Computational modelling for molecular dynamics of TLR2 that regulates sperm-uterine immune crosstalk in cattle

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ウシの精子–子宮免疫クロストークを調節する TLR2 の分子動力学のコンピューターモデリング

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Abbreviations

AI	Artificial insemination
ART	Assisted reproductive technologies
BEEC	Bovine endometrial epithelial cells
BFE	Binding free energy
BHABP	Biotinylated HA-binding protein
CD44	Cluster of differentiation 44
CL	Corpus luteum
COC	Cumulus cell-oocyte complexes
COM	Center of mass
DAMP	Damage-associated molecular patterns
DMEM	Dulbecco's Modified Eagle Medium
E2	Estrogen
ELISA	Enzyme-linked immunosorbent assay
ET	Embryo transfer
FCS	Fetal calf serum
FRT	Female reproductive tract
HA	Hyaluronan
IF	Immunofluorescence
IL10	Interleukin 10
IL1B	Interleukin 1 beta
IL8	Interleukin 8
INOS	Inducible nitric oxide synthase
IVF	In vitro fertilization
KC	Keratinocyte chemoattractant
LH	Luteinizing hormone
LRR	Leucine-rich repeats

MCP-1	Macrophage chemoattractant protein-1
MD	Molecular dynamics
MIP	Macrophage inflammatory protein
MM/PBSA	Molecular mechanics/Poisson–Boltzmann surface area
MME	Matrix-modifying enzymes
OD	Optical density
P4	Progesterone
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PG	Peptidoglycan
PGES	Prostaglandins E synthesis
PMN	Polymorphonuclear neutrophils
PPI	Protein-protein interaction
PRR	Pattern recognition receptors
PTM	Post-translational modification
qRT-PCR	Quantitative real time polymerase chain reaction
RDF	Radial distribution function
RT	Room Temperature
SEM	Standard error of the mean
Th	T Helper
TIR	Toll/IL-1R
TLR	Toll-like receptor
TM	Template modelling
TNFA(TNF-a)	Tumor necrosis factor alpha

General introduction

Nowadays assisted reproductive technologies (ARTs) such as artificial insemination (AI), in vitro fertilization (IVF) and embryo transfer (ET) have been intensively taken into account to improve fertility. As a matter of fact, agriculture and animal husbandry are still fundamentally required to be significantly developed to satisfy the rapid population growth around the world day by day. On the other hand, beef and dairy products heavily depend on the reproduction of cows. Therefore, ARTs play a pivotal role in human daily life. In spite of huge studies regarding ARTs, the farmers still encounter quite a number of problems including the low-pregnancy rate of AI, IVF, and ET. To tackle the above problem, researching the physiological changes during or after insemination (in particular sperm interaction with the female reproductive tract (FRT)) is essential to uncover the mechanism of FRT preparation for a successful pregnancy and how this process can impact on embryo receptivity. Therefore, obtaining the details of the immune cross-talk of sperm with the uterus at molecular-level could assist in development of ARTs.

Our laboratory, quite recently, explored a series of evidence on sperm-induced inflammation to initiate uterine immunity under the physiological condition. This inflammation is mediated *via* Toll-like receptor 2 (TLR2) pathway. In general, TLR2 signaling mainly depends on its dimerization with TLR1 (TLR2/1) and TLR6 (TLR2/6). Activation of TLR2/1 signaling pathway has been reported to be involved in pro-inflammation compared to pro- and anti-inflammation through TLR2/6.

In addition, Hyaluronan (HA) and its receptor (Cluster of differentiation 44: CD44) have an important role in FRT, in particular inflammation response as a TLR2 regulator. HA is the most abundant polysaccharide of the extracellular matrix of connective tissues in uterus. HA has emerged as an important regulator of inflammation, including TLR2 mediated inflammatory response. HA supposed to be an important regulator for sperm interaction with the bovine uterus.

Recently, *in-silico* approaches are widely used to detect and discover such information that cannot be achieved through experiments, for example protein-protein interaction (PPI), protein-endogenous or protein-exogenous molecules interaction etc. PPI plays a pivotal role in protein dimerization. *In-silico* experiments are usually employed to study TLR dimerization process at the molecular-level in different models. Using *in-silico* approaches, possible parameters involved in dimerization and the impact of other molecules on the process can be detected.

1. Artificial insemination (AI)

Artificial insemination (AI) was the first great biotechnology applied to improve reproduction and genetics of farm animals. In this process, sperm are collected from the superior male, next the sperm are processed and stored in liquid N2 tank until artificially introduced into the FRT. In practice,

inseminators who are well-trained, motivated, and have reasonably large herds tend to achieve good results.

2. Sperm communication with female reproductive tract (FRT)

2.1. Bull sperm

Sperm is known as the male reproductive gamete in anisogamous forms of sexual reproduction. Mammalian as well as bovine species could generate motile sperm consisting of a flagellum (a tail), sperm head and midpiece. Sperm is usually generated through a well-known process named spermatogenesis, which happens in the seminiferous tubules located in the testes. This process contains the construction of numerous sperm cell precursors, which usually start with spermatogonia and differentiate into spermatocytes. After that, the generated spermatocytes undergo meiosis process by decreasing their chromosome number by half (haploid male gamete: spermatid). Next, the spermatids become mature by constructing a tail (flagellum for motile sperm). Sperm are not able to divide and they usually have a limited lifespan. However, after interaction with oocytes during fertilisation, a new organism can be developing and start as an embryo. The bull sperm cell is haploid, therefore these 30 chromosomes can accumulate with the 30 chromosomes of the oocyte to form a diploid cell with 60 paired chromosomes. In mammals, in particular cattle, sperm cell can be stored in the epididymis and released from the penis during ejaculation with the seminal plasma (known as semen).

2.2. Sperm journey in the female reproductive tract (FRT) in cattle

Millions or billions of sperm could be deposited into FRT through natural mating or AI, however a few can arrive at the site of fertilization and only one fertilizes an oocyte. The journey is long, tortuous, and includes movement through viscous fluid, interaction with epithelial cells, avoiding dead ends and hostile immune cells. The successful and healthy sperm could reach an oocyte. The privileged sperm that finish this journey pass selection steps in the vagina, cervix, uterus, utero-tubal junction and oviduct. In many locations in FRT, sperm require to interact with the epithelium and the luminal fluid. This kind of interaction could impact on sperm motility and function. Sperm need be tolerated by the immune system in FRT for an adequate time to allow fertilization to occur.

2.3. Sperm-Uterus Immune Crosstalk

During normal insemination or AI in cattle, millions of sperm (nearly 100 million) are released into the uterus to start fertilization. Although, the majority of these ejaculated sperm are removed through different ways including (a) backflow, (b) degradation, and (c) phagocytosis process by PMNs,

only a few thousands of sperm can reach the oviduct (1). After the insemination, sperm require about 6 or 8 h to pass through uterus and reach oviduct in adequate numbers for interacting oocyte, consequently fertilization (2). Quite recently, the molecular-level detail about the interactions and immune crosstalk between bull sperm and bovine endometrial epithelial cells (BEECs) were intensively investigated by *in-vitro* co-culture models and *ex-vivo* bovine uterine explant culture models (3,4). The results of *in-vitro* models suggested that live sperm could stimulate an inflammatory pathway in BEECs through upregulating the gene expressions of Tumor necrosis factor alpha: TNFA, Interleukin 1 beta: IL1B, Interleukin 8: IL8, and Prostaglandin E synthase: PGES (3) **(Figure 1)**. In addition, blocking TLR2/4 of BEECs prior sperm co-culture considerably suppressed sperm-induced inflammation. These findings clearly indicated that sperm though employing TLR2/4 of BEECs initiate inflammation in bovine uterus (5). In *ex-vivo* models, the experimental findings demonstrated that sperm could glide over the surface of endometrial epithelium, then enter uterine glands, afterwards, inducing the process of uterine inflammatory. Therefore, uterine gland act as a sensor for sperm communication and trigger innate immune responses in bovine uterus (4). After inflammatory response, PMNs are the first cells that rapidly migrate form the blood to the uterine lumen. This rapid migration then relatively short period of PMNs existence in uterine lumen guarantee the removal of excess sperm and bacteria before receiving the embryo and implantation (1,4) **(Figure 1)**.

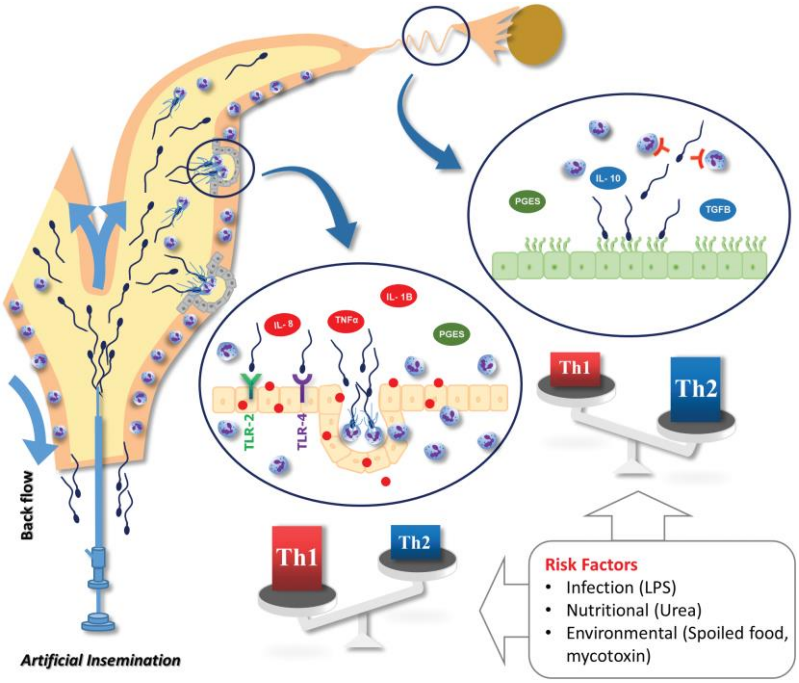

Figure 1. A schematic representation of the impact of bovine sperm interaction with the uterine and oviductal mucosa for physiological changes in the local immunity during the period from insemination until ovulation.

2.4. Sperm-Oviduct Immune Crosstalk

Compared to the uterus, the bovine oviduct has been reported as a safe shelter for sperm from PMNs (1,6–9). In fact, the oviduct is the main place for bull sperm to undergo physical changes, (sperm capacitation) and interact with oocyte (6). A small number of sperm from several hundreds to thousands, which could successfully escape from phagocytosis by PMNs in the uterus, swim towards the oviduct. In this site, sperm must undergo capacitation, followed by hyperactivation and finally meet oocyte for fertilization (1). Quite recently, with regards to sperm–oviduct immune crosstalk, plenty of studies and researches have been conducted to understand and discover the local innate immune system. Basically, it has been indicated that the main anti-inflammatory cytokines, such as Transforming growth factor beta 1: TGFB1 and Interleukin 10: IL10, could be significantly expressed in the bovine oviduct after communication with sperm (6). Such anti-inflammatory (T-Helper 2: Th2) environment should result in ideal environment for sperm and embryo in the oviduct (1). Importantly, as for pro-inflammatory gene expression after oviduct–sperm binding, there is a slight decrease in TNFA, IL1B, IL8, PGES and Th1 gene expression (the main pro-inflammatory markers), which could lead to the immune tolerance in the bovine oviduct (Figure 1) (1). These experimental evidences suggested the bovine oviduct provides an ideal environment (a stable and strong anti-inflammatory) for sperm and embryo under physiological conditions.

3. The role of Toll-like receptor 2 (TLR2) in reproduction

Toll like receptors (TLRs) have been reported to play an essential role in induction of inflammatory responses in the female reproductive tract (FRT) (5,9,10). Previously, it has been indicated that just active sperm can attach to the bovine endometrium to induce a pro-inflammatory response such as TNFA, IL1B, IL8, and PGES gene expression, which interestingly mediated via TLR2 pathway (4–6). In contrast, sperm binding to bovine oviduct cells through TLR2 could induce and release anti-inflammatory cytokines (such as TGFB1 and IL10) (9). Thereby, TLRs, especially TLR2, have an important role in immune-cross talk between FRT and sperm.

TLRs are transmembrane proteins, consisting of three main domains: Extracellular domain or leucine-rich repeats (LRR), Transmembrane domain and Intracellular domain or Toll/IL-1R (TIR) domain. Ligands, which bridge between two TLRs, have a pivotal role in initiating signaling through TLRs, specifically TLR2 and TLR4, by reason of a necessity for two TIR domains in the near each other (11,12). Binding to the bridging ligands, TLR2 can be formed as heterodimerization with TLR1, TLR6. TLR2/TLR6 complex has been more associated to strong pro-inflammatory response (and an

anti-inflammatory response in immune cells) than TLR2/TLR1 related to weak pro-inflammatory response (11–13) **(Figure 2)**.

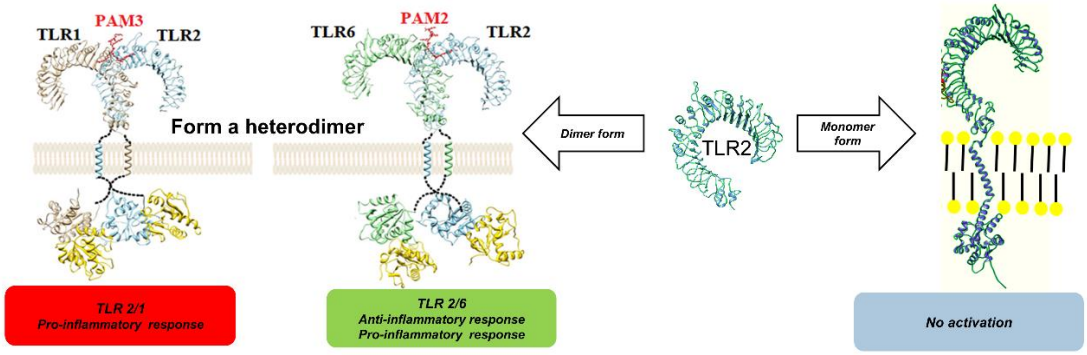

Figure 2. Activation of TLR2 signaling pathway after interaction with bridging ligands and making dimerization.

