2 billion people in the next 3 decades, it is imperative that food production be increased to maintain food security. As the dairy and beef industry are at the forefront as sources for protein and other animal by-products, the goal of the said industries is to increase production through improvement in fertility of the cow and bull. Our current practices employed in reproduction are based on previous information gained from prior studies. It is possible to optimize and improve our existing techniques in natural and assisted reproduction by looking for solutions to overcome reproductive failures. A key area to focus on is to find more information on the physiological and immunological events that transpire throughout the peri-ovulatory period. Elucidating the detailed mechanisms of maternal immune response to the arrival of sperm in the female reproductive tract (FRT), particularly in the ampulla where fertilization occurs, may help in the improvement of cattle reproduction. New treatment modalities may be developed, while assisted reproduction technologies, and processing of bull semen maybe improved using this new information.

1. Sperm

Gametes are an organism's reproductive cells and male gametes are called spermatozoa or sperm and are produced in the testis. These spermatozoa are then transported using a series of tubular structures directly connected with the testis and the penis. The associated tubular structures are also involved in the maturation as well as storage of the spermatozoa [1].

The spermatozoon has a head which highly varies among different animal species [4, 5]. The spermatozoon has a flagellum, which is a tail-shaped structure that allows it to propel and move [2]. A wide degree of diversity in the sperm morphology is found across animal species. These differences are in terms of the size and shape and might have been influenced by the female reproductive tract [3]. The female reproductive tract also selects and transports only the morphologically normal spermatozoa [5].

1.1. Sperm morphologic variation and capacitation process

The morphology of sperm has a high variability in animals in terms of the shape and size. In the bull, spermatozoa have a paddle-shaped and flat head [3,4,6] while the sperm of mouse and rat have a narrow and hook-like shape [6]. All normal spermatozoa are composed of the head, mid-piece, and a tail

[4,6]. It was however found via geometric measurements that the bull sperm show a slight variation in head size [4].

The length of the head of bull spermatozoa is approximately 10 microns, and the width of the head is around 5 microns [4]. The acrosome region consists almost half of the bull sperm head located in the anterior part. When compared to the sheep and pig, the bull sperm possesses a smaller acrosomal area [4]. A plasma membrane covers the entire surface of the spermatozoa. The middle part of the spermatozoa is made of cytoskeleton densely packed with mitochondria where the spermatozoa derives the energy for motility [1]. The tail or flagellum helps propel the bull sperm progressively [6].

After the maturation process, the matured spermatozoa are eventually released from the testes, towards the accessory sex glands, and finally deposited to the FRT [7]. However, sperm must prepare for another process of physiological and metabolic maturation which prepares the morphologically mature sperm to obtain the capacity to fertilize the egg. This process is called capacitation [8, 9]. A comprehensive study of mammalian sperm capacitation process [11] showed that no major structural changes were found to occur in the sperm after the capacitation process [10]. It was found that the spermatozoa did not undergo any changes in its morphological state from introduction into the FRT until acrosome reaction stage [11].

It was described that the capacitation process involves the removal of the sperm glycoprotein coat and hyperactivation or improved motility, and that the acrosome reaction is considered to be distinct from sperm capacitation [12]. The capacitation is a very important concept and is fundamental in the study of the fertilization process. However, some confusion might exist due to the differences in the methodologies of studying capacitation [13.14]. The detailed studies of capacitation provided comprehensive knowledge and understanding of the mechanisms undergone by the spermatozoa prior to the fertilization event. The knowledge gained from these studies contributed helped in the advancement of the assisted reproduction technologies like the *in vitro* fertilization or IVF [13, 14].

1.2. Induction of sperm capacitation by heparin

For successful fertilization to occur, matured oocytes must be incubated with already capacitated sperm or sperm that are undergoing the capacitation process since the capacitated sperm have undergone

biochemical alterations that permit the acrosome reaction to transpire upon contact with the zona pellucida (ZP), the cumulus cells, or oocyte-associated molecules [14, 15].

Many methods to induce sperm capacitation were developed. The most widely used and accepted method of inducing sperm capacitation *in vitro* is by the use of heparin [10]. Binding of heparin molecules to bull sperm membrane must occur before it can induce sperm capacitation [14, 15]. The ability of heparin to induce bull sperm capacitation is primarily due to the charge-dependent nature of heparin-sperm membrane binding [14, 16,17] because it was shown that the heparin binding to sperm membrane can be inhibited by protamine sulfate [15].

It was also found that the binding of heparin molecule to several bovine seminal plasma proteins or BSPs is crucial [18]. The BSPs come from the seminal plasma and attach to the sperm from the epididymis during the ejaculation [18] which are named BSP-A1, BSP-A2, BSP-A3, and BSP-30-kDa [19, 20]. First, the BSPs must have an interaction with the cholesterol and phospholipids present in the plasma membrane of the spermatozoa. After the heparin molecule binding, there is an observed loss of sperm surface components which is seen as an apparent loss of lectin binding to bull sperm [19, 20]. These surface components- associated modifications maybe due to a loss of the BSPs over time induced by heparin binding, which lead bull sperm membrane cholesterol and phospholipid loss [20]. Aside from the loss of BSPs due to heparin binding to sperm membrane, heparin induces changes in the sperm intracellular pH, calcium, and cAMP concentrations [14], which are also needed during the capacitation process.

2. Bovine female reproductive tract

The FRT of a cow is anatomically composed of the vulva, vestibule, vagina, cervix, uterus, oviducts, and ovaries, which are located ventral to the rectum of the animal. The FRT is the organ involved in oogenesis, male and female gamete transport, fertilization, embryonic development, and parturition [21]. The function of male and female gamete transport, the fertilization process, and the development of the early embryo happens specifically in the bovine oviduct.

2.1. Oviduct

The oviduct, also known as the uterine tube, plays many important functions in the reproduction process. This dynamic organ can selectively allow a few sperm to capacitate and assist in the sperm

transport reach the continuously maturing oocyte after its ovulation [21]. Numerous studies were conducted in order to characterize the morphological changes in the oviduct of several domestic species [30] including cows [22, 30], goats [23], pigs [24] and bitches [25] in relation to estrous cycle. The bovine oviduct length may range from 21 to 28 cm and is morphologically differentiated into the infundibulum, the ampulla, and the isthmus [26]. The epithelium lining of mammalian oviduct is made of ciliated and non-ciliated secretory cells [30]. These epithelial cells are dynamic and can undergo atrophy or hypertrophy depending on the hormonal concentrations, and thus the ratio of these cells may vary in different phases of the estrous cycle [27, 30]. There are many variations in the characteristics of the oviduct epithelial cells in many mammalian species in terms of ultrastructural, histochemical, and physiological features [27, 30].

2.2. Ampulla

The ampulla makes up about two-thirds of the oviduct and is thin walled with many easily distensible mucosal folds. The ampulla is made up of the mucosa consisting of luminal epithelial cells, the lamina propria which is the framework for the muscular folds, a muscularis layer which consists of the longitudinal and circular muscle layers, and the outermost serosa [25].

The mucosa of ampulla was thrown into numerous elaborately branched leaf-like folds that formed of primary, secondary and tertiary ones [27, 28, 30]. These folds may be interconnected with one another in a complex manner [30]. During the follicular phase was, the average number of oviductal ampullary folds is 42, while the average number of ampullary fold during the luteal phase is 3 [28, 30]. There is an observed decrease in the number of the folds from infundibulum to ampulla [30]. The lamina propria–submucosa consisted of loose connective tissue with abundant collagenous fibers [30]. The tunica muscularis layer consisted of bundles of smooth muscle cells interspersed with numerous blood vessels between bundles [30].The outermost connective tissue layer is the serosa [27, 30]. The columnar-type lining epithelium has to distinct types of cells: ciliated and non-ciliated secretory cells [21, 30]. The height of the cells in the ampullary epithelium in the follicular phase was higher than in the luteal phase of the estrous cycle [30]. There is abundant cellular secretory activity during the luteal phase, with the secretory cells presenting bulging apical surfaces and constrictions, while some of the secretory products were seen separated from the apical surfaces [30].

Ciliated cells with numerous and prominent cilia predominate the ampullary epithelium during the follicular phase of the cycle [27, 30]. The ampullary epithelium at the follicular phase was characterized by an extensive distribution of ciliated cells and was characterized by its prominent cilia that were protruded into the uterine lumen with decrease in the number of secretory cells [27, 30].

3. Sperm-female reproductive tract interactions

The bull sperm transport along the different parts of the FRT allows sperm to have direct and indirect interaction with the FRT mucosa and components present in the luminal areas. The anatomical characteristics of each portion of the FRT is designed in order to allow only morphologically normal sperm with strong motility to reach the lower ampullary region which is the site of fertilization.

The bull sperm must pass through the hostile uterine environment and reach the isthmic region of the oviduct, where they lie in wait for the arrival of the mature ovulated oocyte. The time the bull sperm may spend in the oviduct until fertilization is completed is however not established. During this time, the sperm must maintain its viability.

3.1. Sperm migration towards the oviduct

After the motile spermatozoa escape the uterus by passing through the utero-tubal junction, they must continue the journey and ultimately reach the oviductal ampulla. One method for migration into the ampulla is by sperm motility. It was observed that active bull spermatozoa can attach and detach repeatedly into the epithelium of the isthmic portion of the oviduct during the migration process [29].

Sperm can also be transported towards the oviduct via peristaltic movements aided by muscular contractions [30, 31, 32]. Blocking the peristaltic movement of the FRT using an anticholinergic drug resulted in lower numbers of sperm reaching the ampulla [31].

Rheotaxis is exhibited by mouse, human, and bovine spermatozoa, by swimming against the oviductal fluid flow which indicates that rheotaxis against oviductal flow is a major determinant of sperm guidance in the oviduct [33, 34]. This is reinforced by an observation that *Catsper1* KO spermatozoa is unable to display rheotaxic behavior and thus cannot migrate through the oviduct efficiently. In addition to rheotaxis, chemotaxis [34, 35] and thermotaxis [34, 36] are also implicated in sperm migration.

Some studies show evidence that the ciliary activity of the epithelial cells in the oviduct plays a major function in the transportation of gamete and early embryo [46]. It has been determined that the frequency of ciliary movement of the human oviductal epithelium increases after ovulation [37, 38, 46].

3.2. Sperm interaction with the oviduct

When the motile spermatozoa arrive in the oviduct, two pathways may be followed. Some of the spermatozoa can immediately migrate to the ampullary region. These spermatozoa are unable to fertilize the oocyte since they have no time to undergo the capacitation process. Conversely, most of the spermatozoa are retained in the isthmic region establishing a sperm reservoir [39, 40, 46]. While within the reservoir, the remaining sperm will retain their viability and their fertilizing ability until ovulation occurs [39, 46].

It was demonstrated in different mammals that spermatozoa sequestered in the isthmus region could attach to epithelial cells [46], suspending capacitation to occur in sperm until signals associated with ovulation allow sperm detachment from isthmus, to continue their ascent towards the lower ampulla [46]. The direct interaction between the bull sperm and the oviductal epithelium involves the sperm head specifically the acrosomal area, and the apical portion of the ciliated epithelial [39, 40, 41, 42, 43, 46]. In domestic animals like the dog, cattle, and horse, sperm-oviduct epithelium interaction may have a function in the sperm viability and state of capacitation [44, 45, 46].

Sperm sequestration in the isthmic portion of the oviduct may function to avert polyspermic fertilization by sequentially releasing low number of sperm to search for the ovulated oocytes in the lower ampulla [26]. Despite numerous studies performed on domestic and laboratory animals, the detailed molecular mechanisms involved in the sperm–epithelium interaction remain unknown [46].

4. Female reproductive tract immunity

The FRT innate immune system involves mechanical, chemical, and cellular components. Examples of mechanical barriers are mucus lining and epithelial cells [109]. The chemical barrier are the natural anti-microbial peptides and pattern recognition receptors such as the Toll-like receptors (TLRs) [49, 109]. Natural antimicrobial peptides are primarily produced and secreted by epithelial cells and blood neutrophils [109]. Microbes such as bacteria and foreign cells are destroyed by rapid changes in pH and ionic concentration gradients [47, 109]. Immune cells, such neutrophils, macrophages, dendritic

cells, endothelial cells, and epithelial cells can express TLRs [48, 109] and in the epithelium lining the uterus [50] and oviduct [51]. TLRs sense the pathogen-associated molecular patterns (PAMPs), then utilize intermediators to signal the immune cells to secrete the produced anti-microbial factors to kill and remove pathogens via intracellular signaling pathways, while modulating the adaptive immune responses.[51,45, 52, 53, 54, 109].

The other component of the immune response involve the cellular components which are composed of inflammatory cells that migrated into the tissues of the genital tract, epithelial cells, and stromal fibroblast cells [46, 109].

The innate immune system found in the mucosa of the FRT is primarily important in complex physiological events, such as fertilization, implantation, pregnancy, and parturition [109]. It also protects the female from sexually transmitted microorganism and supports the allogeneic spermatozoa and an immunologically distinct fetus [109]. In order to competently meet multiple challenges, a complex and multimodality approach is used by the FRT as mentioned above.

4.1. Sperm immunity in the oviduct

The oviduct evolved in order to provide the ideal and optimal environment to allow for fertilization to occur, to maintain the viability of the sperm and the mature oocyte, to initiate and ensure that sperm undergoes capacitation, and to nourish the development of the early embryo. The oviduct immune system maintains a dual role by being able to destroy invading pathogens while maintaining the viability of sperm and early embryo [54].

Cultured oviduct epithelial cells (BOECs) produced an anti-inflammatory or Th2 immune response via the production of anti-inflammatory cytokines [55]. It was also found that when bull sperm are incubated in BOECs, the attachment of the sperm results in a stronger Th2 response [55]. Sperm-induced transcription of novel genes are observed in mouse oviductal tissue which was evidenced by the modification in the oviduct biochemical composition [56]. The presence boar sperm and the arrival of oocytes into the porcine oviduct resulted in the alteration in the gene transcription of the porcine oviduct [57]. The alteration in the gene transcription may have resulted in the *in vivo* modulation of the oviduct fluid composition, and maybe a sperm/oviduct sensing system within the porcine oviduct [57]. Insemination in rabbits resulted in a morphologic change of the oviductal epithelium 1-2 h post-

insemination [58]. It was observed that surface protein transcription and secretion in the oviduct epithelial cells in rabbits, were directly influenced by semen and some proteins [58].

5. Molecules involved in sperm-oviductal interaction.

Heparin and some sulfated glycoconjugates, which were found to be present in oviductal fluid, were shown to stimulate the synchronous release of sperm bound to cultured bovine oviduct epithelial cells [46, 56]. When compared with the sperm attached to the oviduct epithelial cells, sperm released with the addition of heparin have shown higher intracellular Ca^{++} concentrations and have increased amounts of tyrosine-phosphorylated proteins [59]. It was found that non-capacitated bovine spermatozoa attached to the oviduct epithelium, and that sperm capacitation status is related to their detachment from the oviduct epithelium [60]. It was postulated that thiol-reducing agents such as sulfated glycoconjugates and disulfide reducing agents present in the oviduct are responsible for the modulation of the surface protein redox status of bull which allow the detachment of bull spermatozoa from the oviductal epithelium [60, 61]. Sperm surface protein disulfides are reduced into sulfhydryls [60, 61] weakening the force and allowing the sperm to detach from the oviduct.

The endocannabinoid molecule, anandamide, is produced by the oviductal epithelial cells and is released into the oviductal fluid, and subsequently binds to the endocannabinoid receptors in the epithelium [62, 63, 64, 65, 66]. In human, porcine, and bovine species, the cannabinoid receptors were detected in male and female reproductive tissues and in the sperm [62, 63, 64, 65, 66]. Variation in the amounts of anandamide present in the bovine oviductal fluid were detected during different phases of the estrous cycle, with the highest amount measured during the peri-ovulatory period, signifying that levels of endocannabinoid maybe under the control of ovarian hormones [65]. Sperm–oviductal epithelium interaction in cattle was found to be modulated by anandamide through the inhibition of sperm binding and induction of sperm release from oviduct epithelial cells [66, 67]. It was also found that anandamide might be involved in the regulation of the oviduct sperm-reservoir function by decreasing the sperm motility and preventing sperm capacitation, and extending the fertility of the sperm [68].

Carbohydrate moieties located on the oviductal epithelial surface may have a significant function in the interactions with the sperm. It was identified that Annexin proteins (A1, A2, A4, and A5) function as bull sperm receptors in the oviduct [29]. These annexins were found to interact with the surface

proteins of the bull spermatozoa and are localized oviductal epithelial surfaces, [69]. Blocking the annexins with specific annexin antibody decreased sperm oviductal binding [69].

Another protein localized in ejaculated sperm was identified as Binder of Sperm (BSP1) [70]. When BSP1 is incubated into oviduct explants and sperm, there is an increase in sperm attachment to the explant [70, 71]. It was also found thatBSP3 and BSP5 found in sperm can improve the attachment of sperm to oviductal epithelium [71].

Recently, the importance of Toll-like receptors (TLRs) in the reproduction process was recognized. It was found that TLR2 and 4 may mediate the interaction between the sperm and COCs in mouse [71]. It was also found that the sperm-induced inflammatory response of the bovine endometrium is activated through the TLR2/4 signaling pathway [48, 49].

While we have discovered numerous molecules involved in the sperm-oviduct interactions, the process involves a highly complex simultaneous cascades of events which are dependent on multiple factors involving both the sperm and tissue, making it difficult for us to perceive the overview of the phenomenon.

Thus, we need to elucidate the mechanisms involving the molecules involved in the interaction between the sperm and oviduct. It is of particular interest to study the detailed dynamics of sperm with the intact oviductal tissue, the immune response of the oviduct to the sperm, as well as the molecule involved in the interaction.

6. Objectives of the study

I hypothesized that the bovine oviductal immune response to bull sperm binding is mediated by TLR2. The general objective of the study was to investigate the sperm-oviduct interaction and its effect on the immune response of the oviduct.

The specific objectives of the study were:

1. To develop a differentiated oviduct explant model to investigate the dynamics of sperm-oviduct interactions.
2. To evaluate the effects of different status of sperm on the oviduct interactions on sperm binding and oviductal immune response.
3. To investigate the role of TLR2 on oviduct ampulla sperm binding and maternal immune.

Chapter 1

Development of a differentiated bovine ampulla explant model to investigate the dynamics of sperm-oviduct interactions

1. Introduction

Sperm is introduced into the FRT during mating or via artificial insemination during the peri-ovulatory phase of the estrus cycle. After passing through the cervix and uterus, the selected sperm are stored into a "sperm reservoir" in the oviductal isthmic region [29] while waiting for the arrival of the oocyte. Meanwhile, the sperm that has reached the reservoir must not only maintain their viability, but must also undergo several physiological changes in a process called "capacitation" to gain fertilizing capacity towards the oocyte [8, 9, 10, 29]. In the peri-ovulatory period, capacitated sperm move up to the lower oviductal ampulla, where fertilization happens [73].

Despite the sperm being a foreign cell, it was observed in several animal species that the interaction of the sperm with the oviduct environment has advantageous effects. It was found that the sperm attachment to oviduct epithelium has beneficial effects on the sperm motility, survival and fertilizing ability [26, 74, 75, 76].

The dynamics of *in vivo* sperm-oviduct interaction has been well studied in mice [77, 78, 79]. Whereas in domestic animals, studies on the sperm-oviduct interactions are largely conducted *in vitro* using cultured monolayers of oviduct epithelial cells (OECs) in animal species such as pig [80], horse [81], and cattle [73]. It was observed that cultured oviduct epithelium monolayers usually contain few ciliated cells [21] and that these epithelial cells rapidly dedifferentiate after several days of culture [82] thereby lacking the complexity in tissue and cellular architecture present in the intact oviduct which might affect the behavior of sperm.

It is therefore not surprising that we have little information on the physiological interaction of sperm with the intact and complex oviduct tissue. Consequently, the current study was aimed to develop a differentiated bovine oviduct explant model using intact ampulla to observe the dynamics of cryopreserved bull sperm interactions with pre-ovulatory phase oviduct epithelium that approximates *in vivo* condition.

2. Materials and Methods

2.1. Ethics statement

All experiments were performed at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. 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

All used chemicals were sourced from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. A modified Tyrode balanced salt solution, Tyrode Albumin Lactate Pyruvate (TALP) [83] equilibrated in a 38.5˚C incubator with 5% CO_2 in humidified air was used for washing and diluting sperm. Ice-cold phosphate-buffered saline (PBS) with 10 µg/mL amphotericin B (Gibco, Grand Island, NY, USA) at pH 7.4 was used as a transport medium for whole oviducts. Dulbecco's modified Eagle medium (DMEM) supplemented with 1 ng/mL progesterone, 50 pg/mL estradiol [83], and 5 µg/mL gentamycin (DMEM-PEG) was used for storing the oviduct explants (30 min before the coincubation) and sperm-explant coincubation.

2.3. Oviduct pre-ovulatory ampulla explants preparation

Reproductive tracts were collected from the local slaughterhouse (Hokkaido Livestock, Doto plant Tokachi Factory, Obihiro, Hokkaido, Japan). The phase of the estrous cycle of the organ was evaluated based on the appearance, weight, and color of the corpus luteum and follicular diameter according to an established protocol previously mentioned [83]. Oviducts were grossly examined to have no adhesions, inflammation and other abnormalities. Oviducts ipsilateral to the ovary possessing the dominant/mature follicle under the preovulatory phase (days 19-22) were isolated and transported on ice-cold PBS to the laboratory. Oviduct ampulla explants were harvested according to a method described previously [88] with modifications. Oviducts were rinsed with ice-cold PBS and kept moist throughout the dissection procedures. The mesosalpinx was removed using straight iris scissors, and the ampullary portion of the oviduct was isolated and placed into a petri dish containing PBS at 4°C until use. Next, the isolated oviductal ampulla opened longitudinally with straight iris scissors (Bonimed, Germany) and placed onto a clean styrofoam board to expose the mucosal surface. PBS was used to moisten the exposed mucosa. Small pieces of pre-ovulatory ampulla explants (about 3 mm x 3 mm) referred to as "explant" (Fig. 1) were removed by cutting from the primary mucosal folds using Cohan-Vannas spring scissors (Fine Science Tools, USA) and the ends were trimmed using a size 20 scalpel blade (Bonimed, Germany) to make the explants uniform. The folds were transferred to an aluminum foil-covered 6-well plate

(Thermofisher, USA) containing cold DMEM-PEG and were kept at 4°C for up to 30 min, prior to initiating experiments.

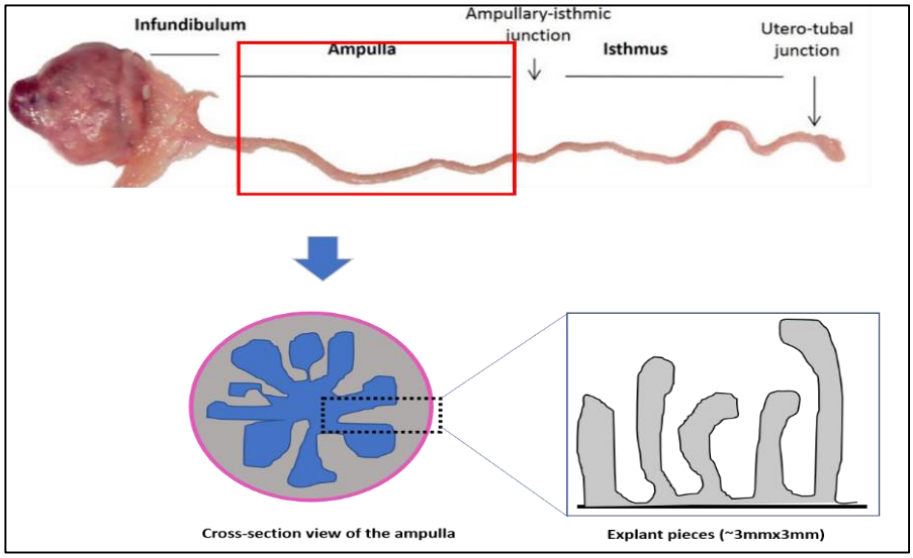

Figure 1. The diagram shows the bovine oviduct attached to the ovary with the dominant mature follicle of the preovulatory phase reproductive tract of the cow. The explants pieces were harvested from the ampulla.

2.4. Sperm sample preparation

Cryopreserved semen from three fertile bulls obtained from Genetics Hokkaido Association, Hokkaido, Japan were pooled and used in the preparation of sperm used in subsequent experiments. A modified swim-up method [83] was performed to obtain viable and motile sperm by layering 0.25 mL aliquots of semen suspension under l mL aliquots of TALP warmed at 38.5° C and 5% CO_2 humidified air in 15 mL Falcon tubes placed in racks at an angle of 45°. Approximately 0.50 mL of the upper layer from each tube was aspirated, pooled and centrifuged at 170 *g* for 5 min after allowing 1 h of incubation. Afterwards, the sperm pellet was reconstituted in warm TALP. To obtain heparin pre-treated sperm ($Hep^{(+)}$ sperm), 10 µg/mL heparin were mixed with the swim-up sperm and further incubated for 3 h. At the end of incubation, heparin was removed by two times centrifugation in 10 mL TALP. The obtained pellet was reconstituted in 1 mL TALP. Control sperm ($Hep^{(-)}$ sperm) were prepared in the same way, except without heparin. A disposable hemocytometer was used in evaluating the sperm concentration (C-chip, NanoEnTek, Korea).