In fact, inflammation induced by sperm under physiological condition is critical for uterus preparation before embryo implantation. The physiological changes in sperm and their interactions with the endometrium (to induce innate immunity) and cumulus-oocyte-complexes (COCs) (to successful fertilization) are the major early reproductive events which lead to a successful pregnancy (14). Therefore, identifying the molecular mechanism of the sperm-uterine inflammatory signaling that regulated by TLR2 is required for better understanding the different immune responses in the uterus.

4. *In-silico* investigations in physiological system

In-silico modelling is computer models which are developed to investigate physiologic process at molecular-level. The aim is extending controlled in vitro experimentation. It is the normal result of the explosive increase in computing power available to the research scientist at continually decreasing cost. *In-silico* modelling combines the benefits of both *in-vitro* and *in-vivo* investigations, without subjecting itself to the ethical considerations and lack of control associated with *in-vivo* experiments. Unlike isolated *in-vitro* investigations, *in-silico* models help the researcher to include a virtually unlimited wide range of parameters, which render the results more applicable to the organism as a whole. *In-silico* modelling is best known due to its ample use in physiological experimentation (15). Moreover, *in-silico* approaches have been employed to provide information which cannot be obtained practically or ethically for pathophysiological problems (15). These computational experiments have

resulted in the development of important insights in different subject matters ranging from pure physiology to pathology (15).

5. Objective of the study

As TLR2/TLR1 complex has been associated to weak pro-inflammatory response compared to TLR2/TLR6 which, more related to stronger pro-inflammatory response, in this study I hypothesized that sperm through employing TLR2/1 heterodimer could trigger bovine uterus to induce a weak and fast inflammatory response **(Q1: Figure 3)**. In addition, hyaluronan (HA: a TLR2 regulator endogenous ligand) as the rich molecules in uterine lumen can effectively act on sperm-communication with bovine uterus **(Q2: Figure 3)**.

The specific objectives of this study were:

Chapter I: To clarify inflammation response through different TLR2 signaling pathway under physiological condition;

a- To investigate which TLR2 signaling pathways (TLR2/1 or TLR2/6) used by sperm to induce weak inflammation

b- To investigate whether the activation of different TLR2 signaling could lead to the identical inflammation response

Chapter II: To examine the impact of HA (as abundant endogenous ligand in the bovine uterus) on sperm-induced inflammation in bovine uterus;

a- To investigate computationally whether HA can be a potential ligand for TLR2 to activate signaling pathway

b- To research experimentally whether HA can enhance sperm-induced inflammation in the bovine uterus.

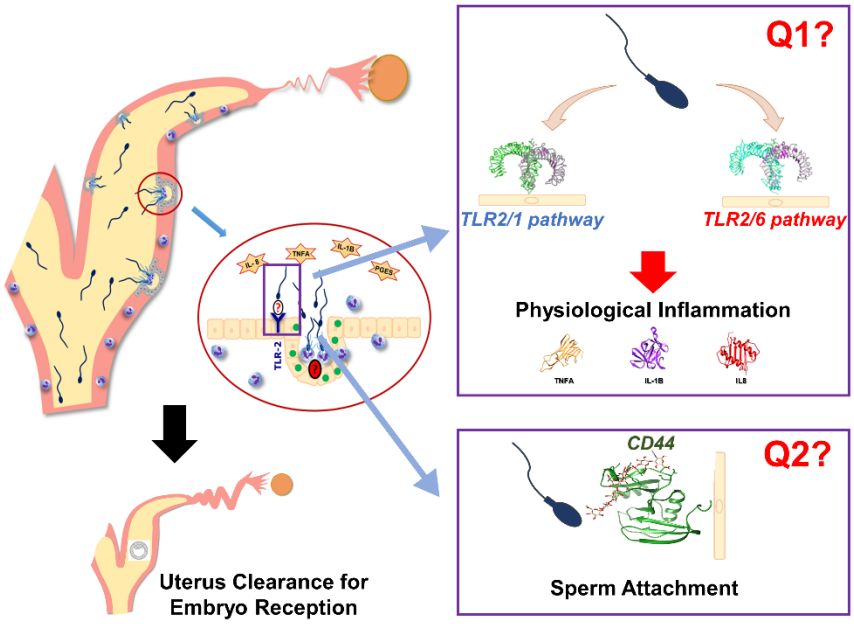

Figure 3. Illustration indicating the hypothesis of this research

Chapter I

Sperm induce TLR2/1 heterodimerization, but not TLR2/6, to trigger the weak proinflammatory response in bovine uterus

1. Introduction

Different Toll-like receptors (TLRs) have been found to be involved in the induction of inflammatory responses in the FRT (1–6,9,16,17). TLR2 play a key role in binding and immune-cross talk between sperm and FRT. Active sperm cells could bind to the bovine endometrium cells through the TLR2 and trigger these cells to induce pro-inflammatory responses (4,5). However, sperm attachment to bovine oviduct epithelial cells is mediated also by TLR2 but leads to an anti-inflammatory response (9). The ovum releasing from the ovary lead to a tightly regulated sterile inflammatory response in bovine oviduct which is rapidly resolved during early corpus luteum (CL) formation (18). Meanwhile, TLR2 is also expressed in cumulus cells of cumulus-oocyte complexes (COCs) and plays immune protective functions critical for cell survival during ovulation and fertilization (17).

Notably, sperm-induced weak inflammation in bovine endometrium has an essential role in uterine clearance prior to accept the embryo (1,14). Despite numerous previous studies, the detailed molecular mechanism of sperm-uterine inflammatory signaling that regulated by TLR2 remains unclear.

Since dimerization is a prerequisite for any TLR2 activation, I hypothesized that the sperm-triggered physiological inflammatory response in bovine endometrium is regulated by different types of TLR2 dimerization (TLR2/1 or TLR2/6), though the structure of TLR2 dimers in bovine is unknown. In-vitro cell cultures using BEECs are considered as the starting point for studying any biological effect in bovine uterus (5). However, this model cannot simulate the anatomical complexity of bovine uterus especially for sperm-uterine interaction (particularly uterine gland). Thus, the *ex-vivo* model using uterine explant has been performed to mimic the *in-vivo* conditions to identify different physiological interactions in bovine uterus (4).

Protein–protein interactions (PPI), including dimerization and protein complex, control all functions of the living cell during physiological and pathological conditions (19). Recently, different in-silico approaches have been used to identify the biological pathways of PPI and highlight possible applications (20,21). TLRs molecular-level responses are extensively studied using computational biology approaches. Hence, *in-silico* model was employed for the first time to investigate dimerization process of TLR2 at molecular-level in bovine model. At first, the binding affinity of TLR2 with TLR1 and TLR6 was evaluated using known crystal structures of both mouse and human. Afterwards, the potential effect of PAM3 and PAM2 (TLR2/1 and TLR2/6 agonists, respectively) on TLR2 dimerization was investigated *via* de novo generated bovine TLRs.

2. Methodology

Study design

In-vitro, *ex-vivo* and *in-silico* investigations were conducted to define the signaling mechanism by which TLR2 is regulated during sperm-uterine immune interactions in non-pregnant cattle (**Figure 4).**

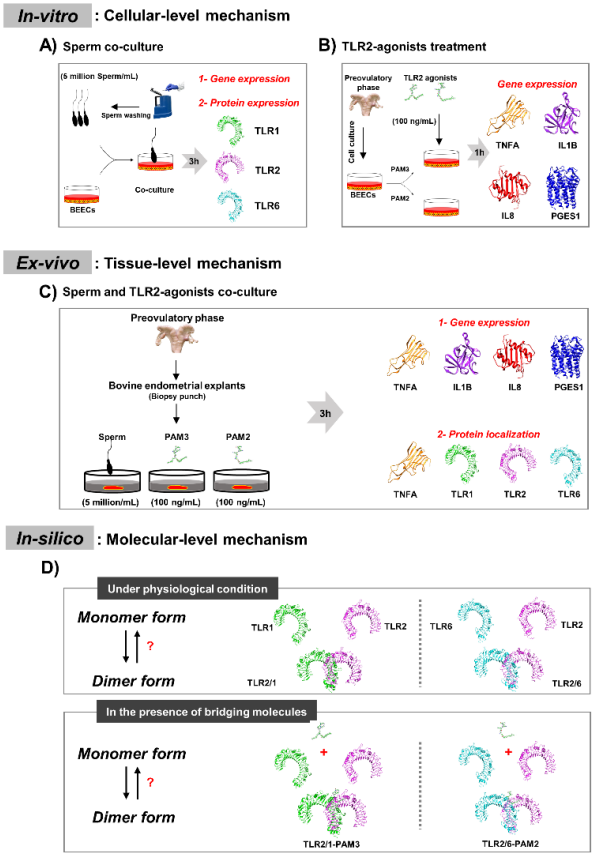

Figure 4. Plan representation of the research design. A and B) *In-vitro* model, C) *Ex-vivo* model and D) *In-silico* model.

2.1. *In-vitro* approaches

Experimental design and *in-vitro* studies

In order to elucidate the heterodimeric form of TLR2 signaling in sperm- induced inflammation in bovine uterus at cellular level, BEECs were co-cultured with sperm (5 million/mL)

for 3h. Furthermore, to investigate the contribution of TLR2 signaling (TLR2/1 and TLR2/6) cascade to uterine inflammation, the following experiments was conducted using different TLR2 agonists. Bovine endometrial epithelial cells (BEECs) were stimulated with PAM3 (TLR2/1 agonist, ab142085, Abcam) and PAM2 (TLR2/6 agonist, InvivoGen, USA) at 100 ng/mL concentration for 1h. The concentration of dose and time point were selected based on our previous reports in which those conditions were investigated in detail (5). In brief, based on dose- and time-dependent investigations (104, 105 and 106 sperm/mL), 5 million/mL of sperm was used to induce the weak physiological inflammation after co-culture with BEECs for 3 h (3). As well, PAM3 (10, 100 and 1000 ng/mL) was applied, and 100 ng/ml was the first to induce the inflammatory response in BEECs at 1h of incubation in the similar level to that of sperm (5). Thus, 100 ng/mL of PAM3 and the same concentration of PAM2, were used in the present study to compare their inflammatory effects with sperm.

BEECs Culture

Initially, macroscopically healthy non-pregnant bovine uteri were carefully observed to be free of inflammation and abnormal color or any pathological lesions in slaughterhouse (Obihiro, Hokkaido, Japan), then collected and directly transferred to the laboratory under sterilized conditions and the uterine horn was used to isolate epithelial cells (5). The isolated cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco, Grand Island, USA) supplemented with 1% amphotericin B, 0.1 % gentamicin (Sigma-Aldrich, Steinheim, Germany), 10 % heat-inactivated fetal calf serum (FCS) (Biowest USA) and 2.2 % $NaHCO_3$ using flask. The culture medium was replaced regularly with new media every 48 h. Upon reaching 70–80 % confluence, the cells were collected with trypsinizing (0.05 % trypsin EDTA; Amresco, Solon, OH, USA), transferred in 24-well and 12-well plates (Nalge Nunc International, Roskilde, Denmark) and cultured up to around 90 % confluence (first passage). Estrogen (E2) and progesterone (P4) were added at preovulatory concentrations in the cell culture media (DMEM/F12, 1% amphotericin B, 0.1 % gentamicin and 5 % FCS) (5).

BEECs co-cultured with sperm

The sub-confluent BEEC monolayers (after first passage) were washed twice with PBS and cultured in a medium supplemented by 0.1 % FCS and gentamicin. The BEECs were co-cultured with 5 million/mL washed sperm, followed by washing cell twice with PBS, lysing with Trizol (Invitrogen, Carlsbad, USA), and storing at −80 °C until RNA extraction. This experiment was repeated seven times using epithelial cells from seven different uteri (n=7). For preparing washed sperm, frozen semen straws (obtained from three Holstein bulls kept in the Genetics Hokkaido Association, Hokkaido, Japan) were thawed at 38.5°C for 30 sec, followed by washing three times at 200g for 10

min using sp-TALP (3,5). The sp-TALP consisted of 99 mM NaCl, 3.1 mM KCl, 25 mM NaHCO3, 0.39 mM NaH2PO4, 10 mM HEPES free acid, 2 mM CaCl2, 1.1 mM MgCl2, 25.4 mM sodium lactate, 0.11 mg/ml sodium pyruvate, 50 µg/ml gentamycin and 6 mg/ml BSA (Sigma-Aldrich, USA) pH 7.4.

Stimulation of BEECs with agonists

The sub-confluent BEEC monolayers (after first passage) were washed twice with Phosphate Buffered Saline (PBS) and cultured in a medium supplemented by 0.1 % FCS and gentamicin. The BEECs were either stimulated by 100 ng/mL PAM3 and PAM2 for 1, 6, 12 h. At the end of BEECs stimulation, cells were washed twice with PBS, lysed with Trizol (Invitrogen, Carlsbad, USA), and stored at −80 °C until RNA extraction. This experiment was repeated seven times using epithelial cells from seven different uteri (n=7).

2.2. *Ex-vivo* approaches

Experimental design

In order to compare the endometrial response toward sperm and TLR2 agonists at the preovulatory phase, an *ex-vivo* model (bovine endometrial explants) was used, due to the advantage of investigating the protein localization in different compartments of the endometrium and the links between whole-animal condition and cellular function.

Sperm and agonist co-incubation with endometrial explants

Bovine endometrial explants were prepared as described previously (4). Briefly, pre-ovulatory bovine uteri were observed to be free of inflammation and abnormal color or any pathological lesions in slaughterhouse (Obihiro, Hokkaido, Japan), then collected and directly transferred to the laboratory for *ex-vivo* investigations under sterilized conditions. Afterwards, using an 8 mm biopsy punch, endometrial explant tissue disks were extracted from the glandular (intercaruncular) endometrial regions. Next, explants disks were placed into a plate with sp-TALP and put in the incubator (38.5°C and 5% CO2) for 15 min (4).

To compare the sperm induced inflammation with agonists, explants were incubated with sperm (5 million sperm/mL) and TLR2 agonists (100 ng/mL) for 3h in the incubator (38.5 0C, 5% CO2, 0.5mL sp-TALP per well in 24-well plate) and at the end of the incubation period the explants were processed for RNA extraction and immunofluorescence analysis. This experiment was repeated five times using explants from five different uteri (n=5).

PCR protocol

Both BEECs and uterine explants were used for RNA extraction, followed by cDNA synthesis and quantitative real-time PCR (4,5). First, to extract total RNA, trizol reagent (Thermo Fisher Scientific) was used, followed by measuring the concentration of RNA using a spectrophotometer (Eppendorf, Munich, Germany), after that the RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at −80 °C until cDNA synthesis step. The cDNA synthesis step was done as previously described (4,5). Quantitative real-time PCR of target genes (TNFA, IL1B, TLR2, TLR1, TLR6, IL8, PGES1 and β-actin, **Table 1**) was carried out by QuantiTect SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) using an iCycler iQ (Bio-Rad Laboratories, Tokyo, Japan) (4,5). The calculated cycle threshold values were normalized using β-actin as an internal housekeeping gene by applying the Delta-Delta comparative threshold method to quantify the fold change between samples.

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

Gene	Primer	Sequence of nucleotide (5′⇒3′)	Accession No.
β-actin	F	TCACCAACTGGGACGACATG	AY141970.1
	R	CGTTGTAGAAGGTGTGGTGCC	
TNFA	F	CAAAAGCATGATCCGGGATG	NM_173966.3
	R	TTCTCGGAGAGCACCTCCTC	
IL1B	F	AATCGAAGAAAGGCCCGTCT	NM_174093.1
	R	ATATCCTGGCCACCTCGAAA	
IL8	F	CCAATGGAAACGAGGTCTGC	NM_173925.2
	R	CCTTCTGCACCCACTTTTCCT	
PGES1	F	AAAATGTACGTGGTGGCCGT	NM_174443.2
	R	CTTCTTCCGCAGCCTCACTT	
TLR2	F	CATGGGTCTGGGCTGTCATC	NM_174197.2
	R	CCTGGTCAGAGGCTCCTTCC	
TLR1	F	ACCCTACTCTGAACCTCAAG	NM_001046504.1
	R	GACTGCACACTGGATTTCTG	

TLR6	F	CTCCGGGAGATAGTCACTTC	NM_001001159.1
	R	GGCCCTGGATTCTATTATGG	

Immunofluorescence (IF) protocol

IF for monolayer cells

At first, monolayer cell was cultured on 24-well plates with 13mm diameter glass coverslips and grow to 90% confluence then co-cultured with 5 million sperm per mL for 3h. Cells were washed with PBS twice and fixed with 2 mL of 4% formaldehyde for 15 min at RT, followed by washing twice with PBS. After that, the cells were permeabilized with 2 mL of 0.1% Triton-X10 in PBS for 15 min on ice, followed by washing three times with PBS. Afterwards, the monolayer cells were blocked using 2mL blocking buffer (5% BSA in PBS) for 1h at RT. The cells were incubated with primary antibodies for TLR1, TLR2 and TLR6 **(Table 2)** in humid chamber at 4ºC overnight. After washing five times with PBS, the cells were incubated with Alexa Flour conjugated secondary antibody **(Table 2)** for 1h at 4ºC. After that, the cells were washed six times with PBS, followed by mounting in VECTASHIELD mounting medium containing DAPI (H-1200, Vector Laboratories, CA, USA).

IF for explant tissue

After the incubation, explants were rinsed in sp-TALP and fixed in 4% paraformaldehyde solution. Then, the fixed tissue samples were dehydrated using ethanol gradient (70, 80, 90, 95 and 100%), cleared in absolute alcohol and xylene, followed by embedding in paraffin and sectioning in 5 µm thick slices. The endometrial sections were deparaffinized and rehydrated through placing on xylene, absolute alcohol and grades series of alcohol, in turn. After that, the tissue sections were blocked with normal goat serum (1:50, S-1000, Vector Laboratories, CA, USA) for 30 min at RT and followed by incubating overnight with primary antibodies for TLR1, TLR2, TLR6 and TNFA **(Table 2)** at 4°C in a humidified chamber. Afterwards, the sections were incubated with Alexa Fluor conjugated secondary antibodies **(Table 2)** for 30 min. Sections were washed, and coverslips were mounted using VECTASHIELD mounting medium containing DAPI. Finally, the sections were observed under fluorescence microscope (BZ-X800, Keyence).

Table 2. List of antibodies used for immunofluorescence (IF)

Target	Antibody	Source (Catalog no.)	Dilution	Working concentration (µg/mL)
TLR1	Rabbit polyclonal anti-TLR1 primary antibody	Biorybt (ORB13722)	× 100	10
TLR2	Rabbit polyclonal anti-TLR2 primary antibody	Biorybt (ORB11487)	× 50	10
TLR6	Rabbit polyclonal anti-TLR6 primary antibody	Biorybt (ORB573911)	× 50	10
TNFA	Mouse monoclonal anti-bovine TNFA primary antibody	BIO-RAD (MCA2334)	× 400	2.5
IgG	Goat anti-rabbit IgG secondary antibody	Invitrogen (A-11035)	× 200	10
IgG	Goat anti-mouse IgG secondary antibody	Invitrogen (A-11029)	× 400	5

2.3. *In-silico* approaches

Preparation of the molecules

In order to investigate TLR2 dimerization under both physiological state and agonist stimulation in a bovine model, an in-silico approach was conducted. To aim that, the TLR2/1-PAM3 (PDB ID: 2Z7X) and TLR2/6-PAM2 (PDB ID: 3A79) were selected for this research. The crystal structures of the TLRs extracellular domain of human (Homo sapiens) and mouse (Mus musculus) species are known (PDB ID of 2Z7X and 3A79, respectively) but have not yet been crystallized in bovine species (Bos taurus). Hence, I carried out Basic Local Alignment Search Tool (BLAST) to calculate the local similarity between bovine TLRs with human and mouse TLRs. It revealed an identity of 77.6 % (Human - Bovine TLR2), 78.5 % (Human - Bovine TLR1), 66.7 % (Mouse - Bovine TLR2) and 72.1 % (Mouse - Bovine TLR6).

Investigating the affinity between TLRs in heterodimer forms

The crystal structure of TLRs in the presence and absence of agonist, and the obtained initial structure from Haddock online server were used to investigate the affinity between TLRs (three repeats). For applying TLRs to the Haddock 2.4 web server, the residues involved in h-bond in TLRs interaction obtained through Ligplot analysis were selected as active residues at the contacting site of TLRs. To obtain the initial structure for MD simulation, cluster 1 was selected as the best docked complex based on the highest HADDOCK score (according to the following formula: Score: 1.0 * Evdw + 0.2 * Eelec + 1.0 * Edesol + 0.1 * Eair). To calculate the binding free energies between TLRs

in heterodimer forms, 150 ns MD simulation were applied to obtain trajectory MD simulation, followed by calculating binding free energy using molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) method (22–24).

The prediction of binding pockets of TLRs in human, mouse and bovine

The possible binding pockets on TLRs were identified through DoGSite Scorer web server, which is a strong tool for investigating potential binding pockets (25). The DoGSite Scorer web server is used for mapping the possible binding pockets based on descriptors calculation (such as depth (A), surface (A2), volume (A3)). Furthermore, the druggability score is estimated through the support vector machine (SVM) method. The score of druggability classified from 0 to 1 while higher values are the potential pockets for the main binding sites. The pocket detection and analysis were performed for TLRs of three mammalian species (crystal structures of human, mouse, and de novo modeling of Bovine TLRs). Bovine TLR protein 3-D structure prediction was carried out after applying amino acid sequences of extracellular domain (Uniprot code: Q95LA9, B5TYW4 and Q704V6 for TLR2, TLR1 and TLR6, respectively) to I-TASSER server (26), followed by optimizing 3-D structures using 100 ns MD simulation.

2.4. Statistical analysis

The statistical analysis was conducted with SPSS® software version 22 (IBM, Armonk, Ny, USA). The data were first tested for normality using Kolmogorov–Smirnov test. A non-parametric Kruskal–Wallis test followed by a Mann–Whitney test were applied for non-normally distributed data of mRNA gene expressions. While One-way analysis of variance (ANOVA) and post hoc Tukey's test were used for normally distributed data obtained from TNFA immunofluorescence analysis. An unpaired two-tailed parametric Student's t-test was performed to evaluate the differences between two unpaired groups. The statistical significance was defined as $P< 0.05$.

3. Result

3.1. TLR2/1 heterodimer employed by sperm to induce inflammation in BEECs

In BEECs, sperm induced TLR2 ($P <0.001$) and TLR1 ($P <0.01$) mRNA expression, but not TLR6 (**Figure 5A)**. Moreover, the immunofluorescence analysis showed similar expression profiles for TLR2 ($P <0.01$) and TLR1 ($P <0.05$) after sperm co-culture with BEECs (**Figure 5B)**.

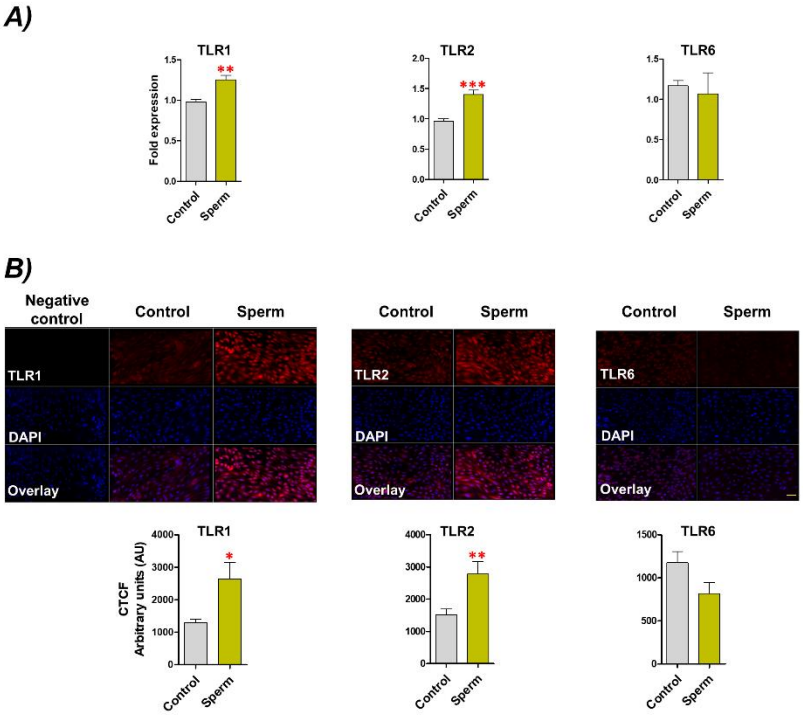

Figure 5. Sperm trigger the bovine uterus to induce TLR2 alongside TLR1 gene (A) and protein (B) expressions.

3.2. The varying degrees of inflammatory reaction following endometrial activation of TLR2/1 and TLR2/6

The PAM3 increased the mRNA expression of TNFA and IL8 ($P < 0.05$) compared to the control. Meanwhile PAM2 significantly increased the mRNA expression of TNFA ($P < 0.01$), IL1B ($P < 0.01$), IL8 ($P < 0.001$), and PGES1 ($P < 0.001$) compared to the control. Compared to PAM3, PAM2 treatment significantly ($P < 0.05$) increased the transcription levels of the pro-inflammatory genes (TNFA, IL1B, IL8, and PGES1). For instance, TNFA, IL1B, and IL8 expressions were roughly 3-fold higher in the PAM2 group than in the PAM3 treatment **(Figure 6)**.

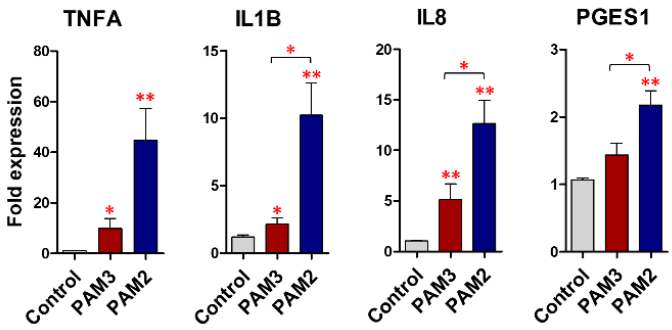

Figure 6. PAM3 and PAM2 induce a weak and strong inflammatory response, respectively, in BEECs at 1 h after stimulation.

Additionally, a time-dependent exposure revealed that PAM2 over PAM3 increased TNFA mRNA expression in BEECs at various time points. (1, 6 and 12h) **(Figure 7)**.