2.5. Sperm and explant viability evaluation

To determine the viability of the incubated explants and sperm, time-dependent observations of beating of the ciliated ampullary epithelial cells, sperm motility, and determination of caspase 3 (*Casp 3*) mRNA expression [84] were performed. Fluorescence microscopy (Keyence, BZ-X800, Osaka, Japan) was used to visualize the surface of the explant and ensure that the epithelium was intact. Hematoxylin and eosin (HE)-stained tissue sections, were prepared as described in section 2.9 to verify normal tissue architecture.

2.6. JC1 labeling of sperm

To visualize the sperm on the epithelial surface of explants, the sperm were labeled with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (AdipoGen, San Diego, CA, USA), which binds the mitochondria in the live sperm midpiece. During immediate observation of $Hep^{(+)}$ sperm - explant epithelium binding (5 min), 10^5 sperm/mL were pre-incubated using 6.4 µM JC-1 in a 38.5˚C incubator with 5% CO_2 in humidified air for 15 min, prior to incubating them to the explant pieces. At 15 min observation, 10^5/ml sperm were added to the explant together with 6.4 µM JC-1 for 15 min prior to observation. Before observation, the explants with attached sperm were transferred into another well with fresh warmed DMEM-PEG to remove the excess JC-1 and debris.

2.7. Incubation of sperm with explant

Explants were incubated with sperm in a 38.5˚C incubator with 5% CO_2 in humidified air using 48-well plates with 0.2 mL DMEM-PEG /well. Explant and 10^5/mL $Hep^{(-)}$ or $Hep^{(+)}$ sperm were co-incubated in under similar conditions for 5 and 15 min and 3h (Fig. 2). The experiments were performed five times using pieces of explants harvested from the oviduct of different animals (five wells for negative control and three wells per treatment per experiment).

2.8. Observation of sperm-explant interaction

To visualize the interaction between the explant incubated with either sperm with or without pre-treatment of heparin, the sperm were labeled with JC-1 prior to observation at 5 min, 15 min, and 3 h. Sperm behavior and pattern of attachment to the epithelium were observed and recorded using fluorescence microscopy (Keyence, BZ-X800, Osaka, Japan).

2.9. Histology

The pre-ovulatory phase ampulla explants and 3 x 10^6 cells/mL heparin pre-treated sperm were incubated for 6 h and prepared for histology. The experiment was performed three times using explant pieces from three independent oviducts. After incubation, explants were carefully washed in PBS and then placed in phosphate buffered 10% formalin. The formalin-fixed tissue samples were transferred to 70% ethanol after 24 h, then dehydrated in increasing grade series of ethanol. The dehydrated tissues were cleared using xylene, and then embedded in paraffin wax, which were serially cut into 5 µm thick slices. The sectioned explants were placed on an aminopropyl-triethoxy-silane- coated slides (S8226, Matsunami Glass Int., Osaka, Japan) and the paraffin wax was melted. The explant sections were then stained with HE for assessment of normal histology of the explant tissue.

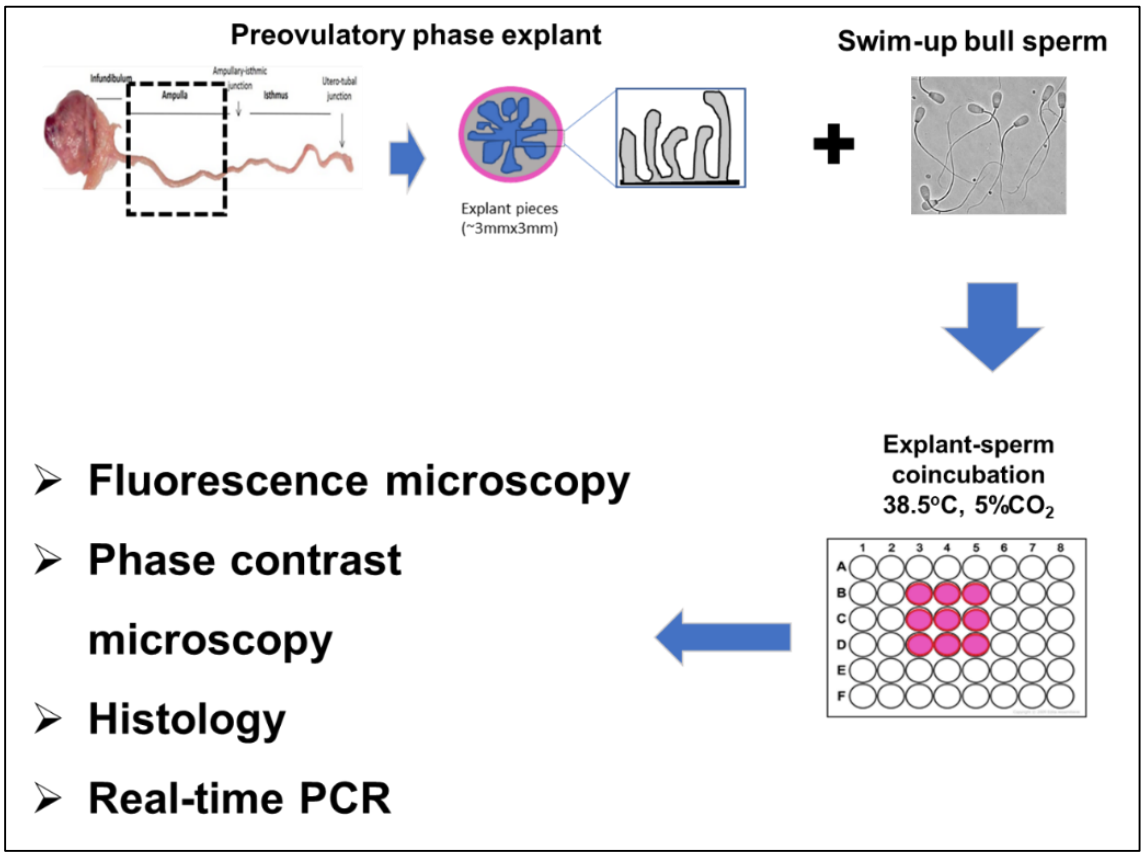

Figure 2. The diagram shows methods performed during the development of the differentiated oviduct ampullary explant model.

3. Results

3.1. Tissue architecture of intact fresh ampulla explants

Fluorescent microscopic observation of the bovine ampulla explants observed showed that the luminal surface of the live explants had a highly folded mucous membrane. Within the primary fold, many smaller secondary folds terminated in a complex system of oval pockets (Fig. 3A). Hematoxylin-eosin (HE) stained sections of the explant showed the complex surface created by numerous folds that were covered primarily by ciliated epithelial cells (Fig. 3B). The explant maintained an intact architecture which was visualized using the green autofluorescence mode of the fluorescent microscope.

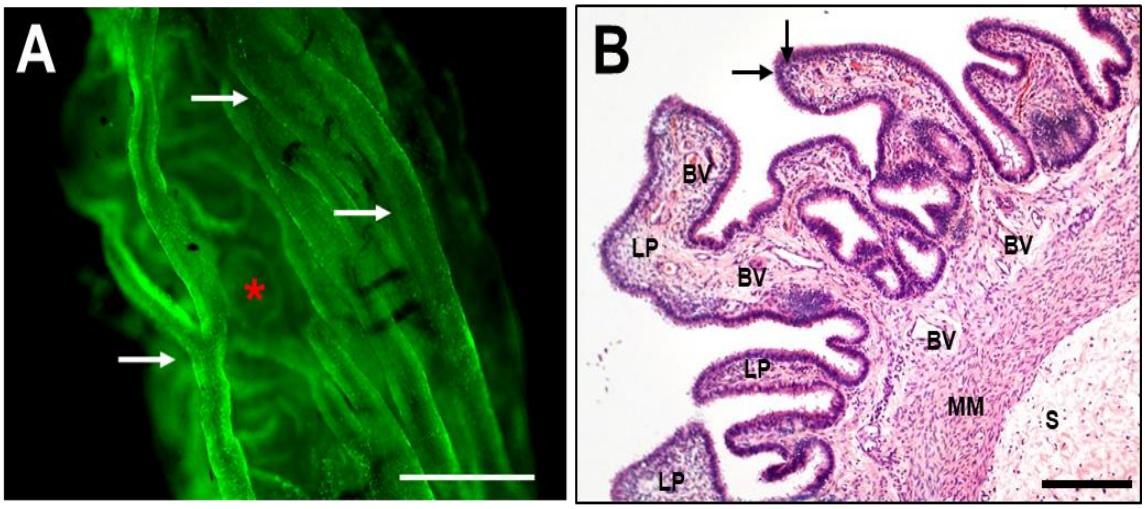

Figure 3. Morphology of the explants of the oviductal ampulla. **(A)** Fluorescence microscopic image of a living explant, showing green autofluorescence. Each explant consisted roughly of a large primary fold. White arrows indicate the apical tips of smaller folds and the red asterisk indicates a pocket at the base of the folds. Bar= 500 µm. **(B)** HE-stained section of explant showing mucosal epithelium (black arrows), blood vessels (BV), lamina propria (LP), muscularis layer (MM), serosa connective tissue (S). Bar= 500 µm.

3.2. Ciliary activity and flow of materials

Phase contrast microscopic observation of the bovine ampulla explants showed that the ciliated epithelial cells moved vigorously (Fig. 4). This synchronous movement created allowed for a unidirectional movement. The vigorous ciliary activity also made the small explants float and move around within the media.

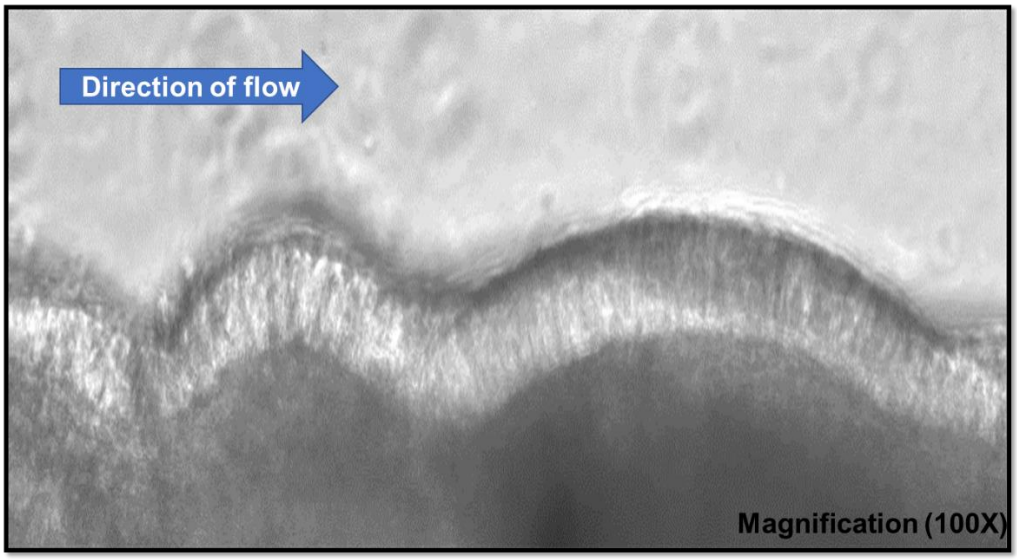

Figure 4. Image of explant taken under phase contrast showing the edge of the explant. The actively motile ciliated epithelial cells created a unidirectional flow of fluid and materials. The blue arrow shows the direction of flow of fluid and materials. Magnification= 100X.

3.3. Sperm head bound to the cilia of ampullary epithelial cells

Upon adding the sperm into the explant, they immediately attached into the oviductal epithelium. The attached sperm bound to the motile cilia of the epithelial cells with the sperm head tightly covered with the cilia. The attached sperm clearly hovers over the epithelium (Fig. 5).