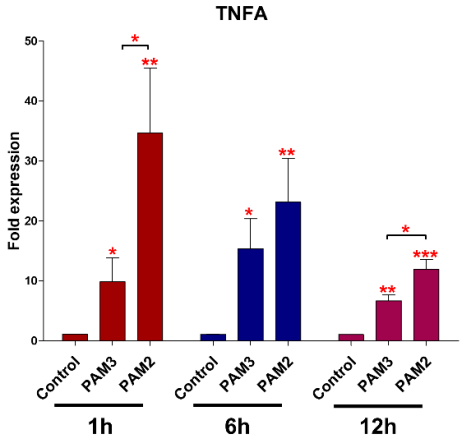

Figure 7. PAM3 and PAM2 induce a weak and strong inflammatory response, respectively, in BEECs at 1, 6 and 12 h after stimulation.

3.3. Sperm induced the TLR2/1 protein expression in endometrial explants

The immunofluorescence analysis revealed that, TLR1, TLR2 and TLR6 is localized in the bovine endometrium, particularly in surface and glandular epithelium. It was obvious that sperm induce the protein expression of TLR2 ($P < 0.01$) alongside TLR1 ($P < 0.05$) in similar manner, in particular in the uterine gland and in the surface epithelium. On the other hand, for TLR6 expression, the intensity was not modulated compared to the control in the uterine gland and surface epithelium after sperm interaction with endometrial epithelia **(Figure 8).**

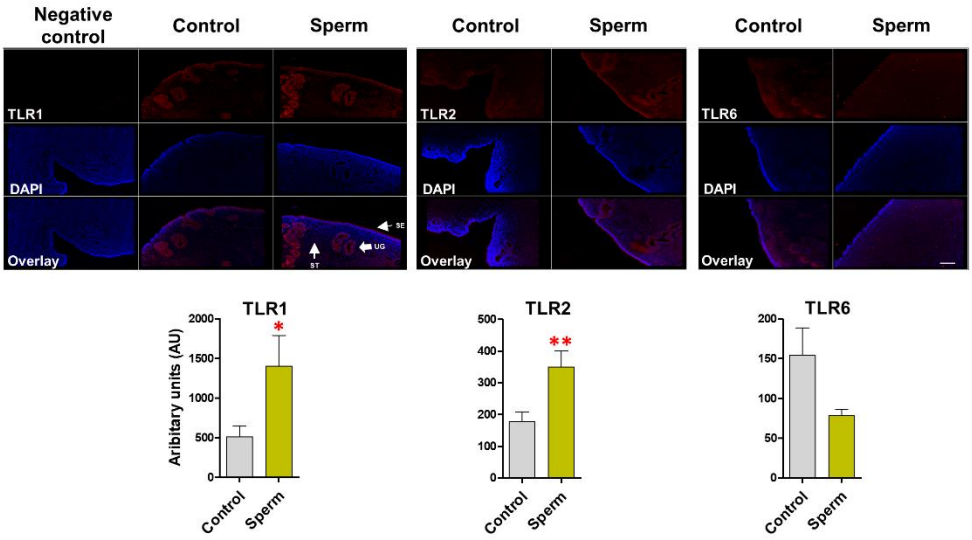

Figure 8. Sperm induce TLR2 and TLR1 (not TLR6) protein expression in bovine endometrial explants.

3.4. Sperm-induced inflammation in bovine endometrium is similar to that of PAM3 (TLR2/1 pathway)

PAM2 induced a higher ($P < 0.05$) mRNA expression of pro-inflammatory cytokines (TNFA, IL1B and IL8) in uterine explant compared to the control and sperm treatments. Of note, there was no significant difference ($P > 0.05$) between sperm and PAM3 groups for mRNA expression of the investigated cytokines (**Figure 9A)**. However, PGES1 gene expression did not show a significant change after stimulating the uterine explant with PAM3, PAM2 or sperm. In the same way, the intensity of TNFA protein expression was highly significant after PAM2 treatment in comparing to sperm and control ($P < 0.05$) particular in uterine gland of the endometrium compared to the control **(Figure 9B)**.

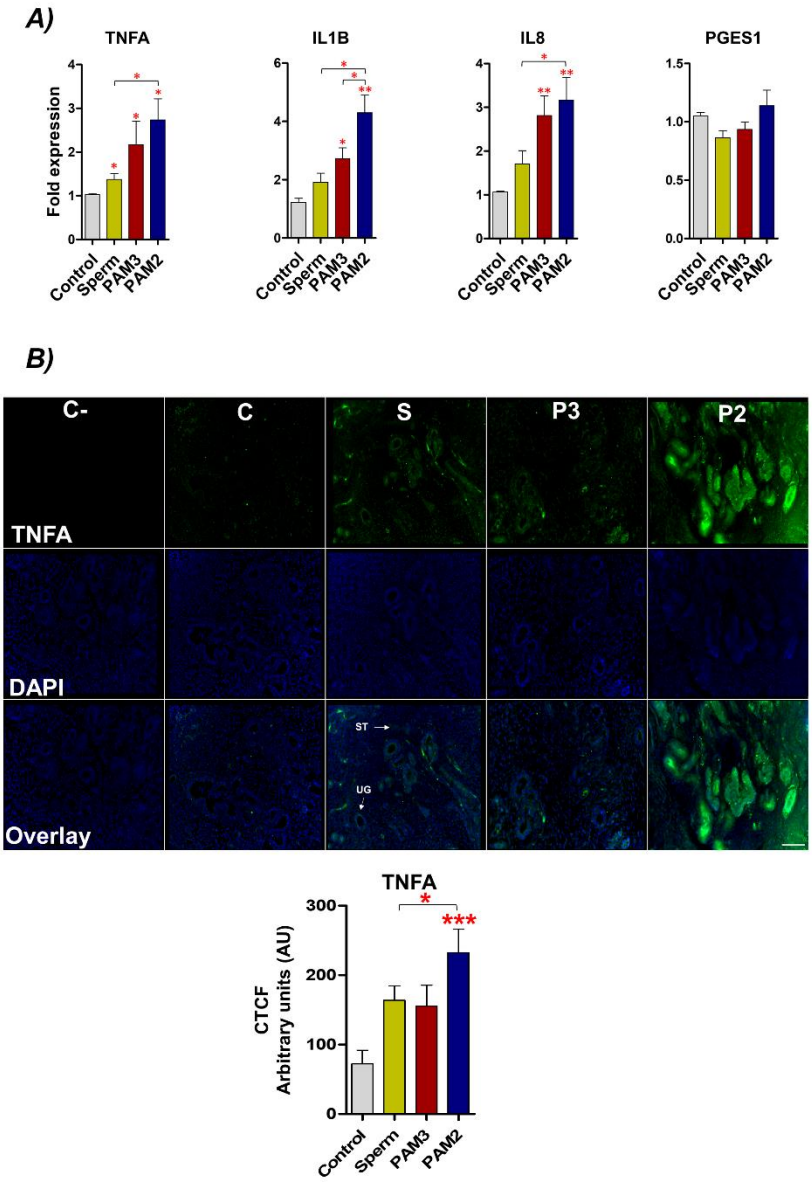

Figure 9. Sperm and PAM3 induce a weaker inflammation in bovine endometrial explants, compared with PAM2. A) Gene expression analysis. B) Protein expression analysis

3.5. Identical TLR2 dimerization process in human, mouse and bovine

In the present study, the in-silico analysis confirmed that TLR2 is not able to interact with TLR1 at cellular level in human and mouse models **(Table 3)**. In contrast, TLR6 has a low affinity with TLR2. My in-silico analyses clearly indicate that the affinity between TLRs is not considerable

to stabilize dimer forms. Thus, the bridging molecules (ligands) are vastly required to stabilize TLR2 dimers **(Table 4)**.

Table 3. Binding free energy (Kcal/mol) calculated by MM/PBSA methods.

PPI	Crystal structure (with agonist)	Crystal structure (without agonists)	Initial structure (by Haddock)	Average	Standard deviation
TLR2-TLR1	104.25	168.8	101.91	124.9	37.9
TLR2-TLR6	-167.04	-198.97	-163.4	-176.47	15.9

Table 4. The result of binding free energy (Kcal/mol) calculated by MM/PBSA methods.

	PAM3	PAM2
TLR2	-57	-64.6
TLR1	-22.8	NA
TLR6	NA	-5.6

The ectodomain of TLRs are split into three subdomains: N-terminal (LRRNT), central and C-terminal (LRRCT) **(Figure 10A)**. The DoGSite Scorer predicted several binding pockets for all TLRs in humans, mouse and bovine **(Figure 10B, C and D)**.

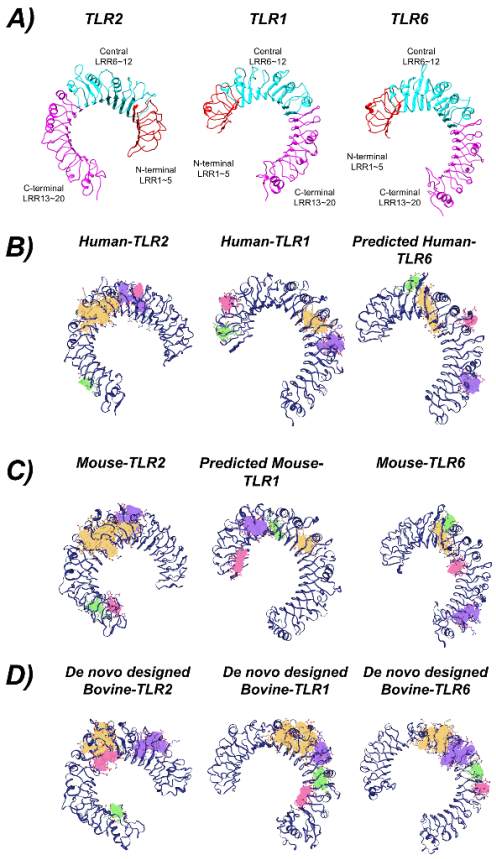

Figure 10. (A) The structure of the subdomains of TLRs. Structural analysis of TLRs in three mammalian classes (B) human, (C) mouse and (D) bovine.

The volume, surface and drugScore of the first predicted binding pockets (yellow pocket) of the all TLR proteins in the three species were indicated in **Table 5**.

Table 5. The physicochemical properties of the first predicted binding site calculated by DoGSite Scorer.

		volume (A^3)	**surface (A^2)**	**drugScore**
TLR1	**Human**	533.93	538.7	0.8
	Mouse	370.52	380.74	0.7
	Bovine	500.76	498.58	0.7
TLR2	**Human**	1803.89	1867.19	0.8
	Mouse	1490.3	1170.56	0.8

	Bovine	1418.18	1434.77	0.8
TLR6	**Human**	496.1	692.31	0.7
	Mouse	460.56	653.29	0.7
	Bovine	1335.24	1671.48	0.8

The druggability score for this pocket in all TLRs is >0.7. Moreover, the physicochemical descriptors showed that TLRs of different species were almost identical **(Figure 10)**. Based on phyciso–chemical properties, the volume (V) of internal pocket was 1803 Å3 (LRR9-12), 1490 Å3 (LRR7-12) and 1418 Å3 (LRR9-12) in human, mouse and bovine TLR2, respectively. Predicting the main binding pocket for TLR1 in the three species showed that the main binding pockets were located between LRR10-12 for human (V~533 Å3) and LRR11-12 for both mouse (V~370 Å3) and bovine (V~500 Å3). The first predicted binding site of bovine TLR1 was almost identical to human and mouse. However, the volume and the surface were slightly different between species for TLR6 in bovine compared with TLR6 in human and mouse **(Table 5)**. **Figure 11** demonstrated that in the main binding site of TLR2 in three species, the type and sequence of amino acids were identical and conserved (in particular, the residue in the entrance of binding pocket).

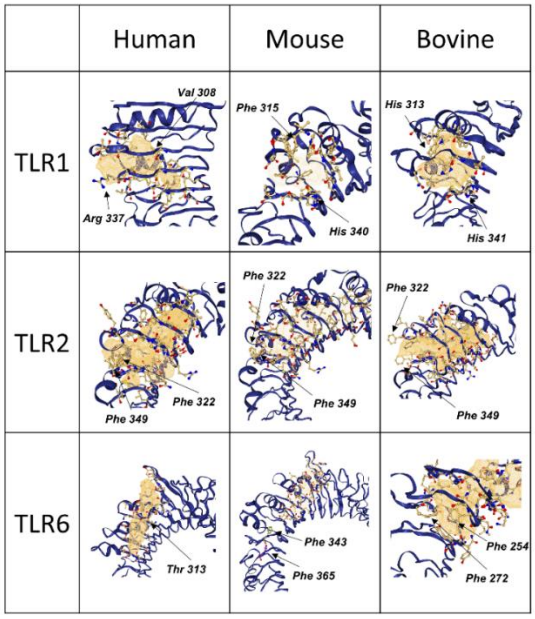

Figure 11. The first predicted binding site of TLRs in three species by DoGSite Scorer.

Looking at the details of the present data, Phe 322 and Phe 349 in three species were selected as the entrance of binding pockets. Additionally, the data obtained from Ligplot analysis indicated that these Phe residues (349 and 322) play an important role in TLRs interaction with agonist and dimerization process (**Figure 11 and 12)**.

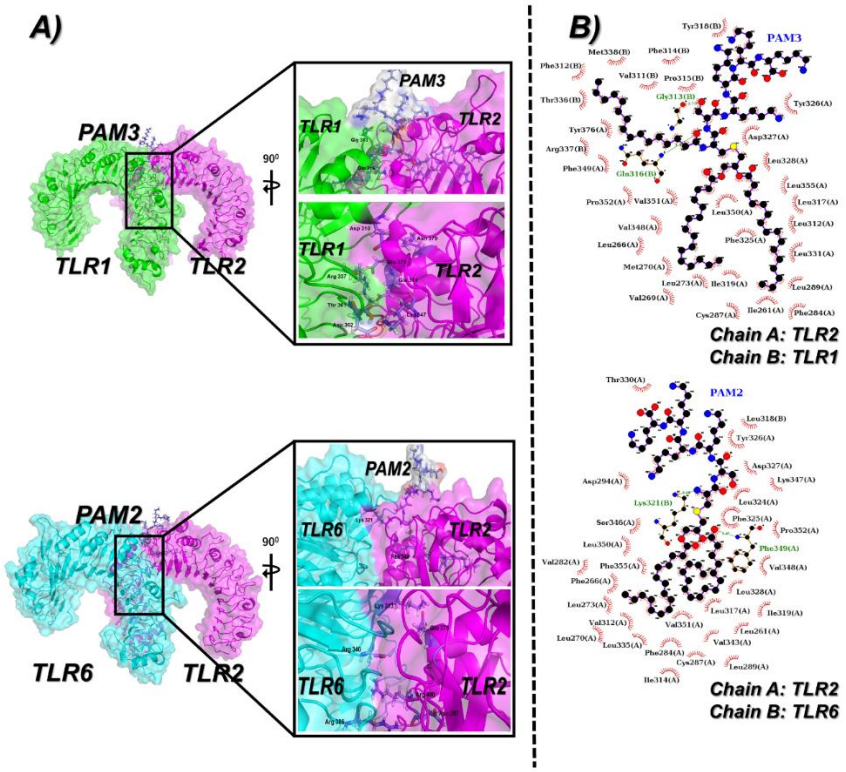

Figure 12. Analyzing the final structure of TLR2/1-PAM3 and TLR2/6-PAM2 complexes after MD simulation. (A) 3-D structure and (B) 2-D structure.

As for TLR1, the predicted main binding pocket for all three species was identical (approximately between Central LRR and c-terminal LRR). With regards to TLR6, in mouse experimentally crystal structure, the Phe 343 and Phe 365 block the internal channel of TLR6, compared with TLR1, consequently, this channel cannot be recognized as main binding site. This structural analysis supposed that the main binding sites of TLRs were similar and located in same place for the studied mammalian species **(Figure 10)**.

4. Discussion

In this chapter, using a combination of experimental and in-silico modeling, I was able to demonstrate that the sperm-induced inflammatory response activates TLR2/1 heterodimer, but not TLR2/6 in bovine endometrium. Importantly, I revealed that sperm could induce a weak inflammatory response in bovine endometrium through ‘‘PAM3-like-weaker’’ TLR2/1 signalling rather than “PAM2-like-stronger’’ TLR2/6 pathway.

In fact, the TLR2 has an essential role in balancing pro- and anti-inflammatory immune responses in different cell types (5,9,13,17,27). We previously reported that sperm induce transient and weak inflammatory response via the regulation of TLR2 signaling in bovine endometrium. (5). On the other hand, heterodimerization of TLR2 with TLR1 or TLR6 has been extensively studied to develop a deep understanding of different immune responses resulted from TLR2 activation. With selective TLR2 heterodimerization, it was important to determine the active TLR2 heterodimer which is involved in sperm-induced inflammation in bovine uterus. Thus, in this study, at first, in-vitro BEECs co-culture was employed to assess the expression of TLR1, TLR2 and TLR6 by sperm. Notably, sperm upregulated the mRNA and protein expression of TLR1 and TLR2, but not TLR6 in BEECs, suggesting that sperm utilize TLR2/1 during sperm-uterine interactions. Moreover, the present data showed that the activation of TLR2/6 signaling pathway could lead to a stronger inflammation compared to TLR2/1 in uterine epithelial monolayer using specific agonists. Similarly, Murgueitio et al. (28) indicated that activating TLR2/6 signaling pathway resulted in 4-fold stronger inflammation than TLR2/1 in human embryonic kidney cells. To get the same level of inflammation in this kind of cells, they used 50 ng/mL of PAM2 versus 200 ng/mL PAM3 for 5h (28). In my in-vitro model, identical concentration from both PAMs (100 ng/mL) was applied to induce inflammation in bovine endometrial epithelia and interestingly, PAM2 induced a 3-fold higher inflammatory response compared to PAM3 and sperm. Altogether, in-vitro studies provided the initial evidence that sperm employ TLR2/1 signaling and activation of TLR2/1 signaling pathway led to a weak inflammatory response in BEECs.

Further, ex-vivo experimental model (i.e., preovulatory endometrial explants that physiologically mimic the intact uterine condition at insemination) was used to investigate the localization of TLR1, 2 and 6 in endometrium and to compare the inflammatory intensities of sperm and TLR2 agonists. We previously reported that sperm interact with the uterine glands to induce an acute and weak inflammatory response. This inflammatory response has been detected by the upregulation of key inflammatory markers TNFA, IL1B, IL8 and PGES as well as by the recruitment of inflammatory cells (i.e., neutrophils) (4,16). Notably, in cattle, sperm trigger the inflammatory cascade primarily via

the TLR2 signaling of uterine glands (4,16). The present ex-vivo protein expression data showed similar upregulation profiles for TLR2 and TLR1, after sperm co-culture with uterine explants. Even though, TLR1, TLR2 and TLR6 localized in surface epithelium and uterine glands, sperm enhanced the expression of TLR1 and TLR2 but not TLR6 in these compartments, which strongly support a co-regulation of TLR2 and TLR1 receptors in particular in uterine glands during sperm-induced inflammatory response. Herein, TLR6 was not modulated after sperm exposure, possibly to prevent strong and long-term inflammatory reactions and tissue damage as high TLR6 that may correlate with higher-risk disease (29). Prominently, the mRNA and TNFA protein profiles of ex-vivo model clearly showed that sperm trigger a weak physiological inflammatory response in endometrial epithelia similar to that of the specific ligand of TLR2/1 heterodimer (PAM3). TNFA is one of the key inflammatory markers in the bovine uterus towards sperm in both physiological and pathological conditions (4,16). Meanwhile, activation of TLR2/6 heterodimers led to a stronger inflammatory response in endometrial explants which assumed to be far from physiological sperm-triggered inflammation. Thus, these ex-vivo evidence reveal that, sperm employ TLR2/1 heterodimerization, in particular in uterine glands of the bovine uterus, to activate the specific inflammatory cascade. In fact, this kind of weak and transient inflammation is required to remove excess and dead sperm remaining in the uterine lumen and to complete this clearance within several hours without tissue damage for providing the ideal uterine environment for acceptance of early embryo and implantation.

Quite recently, Kar et al. (30) predicted the 3-D structure of bovine TLR2 as a reliable computational tool. However, the structures of bovine TLR1 and TLR6 are unavailable. Hence, in the present study, the binding affinity of TLR2 with TLR1 and TLR6 was evaluated using known crystal structures of both mouse and human. The in-silico models showed the evidence for the stabilized interactions between TLR2/1 and TLR2/6 heterodimers in presence of their agonists. Notably, the present study provided the first insight for bovine TLR2 heterodimerization at molecular level. Furthermore, the data revealed that the main binding sites of bovine TLRs were identical to human and mouse and occupied by their specific ligands. Therefore, in complementary with the experimental investigations, it could be concluded that TLR2/1 dimerization occurs in bovine uterus in the presence of sperm. However, the sperm surface molecule/(s) which could initiate and/or regulate this TLR2/1 dimerization process for the specific inflammatory cascade remained to be investigated.

My results revealed that sperm utilize TLR2/1 pathway to induce a weak inflammatory response in bovine endometrium. Activation of TLR2/6 heterodimer could lead to an excessive inflammatory response compared to that of sperm or TLR2/1 **(Figure 13)**. Further, in-silico findings revealed that the ectodomains of bovine TLR2 formed a hetero-dimer upon ligand binding to initiate cell signaling pathway. Altogether, my data strongly suggested that sperm utilize TLR2/1, but not TLR2/6, heterodimerization to induce the weak physiological inflammatory responses in the bovine uterus.

However, further investigations are required to define the specific ligand(s) on the sperm cell membrane that is required to bridge and stabilize the TLR2/1 heterodimer.

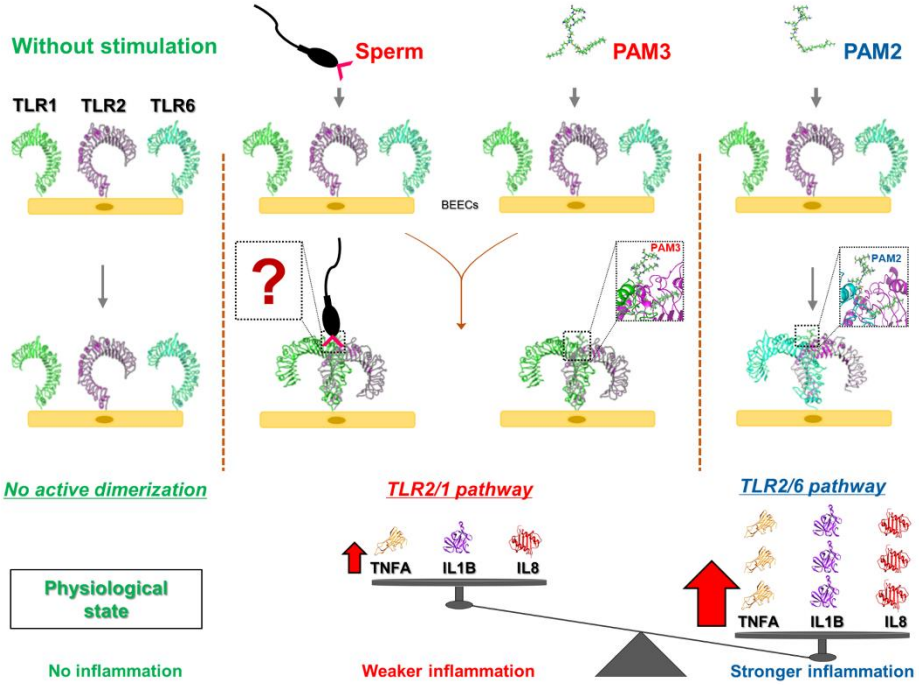

Figure 13. A graphic demo of my working hypothesis in chapter 1.

Chapter II

Impact of hyaluronan (HA) and its receptor (CD44) on regulating the TLR2-mediated inflammation toward sperm in bovine uterus

1. Introduction

Previously, our group has indicated that sperm could interact with BEECs and consequently induce a weak pro-inflammatory response through TLR2 signaling pathway, (in vitro- (5) and ex vivo (4) investigations). However, which molecule(s) linking sperm to TLR2 pathway in BEECs is still unclear.

Different exogenous or endogenous molecules have been reported as ligands and/or regulator for TLR2 signaling (31); one of these molecules is hyaluronan (HA) (32). HA, a non-sulphated glycosaminoglycan, is normally present in most of mammalian tissues and fluids including those of reproductive system (33). Definitely, HA is secreted in the seminal-, oviductal-, uterine-, and cervical fluids of different species including humans (34–37). As well, HA is localized to both granulosa- and cumulus cell layers of the ovarian follicles (38,39). So far, HA has gained a special relevance in the field of reproductive biology due to its participation in numerous physiological events such as ovulation, fertilization, and cervical ripening prior to labor induction (17,40). In the last few decades, several reports have linked HA to TLRs activation with a consequent initiation of a pro-inflammatory cascade in a variety of cell types including endothelial cells, epithelial cells, fibroblasts, dendritic cells, as well as macrophages (41,42). In regard to sperm physiology, it has been shown that HA fragments, produced by sperm-released hyaluronidase, activate TLR2/4 signaling pathway with subsequent cytokine/chemokine production in the cumulus cells of cumulus-oocyte complexes (COCs) which is essential for accomplishment of fertilization process (17,40).

Cluster of differentiation 44 (CD44), a transmembrane protein with multiple isoforms due to its frequent alternative splicing and post-translational modifications, is a major HA receptor (43). CD44 proteins, a class of cell surface adhesion molecules, are ubiquitously expressed throughout the body. Other than its well-known function in facilitating cell adhesion and migration, CD44 can mediate numerous cellular pathways via recruitment and assembly of signaling proteins. Further, CD44 with its ligand HA are involved, either via cell-cell interaction or cell-matrix interaction, in promotion of inflammatory process. Ligation of CD44 to HA is crucial for leukocytic infiltration, T-cell-proliferation and activation, as well as cytokines and chemokines production (41).

Lately, our group have shown that sperm upregulate the gene and protein expression of CD44 adhesion molecule, in both BEECs- and uterine explant models, in the course of sperm-induced uterine inflammation in bovine (16). Altogether, HA could be a good candidate to act as a bridging ligand between the sperm cells from one side and CD44 of BEECs from the other side for regulating sperm attachment, and TLR2-mediated inflammation induced by the sperm. To test the above hypothesis, I first performed in-silico approaches to detect the binding affinity of HA molecules for CD44 and TLR2.

Then, I determined the existence of HA in the uterine environment via immunofluorescence and enzyme-linked immunosorbent assay (ELISA). Further, sperm-BEECs in-vitro co-culture model was applied to investigate the impact of BEECs pretreatment with exogenous HA on sperm attachment and subsequent immune response.

2. Material and methods

2.1. *In-silico* investigations

2.1.1. Phase I: Preparation of CD44, TLR2 and HA molecules

For conducting the *in-silico* investigations, human HA-binding proteins [i.e., CD44 (PDB ID: 1UUH) and TLR2 glycoprotein (PDB ID: 2Z7X)] were applied; the sequence identity between human- and bovine HA binding proteins is 92.5 and 72.27 for CD44 antigen and TLR2 extracellular domain, respectively. Additionally, template-based modeling (44) showed that both proteins in human and bovine species have similar folding, as the template modelling (TM) scores of 0.8 and 0.86 were obtained for TLR2 and CD44, respectively (**Figure 14**).