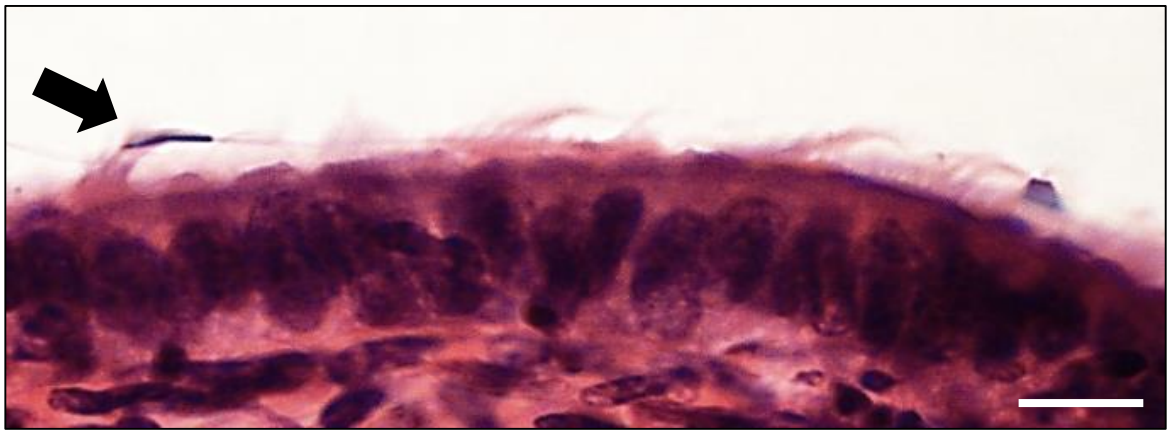

Figure 5. High magnification of an HE-stained section of explant showing an attached sperm (thick arrow) with the head associated with the cilia of an epithelial cell. Bar= 50 µm.

3.4. Fluorescence observation showed sperm attached uniformly on the surface of the explant epithelium.

The green autofluorescence allowed the visualization of the intact explant surface while the JC-1 labelled sperm showed as red rods or dots all over the explant surface. Fluorescence microscopic images showed that sperm uniformly bound all over the surface of the explant. The fluorescent images also allowed for the accurate counting of the bound sperm on the explant (Fig. 6).

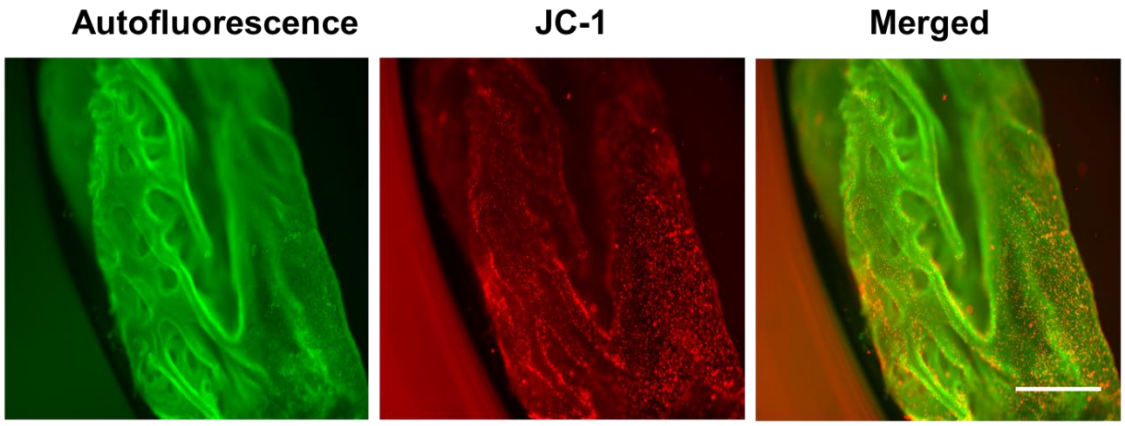

Figure 6. Fluorescence microscopy of explant (green autofluorescence) with attached JC-1-labelled sperm (red fluorescence)**.** The merged fluorescent images showed that the sperm uniformly attached to the explant surface. Bar= 500 µm.

3.5. Explant viability evaluation

The viability of the explant was established by the presence of active ciliary beating, and binding of motile sperm to the explant. No upregulation of caspase 3 (*Casp3)* mRNA was observed, indicating that cells were viable and were not undergoing apoptosis until after 6 h of incubation (Fig. 7).

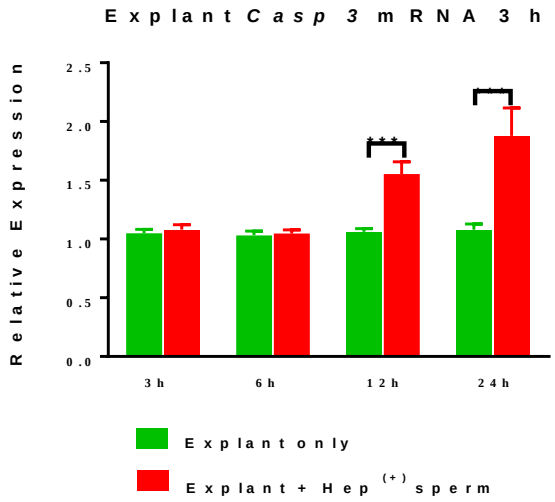

Figure 7. Explant relative mRNA expression for *Casp 3* shows that explants viable and not undergoing apoptosis until 6h. Data collected are shown as mean ± SEM of 3 different experiments. Data were pooled for two explants for each experimental treatment within a replicate. ****P<0.0001* denotes significant differences between two treatment groups.

4. Discussion

This study performed real-time visual observations on the intact pre-ovulatory ampulla explant surface and its associated structures using fluorescent microscopy. Labeling the sperm with JC-1 allowed the localization of attachment on the explant surface, facilitated accurate counting of the number of attached sperm, and provided illumination to the explant surface [53]. The explant model showed physiological interaction between sperm and oviduct that approximates *in vivo* condition.

The differentiated explant model was derived from the ampullary part of the bovine oviduct in the pre-ovulatory phase. The explant consisted of one whole primary fold of the ampulla, and is about 3 mm x 3 mm in dimensions. The fold includes the intact epithelium and lamina propria. Observations of the pre-ovulatory phase explant were performed using phase contrast microscopy. Only the edge of the explant was clearly visible and showed that the explant epithelium contains ciliated and non-ciliated secretory cells. The cilia in the apical surface of the epithelial cells are actively beating synchronously which made the explant move around the incubation media. The synchronous ciliary beating of the cells resulted in a unidirectional flow of fluids and materials. This also allowed the identification of cranial (part which is nearer the ovary) and caudal (part which is nearer the uterus) portion of the explant.

Materials and fluid flow towards the uterus and away from the ovary, which helps in moving the oocyte towards the ampulla around fertilization and the early embryo to the uterus for implantation [89, 91].

Since phase contrast microscopy only allows limited observation of the explant, fluorescence microscopic observations were performed to visualize the intact surface and architecture of the explant. Under the green autofluorescence mode, the explant surface has a highly complex architecture. The primary fold, consists of numerous smaller secondary folds which terminates into deep round pockets. The highly folded explant surface considerably increased the surface are of the explant. However, this also made the lumen very narrow. The morphology of the fresh live explant appears to be similar to HE preparations and scanning electron microscopy (SEM) in bovine [24] and horse [92]. Taken together, the over-all anatomical structure of the explant facilitates direct interaction between the bull sperm and oviductal ampullary epithelium.

To observe the interaction of the explant epithelium and sperm, the sperm was labeled with mitochondrial stain JC-1[93, 53]. The midpiece emits a red fluorescence which contrasts with the green autofluorescence from the explant [53]. Observations of the interactions show that the sperm immediately attaches to the explant epithelium. Further observations under fluorescence for fresh samples and HE-stained tissues showed that sperm attaches to the ciliated epithelial cells. The head of the sperm appears to be tightly bound by the cilia of the epithelial cells (Fig. 5). Sperm appears to hover on top of the epithelial cells and are attached in a very organized manner. The sperm also attached all over the surface of the live explant. This differs in SEM observations in similar tissues which showed that sperm are attached to the grooves separating the folds [24].

Viability evaluation of the explant showed that the explants and sperm maybe viable for 24 h. The presence of ciliary activity of the explant and attachment of sperm was recorded to indicate viability. During initial incubation, the explant ciliary activity is very vigorous and shows almost all ciliated cells actively moving. There is a progressive loss in the ciliary activity as the incubation time increases and the explants eventually lose all movement when the explant dies.

The sperm viability was also determined by its attachment to the cilia of explant epithelial cells. There is vigorous movement of the sperm tail which is tangentially aligned with the epithelium of the explant [91] initially which then loses in speed as the incubation time increases. Once the sperm becomes non-viable, it detaches from the explant and is washed into the media.

In order to accurately quantify the immune response, the explant must be physically and immunologically viable. Cells which are dying and are undergoing degeneration and apoptosis [95]. To make sure that the explants are immunologically viable, the mRNA transcription for *caspase 3* (*casp 3*), which is a marker for apoptosis [95] was evaluated. The *casp 3* expression for explant and explant incubated with sperm were stable until 6 h.

The developed differentiated explant model using the pre-ovulatory phase bovine oviductal ampulla can be used to observe the interaction between sperm and the epithelium, quantify the attachment of sperm to the explant, and evaluate the immune response of the oviductal tissue to sperm. This model provides an alternative method to observe the sperm-oviduct interaction which approximates the *in vivo* condition. However, this explant model can only be used in short term incubation conditions due to the short viability of the explant (6h). Therefore, we can only view the acute physiologic and immunologic response of the oviductal tissue to sperm. It is therefore recommended that other established models be used such as *in vitro* culture and *in vivo* be utilized to observe the long-term physiologic and immune response of the oviduct to sperm.

Chapter 2

Evaluation of the effects of sperm-oviductal ampulla interactions on sperm attachment and oviductal immune response

1. Introduction

After insemination into the FRT, the sperm must reach the oviduct to fertilize the mature ovulated oocyte. However, before the sperm can fertilize the oocyte, it must undergo a complex event involving physical and physiological changes to attain fertilizing capability in a process is called "capacitation" [9, 10]. Although stimulation of capacitation in the female reproductive tract is not completely understood, it has been recognized that heparin enhances bull sperm fertilization *in vitro* [15, 96]. Incubation of bull sperm with heparin results in the loss of cholesterol and some membrane phospholipids, which is a fundamental part of the process of sperm capacitation [96]. Furthermore, heparin can bind to Binder of Sperm Proteins (BSP1, BSP3, and BSP5), which were found on the sperm surface, and have been found to be involved in capacitation [97, 98, 99].

Using the developed differentiated explant model, we compared the interactions of sperm that had been stimulated to capacitate with those that had not. We used explants of mucosal folds of the pre-ovulatory oviductal ampulla to study this response, because the tissue was fully differentiated and prepared for fertilization. Furthermore, in these explants, the epithelium remained in contact with underlying connective tissue, which could provide a more comprehensive information on the oviductal immune response to sperm *in vivo*.

2. Materials and methods

2.1. Non-treated ($Hep^{(-)}$) and heparin-treated ($Hep^{(+)}$) sperm preparation

Sperm preparation was described in detail in chapter 1 and the concentration of sperm was adjusted depending on the type of experiment performed.

2.2. Incubation of sperm with explants

Explants were incubated with sperm in an incubator with a 5% CO_2 level in humidified air. Explants were placed in 48-well plates containing 0.2 mL DMEM-PEG for each well. To observe the explant epithelium immune response to the presence of sperm, either 10^6 /mL $Hep^{(-)}$ sperm or $Hep^{(+)}$ sperm were co-incubated with the explants for 3 h. Explants without sperm were assigned under control groups.

Explants were incubated with 10^5/mL $Hep^{(-)}$ or $Hep^{(+)}$ sperm, for counting attached sperm, or 10^6/mL $Hep^{(-)}$ or $Hep^{(+)}$ sperm for mRNA expression evaluation, in DMEM-PEG. All the performed experiments were repeated five times using explants harvested from the oviduct of different animals (five replicates for negative control and three replicates for each treatment for every experiment conducted). The explants were harvested and were rinsed three times in PBS. TRIZOL (Invitrogen, Carlsbad, CA, USA), was used to preserve the explants and frozen under -80˚C before total RNA extraction was performed.

2.3. Counting of attached sperm after coincubation with ampulla explants

Counting of the attached sperm was performed after 5 min and 15 min of sperm- explant incubation. Labeling of the sperm with JC-1 was described in the previous chapter. Videos were taken using a fluorescence microscope (Keyence, BZ-X800, Osaka, Japan) in a temperature-controlled thermal plate (TPi-SQX, Tokai Hit, Japan). After the sperm-explant coincubation, videos and images were taken under the BZ-X Texas Red (OP-87765, 560/40nm and 630/75nm excitation and emission wavelengths respectively) and BZ-X GFP (OP-87763, 470/40nm and 525/50nm, excitation and emission wavelengths respectively) filters set for red (JC-1 label of sperm mitochondria) and green (autofluorescence of epithelium) wavelengths respectively. The video focus was adjusted to allow counting and visualization of all the sperm attached on the folds during the video recording. The software ImageJ (Version 1.51j8) was used to produce a grayscale rendition and to improve contrast on all videos taken before quantifying the attached sperm. All counts were performed by choosing three places on the ciliated epithelial surface of the explant, each with an area of 1 mm^2. Explant edges were avoided. The experiment was replicated 5 times, each time using explants from an ampulla from one cow for all treatments. Data were pooled for two explants for each experimental treatment within a replicate.

2.4. mRNA isolation and analysis using quantitative real-time PCR

Prior to RNA extraction, the stored explant tissues were sonicated in TRIZOL media with a handheld ultrasonic processor (Sonics and Materials, CT, USA) in an ice bath. Thereafter, total RNA extraction procedure was done using TRIZOL media according to a methodology previously mentioned [101]. Total RNA concentration and quality were quantified using a NanoDrop Spectrophotometer (2000c, Thermo Scientific, Waltham, MA, USA) at 260 nm absorbance and a ratio of 260/280 nm

respectively. RNA was diluted in RNA storage solution (Ambion, Austin, TX, USA) and was frozen at -80˚C storage until further use.

cDNA was synthesized using a methodology previously mentioned [58] . The quantitative real-time PCR was performed in a CFX Connect™ real-time PCR detection system (Bio-Rad Laboratories, Tokyo, Japan). 10 µl of reaction mix containing 2 µl of the sample cDNA, 5 µl of SYBR Green PCR Master Mix (Bio-Rad Laboratories, USA), 0.2 µl of target primers (Table. 1), and 2.8 µl of nuclease-free water (Invitrogen) was mixed. The amplification process was programmed with primary activation step for 15 min at 95˚C and then proceeded by 40 PCR cycles with 15 sec of denaturation at 95˚C, 15 sec of annealing at 55-58˚C, and 30 of sec extension at 72˚C). Nuclease-free water or non-reverse transcribed RNA were included for each run to serve as controls. Primer Express® Software v3.0.1 (Thermo Scientific) was used to design individual primers used for the real-time PCR. Amplification specificity was checked using the melting curve at the end of the reactions. *β-actin* as control gene to normalize the calculated cycle threshold (Ct) values. Delta-Delta comparative threshold method [95] was used to calculate the relative fold change in expression of mRNA. In each experimental replicate, five explants used for control and three explants used for each treatment were all obtained from one cow.

Table 1. List of the primers used in quantitative 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		
IL10	Forward	GAGATGCGAGCACCCTGTCT	NM_174088.1	51
	Reverse	GGCTGGTTGGCAAGTGGATA		
TNFA	Forward	CAAAAGCATGATCCGGGATG	NM_173966.3	51
	Reverse	TTCTCGGAGAGCACCTCCTC		
PGES	Forward	AAAATGTACGTGGTGGCCGT	NM_174443.2	51
	Reverse	CTTCTTCCGCAGCCTCACTT		
TGFB1	Forward	CTTTCTTCAAATGCAGCATTGG	NM_001166068.1	51
	Reverse	GGGTCTGGGTGATACAACGAA		
TLR2	Forward	CATGGGTCTGGGCTGTCATC	NM_174197.2	51
	Reverse	CCTGGTCAGAGGCTCCTTCC		

2.5. Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). A Students t-test was used to compare the mean differences between two groups while one-way analysis of variance (ANOVA), followed by Tukey's tests were used to compare the means for more than two groups. All values are presented as mean ± standard error of the mean (SEM). The statistical significance was set at $p<0.05$.

3. Results

3.1. Sperm treatment with heparin did not affect the binding to oviductal epithelium

No differences were detected between the numbers of $Hep^{(-)}$ (control) sperm and $Hep^{(+)}$ sperm attached to the explants after incubation for 5 and 15 min (Fig. 8). Both types of sperm attached uniformly over the explant surface, with the heads oriented in a similar direction, while orientation of the beating flagella of attached sperm seemed to be influenced by the orientation of the beating of the cilia on epithelial cells. Note that unbound heparin was removed by washing sperm prior to adding them to the explants and controls were handled identically to treated sperm.

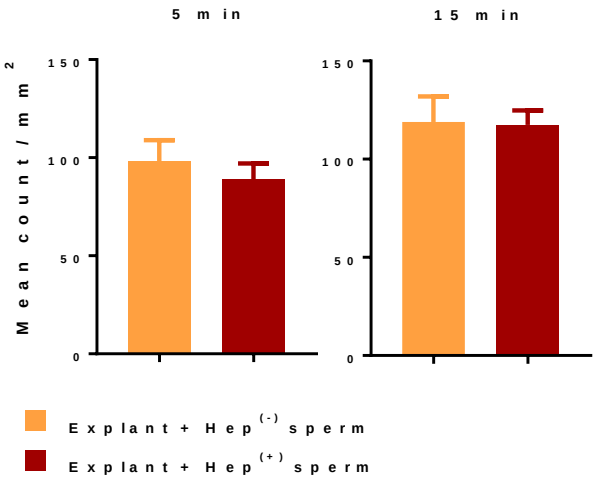

Figure 8. Numbers of JC-1-stained sperm attached to explant epithelium after 5 and 15 min of coincubation. Data are presented as mean ± SEM of five different experiments. Data were pooled for two explants for each experimental treatment within a replicate.

3.2. Heparin treatment of sperm upregulated anti-inflammatory genes

The impact of sperm binding on transcription of targeted genes after 3 h of coincubation was quantified using quantitative real-time PCR. The results showed that the Hep$^{(+)}$ sperm binding upregulated mRNA expression of *TLR2*, interleukin 8 (*IL8)*, prostaglandin E synthase (*PGES)*, and transforming growth factor beta 1 (*TGFB1)*, without affecting tumor necrosis factor alpha *(TNFA)* and interleukin 10 (*IL10)* mRNA expression in explants. Upregulation of transcription of these genes indicates a primarily anti-inflammatory response. In contrast, Hep$^{(-)}$ sperm binding upregulated only *PGES* mRNA expression in explants (Fig. 9).

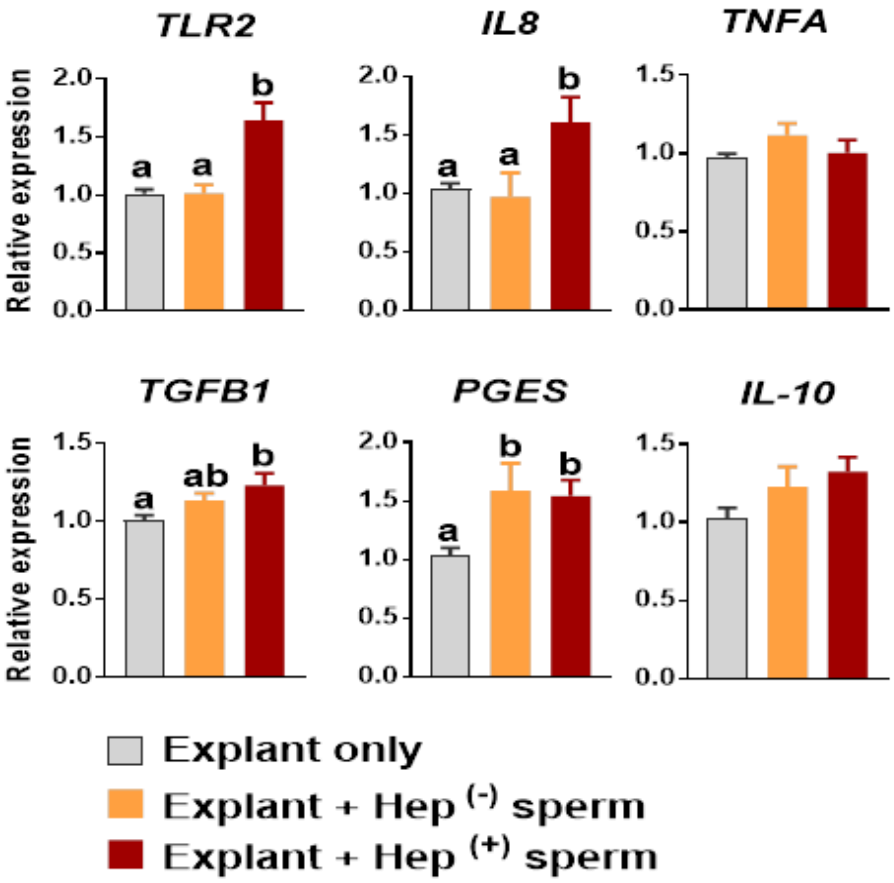

Figure 9. Relative mRNA expression of evaluated genes in explants incubated with Hep$^{(+)}$ sperm or Hep$^{(-)}$ sperm or without sperm (control) for 3 h. The data are presented as mean ± SEM of five different experiments. Five explants for control and 3 explants for each treatment were used from an individual cow in each experiment. Letters in lower case denote a significant difference at $p < 0.05$.

4. Discussion

With the developed differentiated explant model, we observed the interactions of sperm under different capacitation status with the pre-ovulatory phase ampullar epithelium and quantify the immune response of the explant to the presence of heparin-treated or non-treated sperm.

Although no difference was detected in the numbers of $Hep^{(+)}$ and $Hep^{(-)}$ sperm bound to explants, the ampullary epithelium was more immunologically sensitive to $Hep^{(+)}$ sperm than to $Hep^{(-)}$ sperm. Binding of $Hep^{(+)}$ sperm to explant epithelium upregulated expression of *TLR2*, *IL8*, *TGFB1*, and *PGES* by 3 h; whereas, $Hep^{(-)}$ sperm only stimulated the expression of *PGES*. Heparin could interact with the sperm surface to somehow stimulate these changes in the oviduct. For bull sperm, it has been established that heparin can bind to the BSPs coating the sperm surface [99]. It has also been demonstrated that heparin stimulates capacitation of bull sperm by interacting with the BSP proteins [104]. Furthermore, putative oviductal receptors for sperm, certain annexin proteins, are known to bind heparin as well [72]. Thus, it is possible that heparin bound to BSPs on the sperm surface acted on oviductal epithelial cells directly through annexins or other heparin binding proteins, or indirectly, through stimulating capacitation events. These possibilities should be investigated in the future.

Heparin has been used to stimulate detachment of sperm from oviductal epithelium *in vitro* [105]. In our explant system, addition of heparin to sperm prior to adding the sperm to explants did not prevent binding. This may have been because unbound heparin was removed by dilution and washing. It has been reported that pretreatment of sperm with heparin did not prevent bull sperm from binding to isthmic or ampullar explants [88]; further, while heparin addition to bound sperm stimulated detachment of the sperm from isthmic explants, it did not significantly detach bound sperm from ampullar explants [88].

We found that the oviduct epithelium did not mount an inflammatory (Th1) response to the attachment of either type of sperm, because the proinflammatory cytokine, *TNFA*, was not transcrribed. Sperm binding to cultured oviduct epithelium has been reported to induce an anti-inflammatory (Th2) or a generally tolerogenic response by the oviduct epithelium [58]. In the present work, an anti-inflammatory response was evident with sperm pre-treated with heparin, as seen from the upregulation of the anti-inflammatory cytokine, *TGFB1*.

Although sperm are immunologically foreign cells to females, the oviduct epithelium has not been found to launch a hostile immune response that destroys sperm, at least not before fertilization

occurs. In fact, it was found that sperm attachment to the oviduct epithelium has beneficial effects on the motility of the sperm, survival, and fertilizing ability [84, 77, 78, 79]. Sperm attachment to oviductal epithelium influences the gene transcription and protein synthesis, which might help increase the sperm storage and sperm fertility [105]. *In vitro* studies demonstrated that the sperm attachment to cultured bovine oviduct epithelial cells induces a sperm-tolerant environment, as evidenced by upregulation of the mRNA for the anti-inflammatory cytokines *TGFB1* and *IL10*, as well as increased PGE2 secretion [54] which protects sperm from being attacked by local neutrophils [50]. Thus, the response of the oviduct epithelium to sperm creates a favorable environment for sperm survival until fertilization. Nevertheless, despite the fact that sperm fertilize oocytes in the ampulla, we do not know how ampullar epithelium specifically reacts to the arrival of sperm.