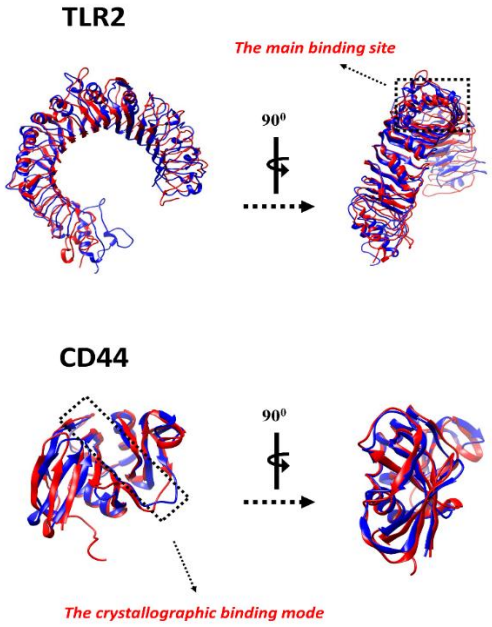

Figure 14. The structural alignment of bovine (blue) and human (red) proteins

The three-dimensional (3-D) structure of HA with 4-, 8- and 32-mers were generated as previously described (45). For optimizing of all the above molecules, molecular dynamics (MD) simulations were operated (22–24,46).

2.1.2. Phase II: Docking simulation of HA to CD44 and TLR2

The optimized HA4, HA8 and HA32 structures, obtained from phase I, were used for docking studies by AutoDock VINA (v.1.2.0) (47). In this study, HA structures without any routable bond were considered to be ligands whereas the binding proteins as rigid entities were considered to be receptors. For docking calculation, the main binding sites of both proteins were selected as the grid box. To compare the interaction of HA molecules to the main binding site of TLR2, the complex of TLR2/1-Pam3CSK4 (PDB ID: 2Z7X) was used to perform MD simulation for 150 ns. PamCSK4 (PAM3) is a synthetic triacylated lipopeptide (LP) and specific agonist for TLR2 which mimics the acylated amino terminus of bacterial LPs. The ligand orientation of HA to the main binding site of receptor with the lowest binding energy were selected for further analysis and MD simulation.

2.1.3. Phase III: Molecular dynamics (MD) simulation of binding proteins/HA complexes

To study the affinity of HA to CD44 and TLR2, 150 ns MD simulation were used to get trajectory, followed by calculating binding free energy using molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) method (23). In addition, radial distribution function (RDF) and center of mass (COM) were calculated during MD simulation to define the atomic interaction between molecules (HA-binding proteins) (48).

2.2. *In-vitro* investigations

Uterine samplings

The uterine samples used for immunostaining as well as isolation and culture of BEECs were brought from a local slaughterhouse (Hokkaido Livestock, Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan). Simply, the bovine uterine horns (contra-lateral to mature follicle) were carefully opened and grossly examined to be free from any abnormalities. Only healthy uterine horns (ipsi-lateral to mature follicle) from the pre-ovulatory phase (Days 19-22) were collected, immersed in physiological saline with antibiotics [1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) and 1% amphotericin B (Gibco)], and then transported to the laboratory within 1-1.5 h on ice. Of note, the phase of estrous cycle was determined on the basis of corpus luteum appearance, size, and color as well as the follicular diameter

Immunostaining of HA

The endometrial tissue explant for immunofluorescence were prepared as previously described (4). In brief, the endometrial tissue explants were dissected from the glandular (intercaruncular) endometrial regions. Then, the explants were fixed in formalin, embedded in paraffin, and 4 µm thick endometrial sections were prepared. HA localization to the bovine endometrium was

determined by immunofluorescence staining using biotinylated HA-binding protein (bHABP) according to (49) protocols with minor modifications. Briefly, the endometrial sections were incubated with 1% BSA for 30 min at room temperature (RT) to block the non-specific binding sites. Afterwards, the sections were incubated with bHABP (5 µg/mL, #385911, Calibiochem) overnight at 4 ºC followed by labeling with Streptavidin-Alexa Fluor conjugate (2 µg/ml, #S11223, Thermo Fisher Scientific) for 1h at RT. The sections were then mounted using VECTASHIELD mounting medium with DAPI (H-1200, Vector Laboratories). Negative controls were performed by pre-digesting sections with hyaluronidase (#H3506, Sigma Aldrich) to ensure the specificity of the reaction. The fluorescence signal was captured using an all-in-one fluorescence microscope (Keyence, BZ-X800) using BZ-X GFP (OP-87763)-, and BZ-X DAPI (OP-87762) filters set for the green- and blue wavelengths, respectively. Exposure time was constant for all sections including negative control. The experiment was repeated three times using endometrial explants from three different uteri.

BEECs isolation and culture

Following the previously described protocols (5) with minor modification, BEECs were isolated (from the obtained uterine horns) and then cultured. In brief, the epithelial cells were detached and suspended in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) (Gibco) supplemented with 22 mM NaHCO3 (Sigma-Aldrich, St. Louis, MO, USA), 0.1% gentamicin (Sigma-Aldrich), 1% amphotericin B (Gibco), and 10% heat-inactivated fetal calf serum (FCS) (Bio Whittaker, Walkersville, MD, USA). Then, cells were seeded in 25 cm2 culture flasks (Nalge Nunc International, Roskilde, Denmark), and cultured at 38.5˚C in a humidified atmosphere of 5% CO2 in air. Upon reaching 70-80% (sub-confluence), the cells were passaged, trypsinized, and re-seeded (at 1 x 105 cells/ml) in 1.5 ml/well culture medium (DMEM/F12, 22 mM NaHCO3, 0.1% gentamicin, 1% amphotericin, and 5% FCS) in 12 well plates (Nalge Nunc International) until sub-confluence. These plated cells were exposed to estradiol-17β (E2; 50 pg/ml) and progesterone (P4; 1 ng/ml) throughout the whole culture period to simulate the pre-ovulatory phase in situ (5). The purity of epithelial cells in my model (>98%) was ensured via immunofluorescence labelling with a monoclonal antibody against cytokeratin (anti-cytokeratin 8+18; ab53280, Abcam, Tokyo, Japan)

Sperm preparation

For getting sperm, frozen 0.5 ml semen straws were obtained from three highly fertile Holstein bulls belonging to Genetics Hokkaido Association, Hokkaido, Japan. Frozen semen straws from three bulls were thawed in a water bath at 38.5°C for 30 sec, pooled, and washed 3 times in a Tyrode's albumin, lactate, and pyruvate medium (Sp-TALP). The progressive motility of the

recovered sperm, as assessed by visual examination using a light microscope equipped with a stage warmer, was around 50%.

Co-culture of BEECs with sperm

Sub-confluent BEECs monolayers in 12-well plates were incubated in 1 ml/well culture medium (DMEM/F12, 22 mM NaHCO3, 0.1% gentamicin, 1% amphotericin, 0.1% FCS as well as E2 and P4 at the above-mentioned concentrations) supplemented with LMW HA (Select-HATM Hyaluronan; mol wt 25-75 kDa, #S0326, Sigma) at different concentrations (0, 0.1, 1, or 10 µg/mL) for 2 h followed by the co-culture without- or with non-capacitated washed sperm (106/ml) for additional 3 h (3,5). This experiment was repeated five times using epithelial cells from five different uteri.

HA levels Quantification

At the end of co-culture period, BEECs-conditioned media were collected, centrifuged twice at 1000 g for 10 min at 4 ºC and kept at -80 ºC for HA quantification. Commercially available HA ELISA kit (DHYALO, R&D Systems, Minneapolis, MN) was used for determination of HA concentrations in BEECs-conditioned media. A 50 µL aliquot of each sample was analyzed according to the manufacturer's instructions. All samples were run in duplicate. Optical density (OD) readings were performed at 450 nm. Control group was run to determine the baseline concentration of HA in DMEM culture media. This experiment was repeated four times using epithelial cells from four different uteri.

Determination of the number of attached sperm

To determine the number of attached sperm, BEECs monolayers in 12-well plates (1 ml/well culture medium without- or with HA) were exposed to washed sperm (106/ml) for 30 min followed by video capturing using a light microscope (at 200 x magnification) equipped with a stage warmer and digital camera connected to EOS utility software® (Canon U.S.A., Inc.); the focus was adjusted during video capturing to visualize all attached sperm. Five random fields were captured per each group. Of note, videos of the different groups within the same experiments were captured under the same field area and video setting (4).

On the other hand, to determine the number of sperm remained attached, BEECs monolayers in 12-well plates (2 ml/well culture medium without- or with HA) were exposed to 106 sperm/well for 3 h. Afterwards, the upper 1.5 ml media were very gently aspirated, centrifuged at 1000 x g, and the pellet was then re-suspended and counted independently by two investigators using a

hemocytometer. To calculate the number of sperm remained attached to BEECs, I subtracted the number of detached sperm (dead and/or floating) from the total number of sperm (106/well) (3).

PCR protocol

At the end of sperm-BEECs co-culture, RNA was extracted from BEECs using Trizol reagent (Invitrogen, Carlsbad, CA, USA), quantified by means of a NanoDrop Spectrophotometer 2000c (Thermo Scientific, Waltham, MA, USA), and then pure RNA samples (i.e., A260/A280 ratio were between 1.8 and 2.0) were kept in RNA storage solution (Ambion, Austin, TX, USA) at -80°C till cDNA synthesis (4,5). The synthesis of cDNA was performed as previously decribed (4,5) with minor modifications. First, the extracted RNA was subjected to a DNase treatment step using RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) to remove residual genomic DNA as well as other contaminants. At such step, 1 µg of extracted RNA was incubated with the first mixure [1 µl of RQ1 RNase-free DNase 10X Reaction Buffer, 2 µl of RQ1 RNase-free DNase (1 unit/ µl), and Nuclease-free water (Invitrogen, Carlsbad, CA, USA) to a total volume of 10 µl] for 30 min at 37°C in a thermal cycler (Eppendorf, Hamburg, Germany), then 1 µl of the RQ1 DNase Stop solution was added for 10 min at 65°C to stop this reaction. Afterwards, the first-strand cDNA was produced via SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer instructions. In brief, the DNase-treated RNA was incubated with the second mixture [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 total volume of 18 µl] at 65°C for 5 min. Then, the third mixture [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 incubated at 42°C for 2 min. Finally, 0.2 µl of 200 units/µl SuperScript II Reverse Transcriptase was added 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. The transcriptional levels of TLR2, Tumor necrosis factor (TNF) alpha (TNFA), Interleukin (IL)-1 beta (IL-1B), IL-8, and Prostaglandin E synthase (PGES) were detected via a quantitative real-time polymerase chain reaction (PCR) by means of an iQ5 real-time PCR detection system (Bio-Rad Laboratories, Tokyo, Japan). To clarify, a total 10 µl reaction mix [i.e., 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 (TNFA, IL1B, TLR2, IL8, PGES1 and β-actin, **Table 1**) , and 2.8 µl nuclease-free water (Invitrogen)] was run in amplification program with an initial denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 51°C for 30 sec, extension at 72°C for 20 sec. A negative control (reactions containing nuclease-free water or non-reverse transcribed RNA) were involved in each run. It should be emphasized that I here used Primer Express Software v3.0.1 (Thermo Scientific) to design the used

primers' pairs. The calculated cycle threshold (Ct) values were normalized against ACTB (β-actin); no significant variances were detected in β-actin mRNA expression among the different treatments. The delta-delta Ct (2–ΔΔCt) method was applied to estimate the fold change between the different samples (4,5).

2.3. Statistical analysis

Each experiment was repeated at least three times using epithelial cells from 3–4 different uteri. In each uterus, 3 replicates were performed (3 wells per treatment per experiment) and data are presented as mean ± standard error of the mean (SEM). Student's t-test was applied to compare the data between two groups, while one-way ANOVA followed by Tukey's multiple comparisons test was used for more than two groups. The results were considered statistically significant at $P< 0.05$ and $P< 0.0001$.

3. Results

3.1. *In-silico* investigations

Docking simulations were performed so as to determine initial structure of HA-receptors complex prior to MD simulations and MM/PBSA analysis. After docking completion, the first three binding orientations of HA with proteins were detected. HA witnessed a potential interaction with CD44 through crystallographic mode. Although, HA could not interact with the internal pocket of TLR2 (TLR2-PAM3 interaction).

All HA polymers (HA4, HA8 and HA32) could not stay in the vicinity of the main binding site of TLR2 during MD simulation **(Figure 15)**.

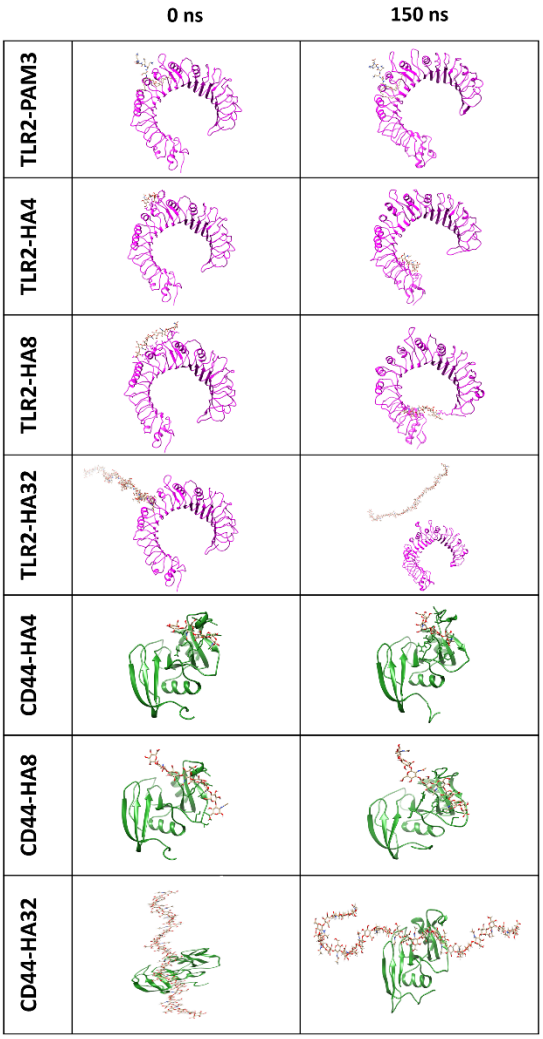

Figure 15. The first and final snapshots of HA/TLR2 and HA/CD44 complexes.