The anti-inflammatory response to bull sperm could serve to protect the sperm as they approach the oocyte in the ampulla. Whereas, surgical ligations of the uterotubal junction and ampullary isthmic junction have provided evidence that bull sperm spend several hours in the oviduct prior to fertilization [103], the time spent migrating in the ampulla to reach the oocyte is unknown. Nevertheless, certain anatomical characteristics indicate that sperm migration to the oocyte in the ampulla could last for hours. When bovine sperm migrate in the ampulla, they move through a very narrow, extremely complex lumen, which is created by large, complicated mucosal folds. The folds are much larger and more complex than those of the bovine oviductal isthmus [104, 88]. The narrow passages of the complex lumen of the ampulla place bull sperm in intimate contact with the epithelium, to which they may bind. All of this may well contribute to the time spent by sperm migrating in the ampulla before they reach an oocyte. Furthermore, the bovine oocyte also moves slowly through the ampullary lumen to the site of fertilization, requiring 8-10 hours or more to reach its destination [25]. Altogether, these factors indicate that bull sperm could spend enough time in the ampulla to benefit from the protection of an anti-inflammatory response of the epithelium.

Collectively, the data show that the oviduct-explant model can be effectively utilized to study sperm-oviduct interaction dynamics and oviduct immune responses. The pre-ovulatory phase oviduct ampulla is more immunologically responsive to heparin-treated sperm and launches an anti-inflammatory response which may serve to protect the sperm.

Chapter 3

The role of Toll-like receptor 2 (TLR2) in the bovine oviductal ampulla immune response to sperm binding

1. Introduction

The FRT is equipped with a well-developed innate immune system to deal with pathogens, sperm, and the semi-allogenic embryo [52, 105]. The innate immune system depends on a large family of pattern recognition receptors (PRRs), which sense diverse and highly conserved structures on pathogens called pathogen-associated molecular patterns (PAMPs) [110]. One of the four known PRR major sub-families are the Toll-like receptors [111].

Toll-like receptors (TLRs) primarily detect potential pathogens to the host and trigger immune responses [108]. TLRs are integral glycoproteins characterized by an extracellular or luminal ligand-binding domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain [110]. These TLRs are expressed on the surface membranes of immune and non-immune cells [110, 111]. In mammals, 11 mammalian TLRs were identified including its specific ligand [113].

The bovine oviduct is known to express TLRs [114] and TLRs have been shown to play roles in the reproductive processes of mice and cattle [75, 115]. In mice, TLR2 activation in cumulus cells of ovulated cumulus–oocyte complex (*COC*) stimulated cytokine production and chemokines that induced the capacitation of sperm and enhanced fertilization; whereas, blocking TLR2 with antibody reduced sperm capacitation and fertilization [75]. TLR2 is expressed by the bovine oviduct epithelium during the follicular and luteal phases, and the expression is regulated by ovarian steroids and luteinizing hormone (LH) [115]. We have shown that TLR2 participates in the inflammatory cascade caused by attachment of bull sperm in the endometrium of the cow [52, 53] which serves to clear the excess sperm in the uterine environment and to prepare for pregnancy.

Whereas there is evidence that a TLR2 response to sperm in the uterus serves to eliminate sperm that have not reached the oviduct, the immune response to sperm in the oviduct is less well understood, particularly in the oviductal ampulla where fertilization occurs. We therefore examined the immunological impact of sperm interaction with the ampullary epithelium and the role of TLR2 in sperm-epithelial binding.

2. Materials and methods

2.1. Observation of sperm dynamics and attachment to epithelium during coincubation with oviduct explants

Explants were incubated with 10^5/mL $Hep^{(-)}$ or $Hep^{(+)}$ sperm, for observation of behavior and counting attached sperm, in DMEM-PEG +/- 100 µM antagonist in a warmed incubator with 5% CO_2 in humidified air for 30 min prior to observation and recording. During immediate observation of sperm - explant epithelium binding (5 min), 10^5 sperm/mL were mixed with 6.4 µM JC-1 in a 38.5˚C incubator with 5% CO_2 in humidified air for 15 min, prior to coincubation explants. At 15 min observation, 10^5/ml sperm were added to the explant together with 6.4 µM JC-1 for 15 min prior to observation. Before observation, the explants with attached sperm were transferred into another well with fresh warmed DMEM-PEG to remove the excess JC-1 and debris.

Videos were taken using a fluorescence microscope (Keyence, BZ-X800, Osaka, Japan) in a temperature-controlled thermal plate (TPi-SQX, Tokai Hit, Japan). After the sperm-explant coincubation, videos and images were taken under the BZ-X Texas Red (OP-87765, 560/40nm and 630/75nm excitation and emission wavelengths respectively) and BZ-X GFP (OP-87763, 470/40nm and 525/50nm, excitation and emission wavelengths respectively) filters set for red (JC-1 label of sperm mitochondria) and green (autofluorescence of epithelium) wavelengths respectively.

The video focus was adjusted to allow counting and visualization of all the sperm attached on the folds during the video recording. The software ImageJ (Version 1.51j8) was used to produce a grayscale rendition and to improve contrast on all videos taken before quantifying the attached sperm. All counts were performed by choosing three places on the ciliated epithelial surface of the explant, each with an area of 1 mm^2. Explant edges were avoided. The experiment was replicated 5 times, each time using explants from an ampulla from one cow for all treatments. Data were pooled for two explants for each experimental treatment within a replicate.

2.2. Coincubation of sperm with oviduct ampullary explants to evaluate the oviduct immune response

The involvement of the TLR2 in $Hep^{(-)}$ or $Hep^{(+)}$ sperm-oviduct epithelium binding and the consequent immune response were evaluated using a specific TLR1/2 antagonist-CU-CPT22 (Merck,

Darmstadt, Germany). Explants were incubated with 10^6/mL $Hep^{(-)}$ or $Hep^{(+)}$ sperm for mRNA expression evaluation, in DMEM-PEG +/- 100 µM antagonist in a warmed incubator with 5% CO_2 in humidified air for 3 h. All the experiments were performed five times using explants harvested from the oviduct of individual animals (five explants for negative control and three explants per treatment). The explants were harvested and rinsed three times in PBS and then frozen under TRIZOL media, at -80˚C until mRNA extraction.

2.3. Histology and Immunofluorescence (IF) staining for TLR2 in explants

To investigate the effects of $Hep^{(+)}$ sperm on TLR2 synthesis of explant epithelium, the explants were incubated with $3x10^6$ $Hep^{(+)}$ sperm /mL for 6 h. Explants without $Hep^{(+)}$ sperm and TLR1/2 antagonist were used as control samples. The experiment was performed three times with explants from three individual oviducts (n=3). After incubation, explants were carefully washed in PBS and then placed in phosphate buffered 10% formalin. The formalin-fixed tissue samples were transferred to 70% ethanol after 24 h, then dehydrated in increasing grade series of ethanol. The dehydrated tissues were cleared using xylene, and then embedded in paraffin wax, which were serially cut into 5 µm thick slices. The sectioned explants were placed on an aminopropyl-triethoxy-silane- coated slides (S8226, Matsunami Glass Int., Osaka, Japan) and the paraffin wax was melted. The explant sections were then stained with HE for assessment of normal histology of the explant tissue.

For immunofluorescence labeling, sections tissues were heated using microwave in target retrieval solution (1:10, S1699; Dakocytomatin, CA, USA) for 15 min. Next, he samples were immersed in methanol containing 0.3% H_2O_2 for 10 min at room temperature (RT) in order to block any endogenous peroxidase activity. Thereafter, to reduce the non-specific staining, normal goat serum (1:50, S-1000, Vector Laboratories, CA, USA) was added into the tissue sections and were incubated for 30 min at RT. All sections except the negative control were incubated for 12 hr with rabbit polyclonal anti-TLR-2 primary antibody (1:50; orb11487, Biorbyt, Cambridge, UK) at 4˚C in a humidified chamber. DAPI (1:50, 340-07971, Dojindo Laboratories, Japan) was used as counterstain for 30 min. The samples were then labeled by adding the secondary antibody for 30 min (1:200, goat anti-rabbit IgG conjugated with Alexa Fluor 546, Invitrogen, Thermo Fisher Scientific). VECTASHIELD mounting medium (H-1000; Vector Laboratories) was added after rinsing the samples and sealing them with a coverslip. Fluorescence

microscope (Keyence, BZ-X800) was used to record the fluorescence signal using the BZ-X TexasRed (OP-87765, 560/40nm and 630/75nm excitation and emission wavelengths respectively) and BZ-X DAPI (OP-87762, 360/40nm and 460/50nm excitation and emission wavelengths respectively) filters. Exposure time was standardized for the primary antibody and its negative antibody control.

2.4. 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 in the previous chapter.

2.5. Statistical analysis

The data were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). A Students t-test was used to compare the mean differences between two treatments, while one-way ANOVA followed by Tukey's tests was applied to compare the means for more than two groups. All experiments were performed five times using explants from individual cows. Each treatment group has three replicates. All the measured values were pooled and subjected for statistical analysis. Values are presented as mean ± standard error of the mean (SEM). Significance was set at $p<0.05$.

3. Results

3.1. The TLR1/2 antagonist reduced binding of $Hep^{(+)}$ and $Hep^{(-)}$ sperm to explants

Comparison of the numbers of bound sperm in the explants from different groups showed that the addition of TLR1/2 antagonist in the coincubation system resulted in significant reduction in both $Hep^{(+)}$ and $Hep^{(-)}$ sperm binding to epithelium at 5 and 15 min (Fig. 10).

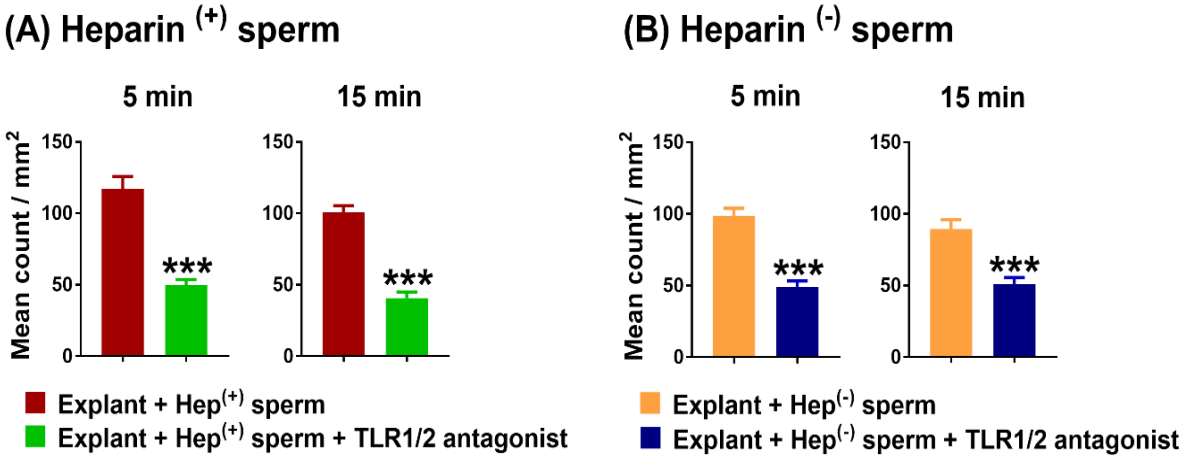

Figure 10. TLR1/2 antagonist reduced sperm binding to explants**.** Numbers of JC-1-stained Hep$^{(+)}$ **(A)** or Hep$^{(-)}$ **(B)** sperm attached to explants at 5 and 15 min of co-incubation. The data are presented as mean ± SEM of five independent experiments. Data were pooled for two explants for each experimental treatment within a replicate. ****P<0.0001* indicates significant differences between compared groups.

3.2. TLR1/2 antagonist inhibited effects of sperm on explant mRNA expression

The observed upregulation of *TLR2* mRNA in the explant after incubation with Hep$^{(+)}$ sperm directed us to investigate the role of TLR2 in Hep$^{(+)}$ and Hep$^{(-)}$ sperm-oviduct interactions. TLR1/2 antagonist was added to explants prior to addition of sperm, after which sperm attachment to explants and explant mRNA expression were quantified. The TLR1/2 antagonist inhibited upregulation of explant transcription of *TLR2, IL-8* and *TGFB1* mRNA by Hep$^{(+)}$ sperm (Fig. 11A). The antagonist did not inhibit the sperm-induced upregulation of *PGES* mRNA. (Fig. 11 A, B).

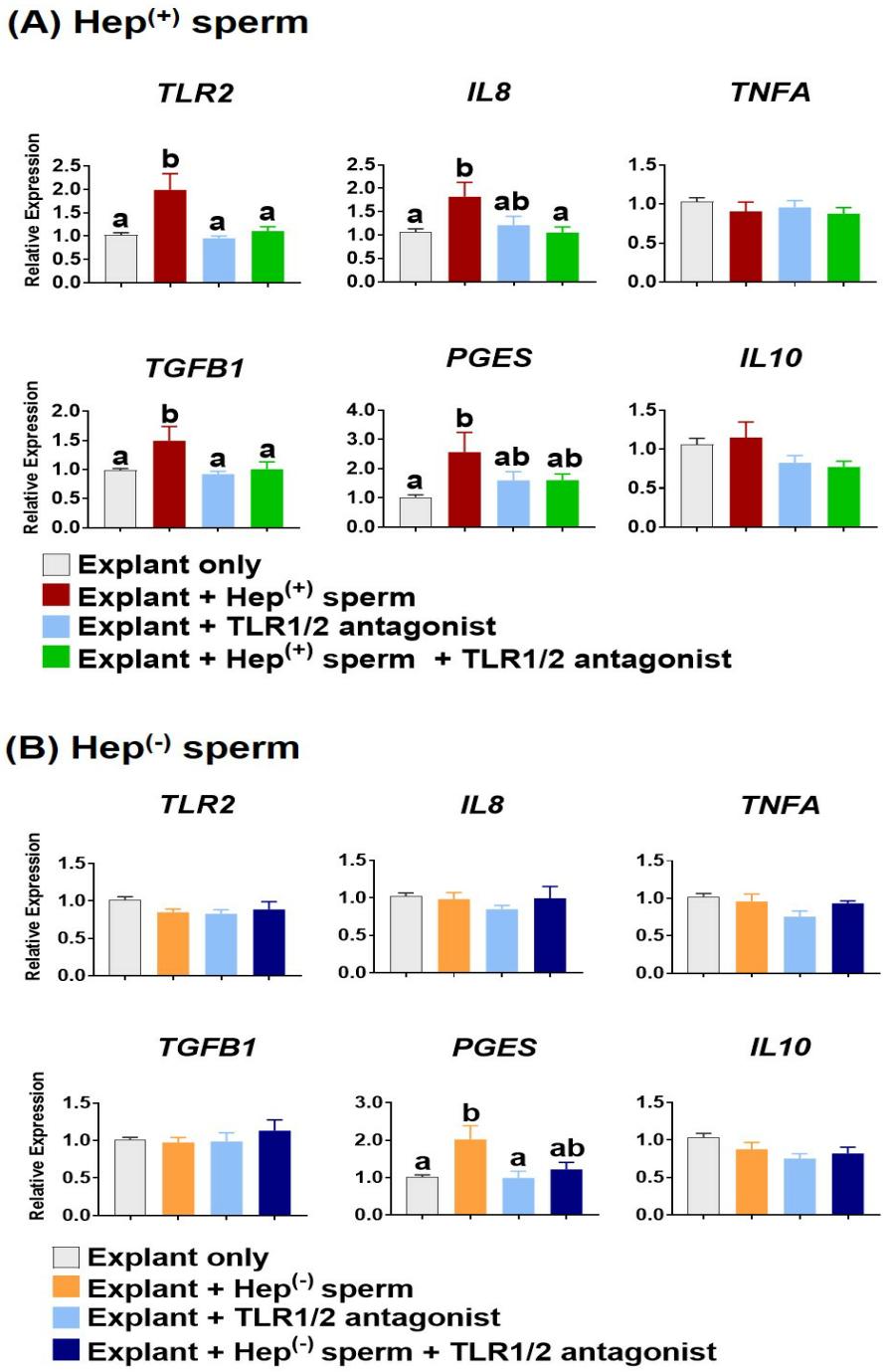

Figure 11. Gene transcription of explants. Comparison of relative mRNA expressions of target genes in explants incubated with Hep$^{(+)}$ sperm or Hep$^{(-)}$ sperm with and without TLR1/2 antagonist. The data are shown as mean ± SEM of five different experiments. Five explants in control group and 3 explants for each treatment groups were used from an individual cow in each experiment. Different letters assigned for compared groups denote significant differences (p<0.05).

3.3. Upregulated TLR2 protein was mainly localized to epithelium of explants

Immunofluorescence labeling was made to examine the localization of TLR2 protein expression in the oviduct epithelium. Explants were coincubated with Hep$^{(+)}$ sperm in with or without the TLR1/2 antagonist for 6 h (n=3). Faint but distinct red fluorescence indicating TLR2 immunoreaction was observed in the oviduct epithelium in the explant tissue without sperm (control group) in the cytoplasmic and ciliary region of the epithelial cells lining the ampullary folds. Some fluorescence was observed in cells within the connective tissues and within the endothelium of blood vessels located in the lamina propria of the explant. Co-incubation of the explant with Hep$^{(+)}$ sperm resulted in a more intense and uniform fluorescence in the epithelium when compared to the control.

The addition of TLR1/2 antagonist to sperm-explant co-incubation markedly reduced the TLR2 fluorescence intensity on epithelium compared to its intensity in Hep$^{(+)}$ sperm-treated explants (Fig.12).

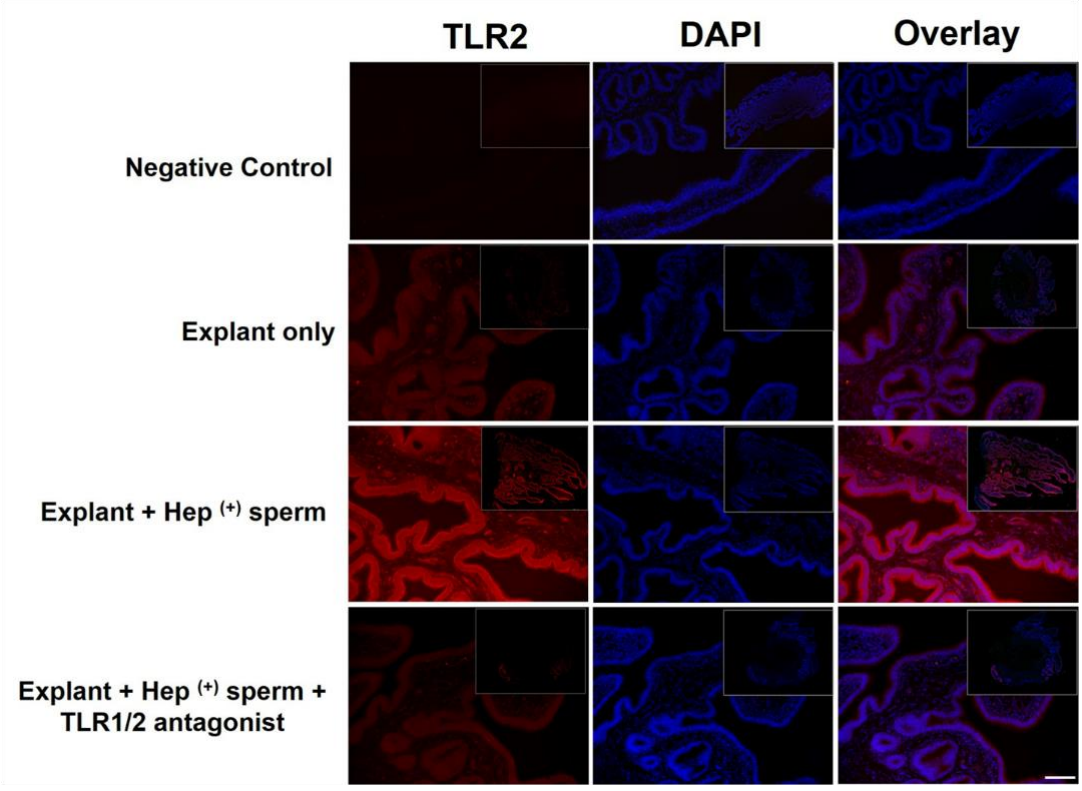

Figure 12. Immunofluorescent detection of TLR2 protein in explants. TLR2 localization in explant after incubation with Hep$^{(+)}$ sperm and TLR1/2 antagonist. Primary antibody for TLR2 was not included in the negative control. Bar = 200 µm.

4. Discussion

The study utilized a differentiated explant model to evaluate the involvement of TLR2 in the immune response of the oviduct ampulla to sperm binding. Blocking the receptor with a specific antagonist resulted in the reduction of sperm binding, explant gene transcription, and TLR2 protein expression in the explant.

It has been discovered that TLR2 is present in various tissues of domestic of domestic animals [116] such as in pigs [117], horses [118] and in cattle [52]. However, it was only recently that the TLRs role in the reproductive process was discovered. In a recent study, it was found that the TLR2 is involved in the modulation of inflammatory response during the sperm-COC interaction at the time of fertilization in mice [75]. It was also found that the TLR2/4 was involved in the inflammatory cascade in the bovine uterus to the presence of sperm [52, 53]. It was also found that the bovine oviduct expresses TLR2 by oviduct epithelial cells during luteal and follicular phases [115].

The oviduct ampulla, being the site of fertilization in the cow [28, 31] and early stage of embryonic development, maintains an environment that promotes sperm survival [29]. It was recently found that cultured BOECs create an anti-inflammatory environment by upregulating genes involved with Th2 response when sperm binds to the cells [115].

Sperm attachment was suppressed by the addition of TLR1/2 antagonist in the system for heparin-treated and non-treated sperm. This is a very interesting finding and maybe related to the observed reduction in the sperm entry to endometrial glands in bovine [49] as both the endometrium and oviduct epithelium were found to express TLR2 [53, 115].

We found that the explant is more sensitive to heparin-treated than non-treated sperm, and that this response is accompanied by upregulation of the mRNA expression for *TLR2*. It is also very interesting that although *TLR2* is upregulated, the over-all response of the oviductal tissue is anti-inflammatory because the mRNA transcript for *TNFA* was not affected but *TGFB1* was upregulated.

Blocking the TLR2 not only effectively reduced the *TLR2* expression, it was accompanied by the reduction in the mRNA transcripts for all genes stimulated by the binding of heparin-treated sperm. Furthermore, even though the oviduct epithelium normally expresses TLR2 [115], the addition of heparin-treated sperm resulted in intense TLR2 protein expression. It is very interesting to observe that

addition of the TLR1/2 antagonist effectively suppressed the TLR2 protein caused by sperm binding to the epithelium.

All data and observations indicate that the TLR2 is involved not only in the bovine sperm-oviduct interaction, but also mediates the immune response of oviductal ampulla to the attachment of sperm. Moreover, TLR2 senses the sperm and the sensing is more sensitive to heparin-treated sperm than non-treated sperm.

General discussion and conclusion

This study developed a differentiated explant model using pre-ovulatory phase bovine oviductal ampulla which provides us with an alternative method to observe the interaction of the sperm with the intact and live oviductal tissue. Imaging of the explant surface using fluorescence microscopy showed us that the explant architecture is preserved and intact throughout the observation period and is very similar with images obtained using SEM. Viability study during the development of the model showed that the explant and sperm remain viable up to 24 h. It was also found that the cells start to undergo apoptosis after 6 h. In order for the immune response to be physiological, all experiments and incubations have been done within 6 h. Due to the limited time for incubation, there are some limitations on the application of the model, because it can only be used for short term incubation studies. Further improvements on the incubation methods is therefore recommended for future research.

Using the model, we found that sperm immediately attaches to the cilia of ampullary epithelial cells. It was also interesting that the sperm cells tend to attach all over the explant surface in a uniform manner. The active ciliary movement also created a gradient which made the sperm orient in a similar direction [4, 41]. The sperm heads are tangentially attached relative to the epithelial cells and sperm tails are beating synchronously [41].

Counting the numbers of attached sperm showed that both heparin-treated and non-treated sperm bound in equal numbers in the explant during 5 min and 15 min observations. It is very interesting that despite the equal numbers attached, the explant is more immunologically sensitive to heparin-treated sperm. The explant epithelium somehow differentiates whether the sperm is capacitated or not and that the response helps in the survival of sperm. The explant promotes a tolerogenic environment for the sperm by generating a primarily anti-inflammatory immune response as seen via the upregulation of *TGFB1* and the non-stimulation of *TNFA*, which is a strong marker for inflammation [53].

The over-all immune response was also accompanied by upregulation of *TLR2*, which made us suspect that the TLR2 has a role in the bull sperm-oviduct interaction. Using a specific TLR2 antagonist to block the receptor, we proceeded with the sperm-explant coincubation. The TLR1/2 antagonist reduced the attachment of heparin-treated and non-treated sperm to the explant. The transcription of heparin-treated sperm-induced genes was also reduced. The mRNA expressions of *TLR2, TGFB1*, and *IL8* were downregulated whereas *TNFA* and *IL10* remained unchanged.

Although the TLR2 protein is continually expressed by the tissue, we wanted to verify whether sperm attachment can stimulate more TLR2 protein expression in the explant. We also wanted to verify whether TLR2 protein expression in the explant is affected by the addition of the TLR1/2 antagonist. The explant TLR2 protein localization was evaluated using immunofluorescence staining. The sperm attachment to the explant epithelium stimulated intense expression of the TLR2 protein in the oviduct epithelium. The TLR1/2 antagonist effectively blocked the sperm-induced increase in TLR2 expression of the explant epithelium compared to the controls. Our findings such as the reduction of sperm attachment to explants, mRNA expression, and TLR2 fluorescence intensity by TLR1/2 antagonist, provides us with evidence that TLR2 in the oviduct ampullary epithelium is involved in the recognition of sperm and the subsequent immune response.