However, CD44 has the main binding domain for all HAs. Moreover, the BFE of HAs-CD44 proposed the high affinity of HA to this receptor (-723.4, -1272.5 and -1680.6 kJ/mol for HA4-CD44, HA8-CD44 and HA32-CD44, receptively). Additionally, HA with higher number of monomer (HA32) showed stronger interaction to the main binding site of CD44 comparing to HA4 or HA8, since more residues in the binding pocket involved in the h-bond interaction. RDF (calculated during the last 10 ns simulation time) of the complex of HA in the main binding site of receptors was showed in **Figure 16A**. RDF calculated for PAM3 and HAs induced individual peaks for PAM3 at TLR2 main binding distance of ~ 5Å. As for HA4 and HA8 in HA-TLR2 complexes, the peaks of RDF were appeared at distance of ~ 30 Å. Regarding CD44 crystallographic mode, the RDF of HA4, HA8 and HA32 has peaks at ~ 7Å which indicating a high tendency of HA to CD44.

Figure 16B illustrates the distance averages of COM between HAs and main binding sites of receptors. Regarding the main binding site of TLR2, there were fluctuations between 3 and 4 nm for HA4 and HA8 and became stable after 60 ns, as they moved and stabilized into another subdomain in TLR2. However, as for HA32, the ligand failed to interact with any pockets of TLR2. Looking at CD44, the fluctuation of COM distance was not changed during 150 simulation time (the amplitude of the oscillations is between around 1.5 and 2 nm for all HA). HA tried to preserve the initial distance (positions obtained from docking simulation) during 150 ns MD simulation time.

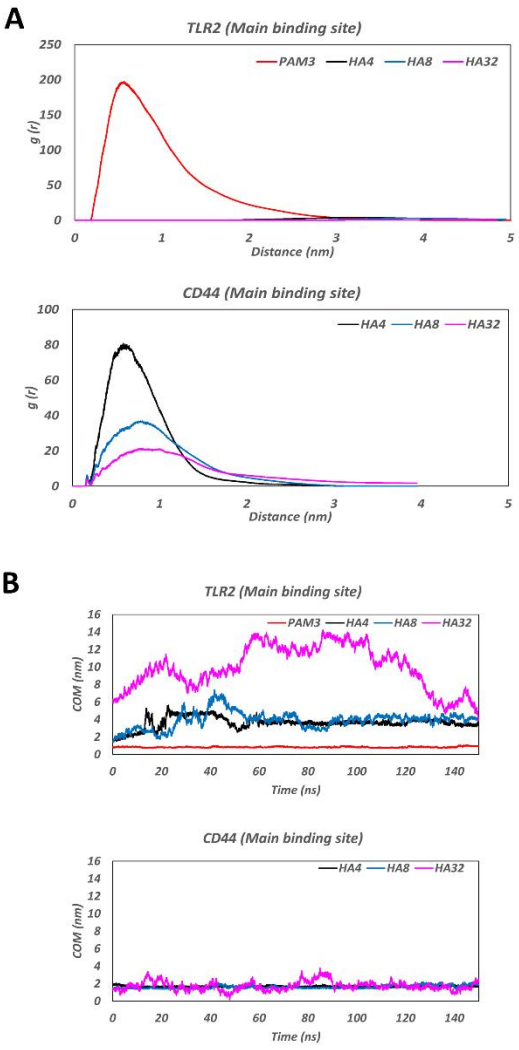

Figure 16. (A) Radial distribution function (RDF) and **(B)** the average of center of mass (COM) distance between of HA and receptors.

3.2. Immunofluorescence and ELISA experiments

To ensure the localization of HA to bovine endometrium, the endometrial sections were subjected to immunostaining using bHABP. Indeed, HA was localized to bovine endometrium.

Although a higher expression was observed in the endometrial stroma, HA was also expressed by luminal- and glandular epithelia of bovine endometrium **(Figure 17).** Negative control sections treated with hyaluronidase showed no staining, demonstrating the specificity of the staining. Concurrently, the conditioned media in-vitro produced via incubating the culture media with BEECs monolayers showed detectable levels of HA with an average of 16.05 ± 2.33 ng/ml.

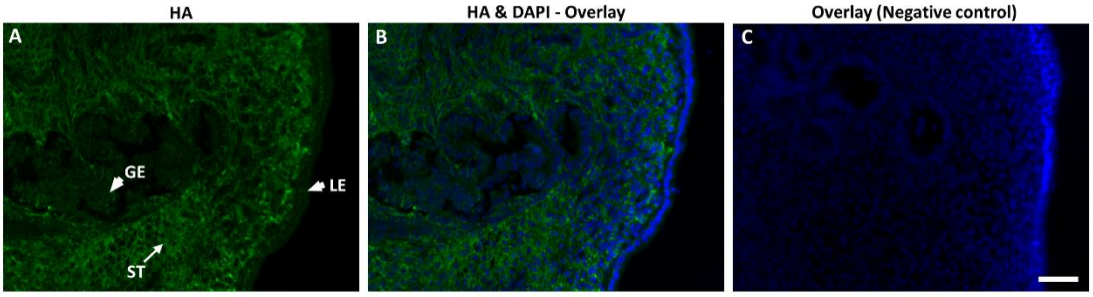

Figure 17. Immunofluorescence localization of hyaluronan (HA) in bovine endometrium.

3.3. Cell culture and gene expression experiments

Accordingly, it was essential to elucidate the possible role(s) of HA in sperm-BEECs crosstalk. BEECs monolayers were enriched with different concentrations of HA (0, 0.1, 1, or 10 µg/mL) for 2 h prior to the co-culture with sperm for further 3 h. At a glance, I observed that the number of attached sperm was significantly higher in BEECs treated with HA (at either 1 or 10 µg/mL) than in the non-treated BEECs **(Figure 18A).** To confirm such observation, I quantified the number of sperm remained attached to BEECs at the end of co-culture period (3 h). Likewise, BEECs pretreatment with HA dose-dependently increased the number of remained attached sperm **(Figure 18B).**

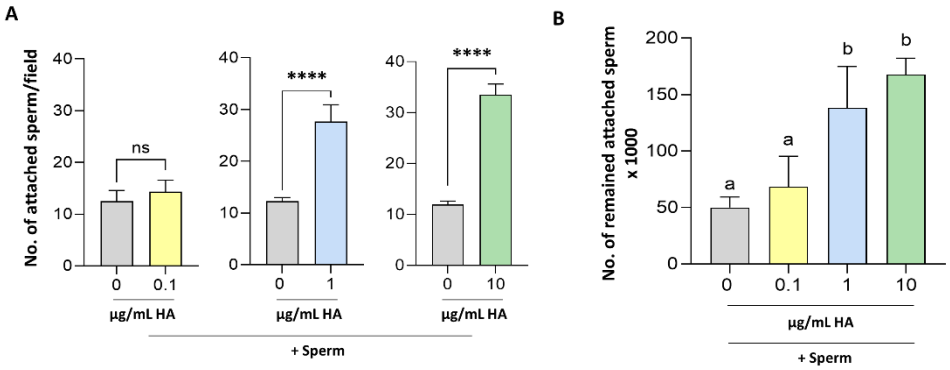

Figure 18. Determination of the number of (A) attached and (B) remained attached sperm to BEECs pretreated without- with HA.

To exclude the possibility that HA could adversely affect sperm dynamics, sperm cells at 106/ml were exposed to HA at the above concentrations for 2 h. Then, the progressive motility of recovered sperm

was assessed at 0-, 30-, 60-, and 120-min post-exposure. Importantly, HA did not affect sperm progressive motility over the exposure period **(Figure 19).**

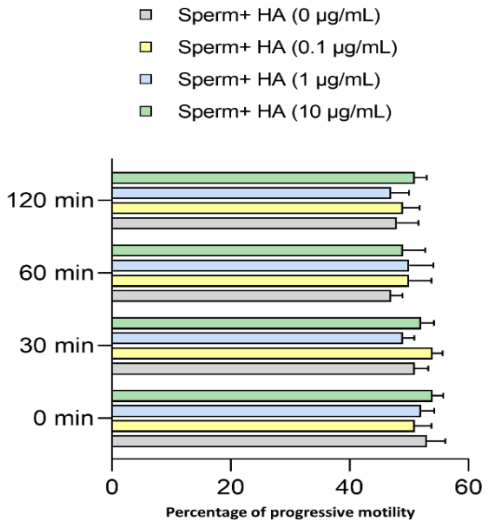

Figure 19. HA has no affect on sperm motility.

Afterwards, it was crucial to investigate the effect of BEECs pretreatment with HA on sperm-induced inflammation. Therefore, BEECs monolayers were co-incubated with HA at either 0, 0.1, 1, or 10 µg/mL for 2 h followed by the co-culture with sperm for additional 3 h. Then, mRNA expressions of TLR2, pro-inflammatory- cytokines (TNFA and IL-1B) and chemokines (IL-8) as well as PGES were quantified in BEECs via a real-time PCR. My data showed that the pre-incubation of BEECs with HA at lower concentration (0.1 µg/mL) has no effect on TLR2, TNFA, IL-1B, IL-8, and PGES mRNA expressions in BEECs triggered with sperm **(Figure 20A),** while HA at medium concentration (1 µg/mL) increases the stimulatory effect of sperm on the abundances of TLR2, TNFA, IL-1B, IL-8, and PGES transcripts in BEECs **(Figure 20B)**. However, I recorded lower transcriptional levels of the aforementioned genes upon sperm co-culture with BEECs treated with HA at higher concentration (10 µg/mL) when compared to the non-treated BEECs **(Figure 20C)**.

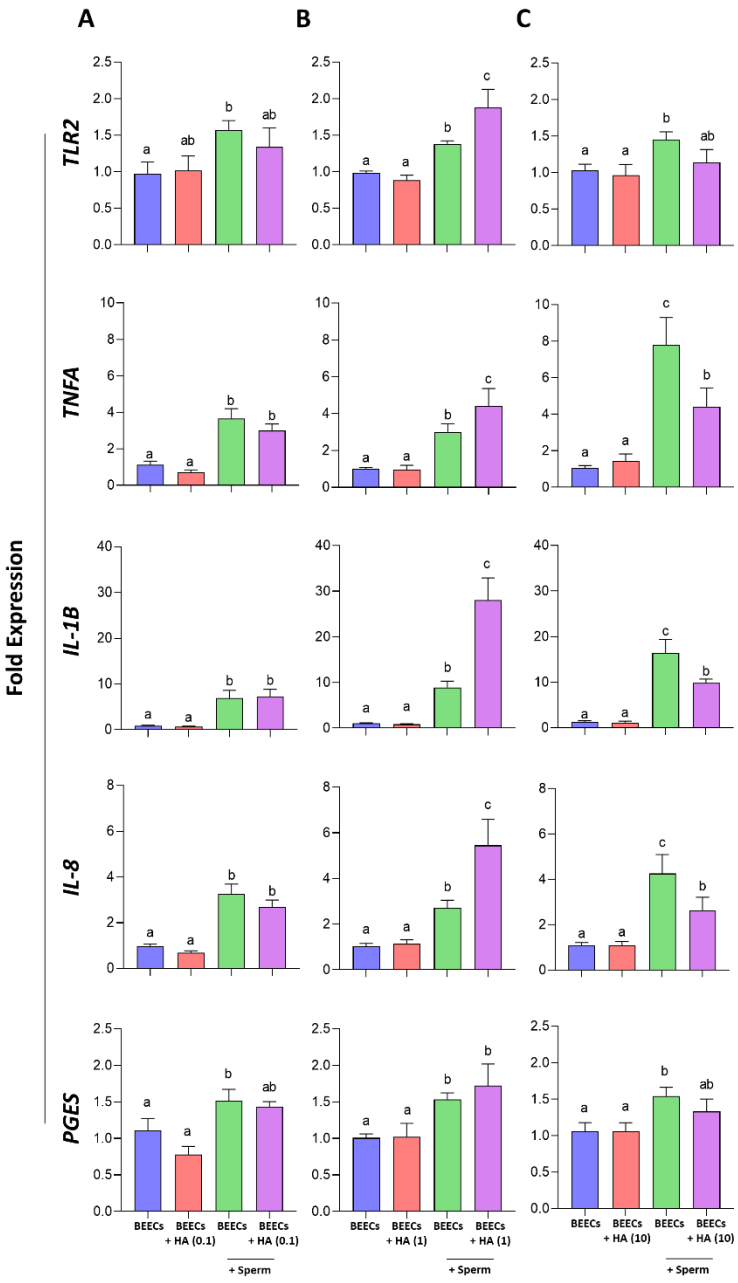

Figure 20. Effect of BEECs pretreatment with HA on sperm- induced weak inflammation.

4. Discussion

In this chapter, *in-silico* analyses combined with in-vitro co-culture model of sperm with endometrial epithelial cells provides several lines of evidence that HA, primarily through CD44

interaction, has the capacity to facilitate sperm attachment to the endometrial epithelia and thereby triggering sperm-induced inflammatory response in bovine uterus via TLR2 signaling transduction. HA, the most abundant glycosaminoglycans in female reproductive organs (33), interact with CD44 but could not interact with the main binding site of TLR2. The present study showed that HA has no affinity to the main binding site of TLR2, compared with PAM3 during MD simulation time. Notably, the main binding site of TLR2 is the internal hydrophobic channel, which highly attract lipoprotein, such as PAM3.