The resulting anti-inflammatory immune response of the oviductal ampulla to capacitated sperm is thought to confer protection to the sperm. Although the time, the sperm spends in the bovine oviductal ampulla is unknown, the complex anatomy of the oviduct allows the sperm to spend enough time in the ampulla to benefit in some sort of protection.

It can be concluded that the differentiated bovine explant model we developed can be used to observe the sperm interaction with the intact and highly complex oviductal tissue. The model provided detailed observations on the dynamics between the sperm and intact ampullary epithelium. Although both heparin-treated and non-treated sperm showed the same affinity for the explant epithelial cells, only the sperm incubated with heparin stimulated an anti-inflammatory or Th2 immune response in the explant which may serve to protect the sperm in the oviduct. The response is accompanied by increased transcription and translation for the TLR2 protein. Blocking the TLR2 allowed us to gather evidence that the TLR2 mediates the immune response of the pre-ovulatory ampullary epithelium to sperm binding. Further studies are indicated in order to clarify the specific mechanisms of the sperm-oviduct interactions and the identification of the ligands that participate in the interaction and subsequent immune response.

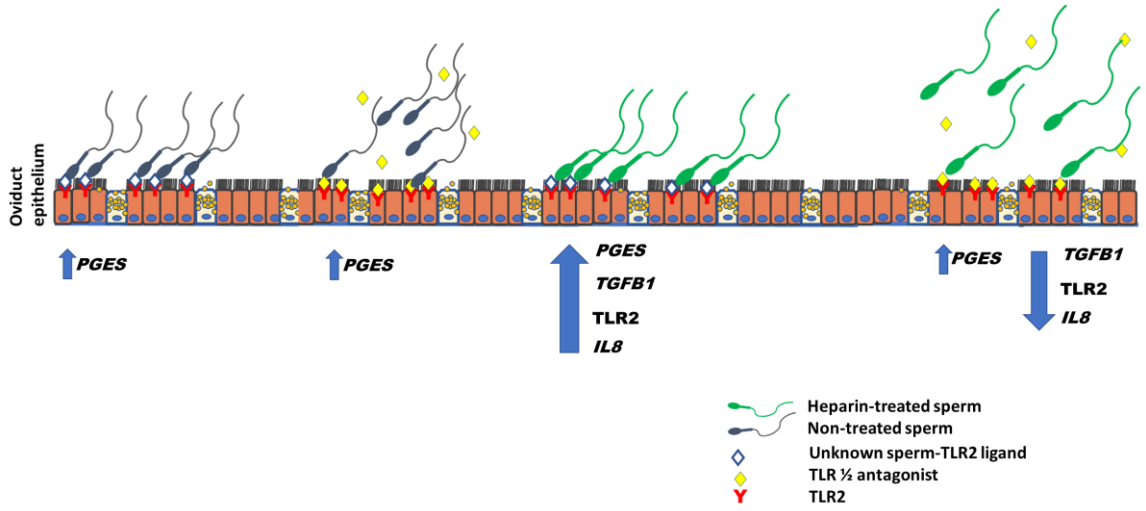

Figure 13. Detailed illustration of the findings of the study. Heparin-treated sperm attaches to the ciliated epithelial cells to trigger the oviductal immune response. The attachment of sperm then stimulates gene transcription of *TGFB1* and other genes which generates a Th2 response. TLR2 mediates the response via an unknown ligand. Blocking the TLR2, prevents the sperm attachment and consequent oviduct ampullary immune response.

Summary

The oviduct is an active and dynamic organ that supports functions critical for reproduction, such as sperm storage, sperm capacitation, fertilization, and early embryonic development [29, 119]. Once sperm reach the oviduct, many attaches to the epithelium that lines the caudal isthmus, forming a sperm reservoir [107, 77, 120, 32]. In the peri-ovulatory period, sperm move up to the ampulla of the oviduct, where fertilization process and the initial several days of embryonic development transpire [41, 37,]. While much has been learned about sperm interaction with the isthmus, there is limited information about the interaction of sperm with the ampulla, despite its role in fertilization.

In chapter 1, a differentiated explant model using the pre-ovulatory phase bovine oviductal ampulla was developed to study in detail the interaction sperm-oviductal ampullary epithelium in bovine. The explants were derived from the primary fold of the oviductal ampulla and is approximately 3mm x 3mm in size. The explant was incubated in PEG-supplemented DMEM under a 38.5˚C incubator with 5% CO_2 in humidified air and was subjected to microscopic observation of morphology under phase contrast and fluorescence microscopy. Hematoxylin and eosin-preparations of the explant was also used for further observation of the complex tissue architecture. The explant is comprised of ciliated and non-ciliated cells. The motile cilia are beating synchronously which caused the explant to move around the media. The explant was incubated with sperm to observe the interaction. Mitochondrial stain JC1 was used to stain the midpiece of sperm for illumination. The sperm attached immediately to ciliated cells with the sperm head bound tightly by the cilia. The sperm bound uniformly all over the explant surface and are tangentially attached to the epithelium. The beating of the ciliated epithelial cells allowed the sperm to orient in similar direction. Viability evaluation showed that sperm and explant optimal condition for incubation is maintained up to 6 h. The results show that the explant model is a suitable model for observation of the interaction between the sperm and the intact oviductal ampullary tissue.

In chapter 2, a detailed study on sperm-oviductal ampulla interaction and its impact on sperm attachment and immune response of the oviductal tissue was carried out using the differentiated explant model comprised of the pre-ovulatory phase bovine oviductal ampulla. Sperm capacitation was induced by incubating the sperm with heparin while non-treated sperm served as the control. Both heparin-treated and non-treated sperm attached in equal numbers to the explant. Despite no detectable difference in the sperm attachment, only the heparin treated sperm stimulated mRNA transcription of *TLR2, TGFB1,*

PGES, and *IL8*, whereas *TNFA and IL10* were not affected. In the non-treated sperm, only *PGES* was upregulated. The results show that the explant epithelium is more immunologically sensitive to the sperm treated with heparin for capacitation. Somehow, heparin allowed modifications on the sperm membrane which facilitated the interaction with the TLR2.

In chapter 3, we used the explant model to study the involvement of TLR2 in the bovine sperm-oviduct ampullary explant interaction and immune response of the explant to the presence of the sperm. The TLR2 was blocked using a specific TLR1/2 antagonist. Afterwards, the sperm attachment to explant, gene transcription, and TLR2 protein expression and localization in the explant were evaluated. Adding the TLR1/2 antagonist in the coincubation system for either types of sperm resulted in a reduction of the attachment at 5 min and 15 mins. The TLR1/2 antagonist also effectively blocked the sperm-induced mRNA expression for *TLR2, TGFB1,* and *IL8.* The attachment of the sperm to the explant also resulted in an intense TLR2 protein expression of the oviductal ampullary epithelium. However, the tissue response to sperm was ablated by the addition of the TLR1/2 antagonist into the coincubation system.

It can be concluded that the differentiated bovine explant model we developed can be used to observe the sperm interaction with the intact and highly complex oviductal tissue. The model provided detailed observations on the dynamics between the sperm and intact ampullary epithelium. Although both heparin-treated and non-treated sperm showed the same affinity for the explant epithelial cells, only the sperm incubated with heparin stimulated an anti-inflammatory immune response in the explant which may serve to protect the sperm in the oviduct. The response is accompanied by increased transcription and translation for the TLR2 protein. Blocking the TLR2 allowed us to gather evidence that the TLR2 mediates the immune response of the pre-ovulatory ampullary epithelium to sperm binding. Further studies are needed to elucidate the detailed mechanism of the sperm-oviduct interactions and the identification of the ligands involved in the interaction and subsequent immune response.

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和 文 要 約

卵管は、精子の貯蔵、精子受精能獲得、受精、初期胚発生など、生殖に不可欠な機能をサポートする活動的で動的な器官である。精子が卵管に到達すると、その多くが卵管峡部を覆う上皮に付着し、精子貯留層を形成する。排卵の時期には精子は卵管膨大部まで移動し、そこで受精し、続く数日間の胚発生が進む。精子と卵管峡部の相互作用については多くのことが知られているが、卵管膨大部が受精の場であるにも関わらず、精子と膨大部との相互作用についてはほとんど知られていない。

第 1 章では、精子と卵管上皮の相互作用を研究するために、排卵前の時期に採取したウシ卵管膨大部を使用した卵管組織片の器官培養モデルを開発した。卵管組織片は卵管膨大部の主要なヒダに由来し、サイズは約 3mm x 3mm であった。卵管組織片は、加湿空気中の 5％CO2 を含む 38.5˚C の炭酸培養器中で PEG を添加した DMEM でインキュベートされ、位相差および蛍光顕微鏡下で形態の顕微鏡観察を行なった。ヘマトキシリンおよびエオシンによる染色で卵管組織片の複雑な組織構造の観察も行なった。卵管組織片は繊毛細胞と非繊毛細胞で構成されていた。運動性の繊毛は同期化して拍動しており、これにより卵管組織片が培養液中を動き回っていた。卵管組織片を精子とインキュベートして相互作用を観察した。活発な精子の中片部を染色するために、ミトコンドリアを染色する JC1 を使用した。精子は頭部から繊毛にすぐに付着した。精子は卵管組織片の表面全体に比較的均一に結合していた。繊毛上皮細胞の鼓動によって、結合した精子が揃って同じ方向に向いていた。生存性の評価により、精子と卵管組織片の培養に最適な状態が 6 時間まで維持されることが確認された。以上の結果から、卵管組織片モデルが精子と自然状態の卵管膨大部組織の間の相互作用の詳細な観察に適したモデルであることを示された。

第 2 章では、第 1 章で確立した卵管組織片モデルを活用して、精子と卵管の相互作用、および精子の付着と卵管組織の免疫応答への影響に関する詳細な観察を行なった。精子をヘパリンとインキュベートすることによって精子受精能獲得を誘導した。未処理の精子を対照として使用した。ヘパリン処理された精子と処理されていない精子の両方が、同数で卵管組織片に付着した。精子の付着数に差異がないにもかかわらず、ヘパリン処理された精子のみが *TLR2*、*TGFB1*、*PGES*、および *IL8* の mRNA 発現を刺激したが、*TNFA* および *IL10* 発現は影響を受けなかった。未処理の精子では、*PGES* 発現のみが刺激された。以上の結果は、卵管組織片の上皮が、受精能獲得のためにヘパリンで処理された精子に対して免疫学的により敏感であることを示している。その詳細は未知であるが、ヘパリンは精子膜構造を改変し、TLR2 との相互作用を促進したと考えられた。

第 3 章では、卵管組織片モデルを使用して、精子–卵管膨大部の相互作用における TLR2 の関与と、精子の存在に対する卵管組織片の免疫応答を検証した。TLR2 は、特異的な TLR1 / 2 アンタゴニストを使用してブロックした。その後、卵管組織片への精子の

付着、遺伝子発現、および TLR2 タンパク質の発現と局在を評価した。ヘパリン処理あるいは未処理の精子の共培養システムに TLR1 / 2 アンタゴニストを添加すると、5 分と 15 分の時点で既に付着が減少した。TLR1 / 2 アンタゴニストは、*TLR2*、*TGFB1*、および *IL8* の精子誘発性 mRNA 発現も効果的にブロックした。卵管組織片への精子の付着は、卵管膨大部上皮での強力な TLR2 タンパク質発現を誘導した。しかし、精子に対するこれら一連の組織の反応は、共培養システムへの TLR1 / 2 アンタゴニストの添加によってブロックされた。

以上の結果から、本研究で開発したウシの卵管膨大部の組織片モデルは、無傷で非常に複雑な卵管組織と精子の相互作用を観察するために効果的に使用できると結論付けることができた。このモデルは、精子と無傷の膨大部上皮の間のダイナミクスに関する詳細な観察を可能にした。ヘパリン処理した精子と未処理の精子の両方が卵管組織片の上皮細胞に対して同じ親和性を示したが、ヘパリン処理した精子だけが、卵管内の精子を保護するのに役立つ卵管組織片の抗炎症性の免疫応答を刺激した。この応答には、TLR2 タンパク質の転写と翻訳の増加が伴った。そして、TLR2 を遮断することで、TLR2 が精子結合に対する排卵前の上皮の免疫応答を仲介するという証拠を示すことができた。精子と卵管の相互作用の詳細なメカニズムと、それに続く免疫反応に関与するリガンドの同定を解明するには、さらなる研究が必要である。