According to the Ligplot result, the main bond types for HA interaction were hydrogen bonds (h-bonds). For instance, HA create h-bonds with residues of CD44 (Arg 41, Tyr 42 etc.) to interact strongly. TLR2 presented h-bonds (such as Arg 508, Arg 447 and Arg 486) in a peripheral subdomain, away from the main pocket, which involved in the HA-TLR2 interaction (for 4- and 8-mers HA). It is obvious that polar and electricity charged amino acids play an important role in HA binding affinity. CD44 has three topographically main binding for HA, however HA has the high potential affinity only to crystallographic mode (45). In harmony to previous literatures (45), my computational analysis confirmed the strong interaction between crystallographic binding mode of CD44 with all HAs (BFE: -723.4, -1272.5 and -1680.6 kJ/mol for HA4, HA8 and HA32 respectively). Hence, my in-silico investigations strongly suggest that HA has higher binding affinity to the main binding site of CD44 than TLR2.

Based on the above computational analyses, it was fundamental to confirm the existence of HA in bovine uterus. Importantly, the immunohistochemical detection of HA in the pre-ovulatory uterus showed its localization to both endometrial stroma and epithelia. The staining revealed that HA were strongly localized within the stroma and weakly expressed by luminal and glandular epithelium of the endometrium. Similar observations (i.e., intense expression in stroma) were reported in other species such as in ovine and mouse endometrium (35,50). To clarify more, in ovine species, HA was expressed strongly in the follicular stage endometrium compared to luteal stage (50). Surprisingly, in bovine oviducts, HA was strongly localized to the stroma of the oviductal villi and no expression was observed in the luminal epithelium (51). The observed lower expression of HA in endometrial epithelium may be due to the release of luminal and glandular epithelial HA into the uterine cavity. It is likely that, the lower level of epithelial HA would favor a diffusion from stroma to epithelium which could lead to a dynamic releasing of HA from stroma to the uterine lumen. In support of this, the present ELISA result demonstrated that BEECs have the ability to release detectable levels of HA into the culture media over the co-incubation period.

Accordingly, I next aimed to investigate the effect of BEECs enrichment with HA prior to the co-culture with sperm on the subsequent sperm-BEECs interaction. Interestingly, BEECs pre-enrichment with HA dose-dependently increased the number of sperm attached to BEECs; such

phenomenon could be referred to HA-CD44 interaction. In light of my in-silico investigations, HA has a much stronger binding affinity to CD44 than TLR2. As well, I have previously reported that CD44 adhesion molecule plays a principal role in sperm attachment to the endometrial epithelia in bovine uterus; the addition of anti-CD44 neutralizing antibody negatively impacted sperm-BEECs interaction (16). Besides, it has been reported that sperm cells could express CD44 (43). Altogether, I speculate that the exogenous HA added to BEECs culture media prior to sperm exposure could act as bridging ligand between sperm from one side and CD44 of BEECs from the other side. Such model has been extensively studied in leukocyte trafficking. Namely, leukocytic infiltration from the bloodstream into inflamed tissues or organs requires binding interactions between adhesion molecules on leukocytes from one side and endothelial cells from the other side; HA-CD44 interaction has been implicated in regulation of rolling-, adhesion- as well as invasion processes ending with leukocytic infiltration (52).

Since sperm attachment to the endometrial epithelia is a prerequisite for promotion of a pro-inflammatory response in bovine uterus via activation of TLR2 signaling pathway (1,3–5). it was necessary to determine whether enhancing sperm attachment by the aid of BEECs pretreatment with HA could stimulate a much stronger inflammatory response in BEECs. Strikingly, my PCR data showed that BEECs pretreatment with medium HA concentration (i.e., 1 µg/mL) upregulates mRNA expressions TLR2, pro-inflammatory- cytokines (TNFA and IL-1B) and chemokines (IL-8) as well as prostaglandins E synthesis (PGES). However, higher exogenous HA concentrations (i.e., 10 µg/mL) added to BEECs prior to exposure to sperm weakened sperm-triggered inflammation in BEECs.

In the past few decades, HA was considered as inert constituent of extracellular matrix, but it is currently categorized as “dynamic” molecule with a continuous turnover to HA molecules of various sizes: high molecular weight (HMW) HA, low molecular weight (LMW) HA as well as oligosaccharides. Under the physiological conditions, HMW HA primarily contributes to tissue integrity (53). Upon tissue injury, HMW HA molecules are rapidly degraded into LMW HA molecules, referred as HA fragments. Via binding with HA receptors, mainly CD44 adhesion molecule (54), the generated HA fragments witness a special capacity to initiate a pro-inflammatory response : upregulation of pro-inflammatory genes expression such as chemokines [keratinocyte chemoattractant (KC), macrophage chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP), and IFN induced protein-10], cytokines (TNFA, IL12 and IL8), inducible nitric oxide synthase (iNOS), matrix-modifying enzymes (MMEs), as well as plasminogen activator inhibitor (41). Simultaneous with this capability to induce inflammation *via* CD44 interaction, HA fragments can also activate TLR-mediated pathways. By employing several murine models, it has been indicated that HA fragments could upregulate the expression of cytokines and chemokines gene expression through activating of TLR2 and TLR4 signaling pathway in various types of cells (17,40).

On the other hand, it has been shown that CD44 plays a role in stimulating TLR2 downstream targets with a subsequent progression of osteoarthritis as evidenced by the upregulation of NFκB-, IL-1B- as well as TNFA gene expression in human macrophages. This study demonstrated that a reduction in CD44 levels in macrophages, via using CD44- specific antibody or knockdown, prior to TLR2 activation downregulates NF-κB transcription and thereby lowers proinflammatory cytokines' (IL-1B and TNFA) production. In addition, they reported that pretreatment of human macrophages with higher doses of HA (100, 250, or 500 µg/mL) dose-dependently inhibits their pro-inflammatory response upon TLR2 ligation (55). Based on the latter finding, I can conclude that BEECs treatment with a higher dose of HA (10 µg/mL) prior to sperm exposure could reduce CD44-meditated TLR2 activation and thereby diminish sperm-induced inflammation in BEECs.

Importantly, it has been shown that both TLR2 and CD44 molecules can function as coreceptors after stimulation of immune cells with TLR2 agonist (Zymosan) (56). Moreover, HA can form a triple complex with CD44 and TLR2 to promote the invasiveness and pro-inflammatory environment in cancer cells (57). Therefore, it seems that sperm-uterine immune-crosstalk could be regulated by CD44 and TLR2 under the effect of HA. However further investigations are necessary to clarify this phenomenon. Moreover, additional studies are required to identify the post-translational modification (PTMs) and how polymorphism affect TLR2 response toward different ligands in bovine species.

In conclusion, the present in-silico and in-vitro investigations proposed a possible cross-talk between sperm and uterine epithelial cells via hyaluronan and hyaluronan binding receptors (CD44 and TLR2), to induce pro-inflammatory response in bovine uterus **(Figure 21)**. The physiological molecule HA increase the sperm attachment to BEECs via CD44 which in turn activate TLR2 signaling pathway ending with transcription of the pro-inflammatory genes in response to sperm. Further investigations are required to define the specific molecule(s) from the sperm side which regulate the TLR2 to induce inflammation after sperm attachment.

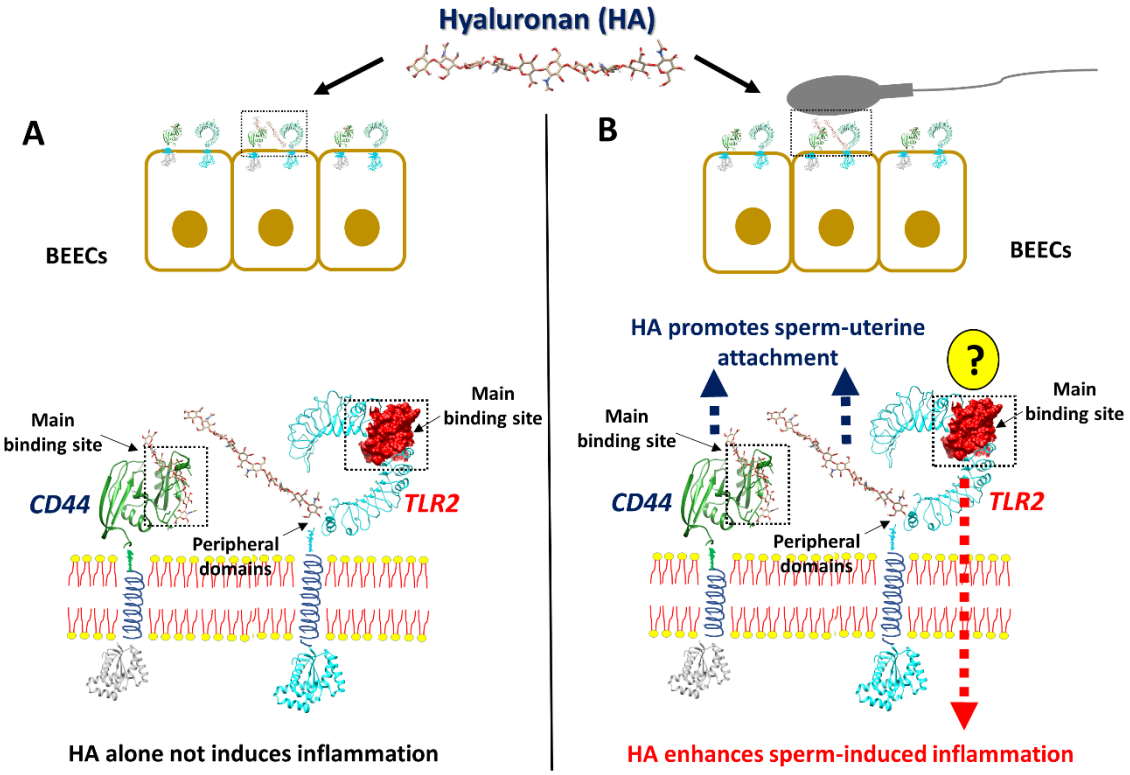

Figure 21. The working hypothesis of the impact of Hyaluronan (HA) on sperm-induced inflammation in BEECs

General discussion

Bovine uterus has a well-regulated immune response to eliminate bacterial contamination after parturition, tolerate the allogenic sperm and accept semi-allogenic embryos (1,2,14). TLR2 has been implicated in the regulation of various physiological functions of the female reproduction including ovulation, fertilization, gestation, and parturition (5,9,10,14,16). Quite recently, our research group has demonstrated, via employing series of in vitro and ex vivo investigations, that active sperm attach to bovine endometrial epithelial cells (BEECs) and thereby stimulate a pro-inflammatory response through activation of TLR2 signaling pathway (4,5,14,16). However, the underlying mechanism of the sperm-induced inflammation in BEECs that mediated by TLR2 signaling is still unclear.

Of note, the TLR2 seems to have an essential role in balancing pro- and anti-inflammatory immune responses in bovine genital tract (1). TLR2 forms heterodimers with TLR1 or TLR6, which is the initial step in a cascade of events leading to significant innate immune responses (12,58). With selective TLR2 heterodimerization, it was important to determine the active TLR2 heterodimer which is involved in sperm-induced inflammation in bovine uterus. In chapter I, I provided the initial evidence that sperm employ TLR2/1 signaling that led to a weak inflammatory response in BEECs. In fact, this kind of weak and transient inflammation is required to remove excess and dead sperm remaining in the uterine lumen and to complete this clearance within several hours without tissue damage for providing the ideal uterine environment for acceptance of early embryo and implantation (1,2,14). Meanwhile, the activation of TLR2/6 signaling pathway could lead to a stronger inflammation compared to TLR2/1 in uterine epithelial monolayer using specific agonists. In harmony with my result, it has been reported that activation of TLR2/6 signaling cascade in human embryonic kidney cells could lead to 4-fold stronger inflammation compared with TLR2/1 (28). Herein, TLR6 was not modulated after sperm exposure, possibly to avoid strong and chronic inflammation and the tissue damage as high expression of TLR6 correlated with higher-risk disease (29).

Additionally, the present in-silico models showed the stabilized interactions between TLR2/1 and TLR2/6 heterodimers in presence of their agonists. Furthermore, the data revealed that the main binding sites of bovine TLRs were identical to human and mouse using de novo protein structure prediction model. Therefore, it could be concluded that TLR2/1 dimerization initiate the inflammatory response that induced by sperm in the bovine uterus. However, the sperm surface molecule which could initiate and/or regulate this TLR2/1 dimerization process is unknown.

For instance, different exogenous or endogenous molecules have been reported as ligands and/or regulator for TLR2 signaling (31); one of these molecules is hyaluronan (HA) (32). HA, a non-sulphated glycosaminoglycan, is normally present in most of mammalian tissues and fluids including

those of reproductive system including the uterus (35). So far, HA has gained a special relevance in the field of reproductive biology due to its participation in numerous physiological events such as ovulation and fertilization (33). Moreover, CD44 with its ligand HA are involved, either via cell-cell interaction or cell-matrix interaction, in promotion of inflammatory process (41,54–56,59). In regard to sperm physiology, it has been shown that HA fragments, produced by sperm-released hyaluronidase, activate TLR2/4 signaling pathway with subsequent cytokine/chemokine production in the cumulus cells of cumulus-oocyte complexes (COCs) which is essential for accomplishment of fertilization process (17).

Thus, in chapter II, I supposed that HA could be a good candidate to act as a bridging ligand between the sperm cells from one side and CD44 of BEECs from the other side for regulating the TLR2-mediated inflammation induced by the sperm. My in-silico investigations showed that HA has higher binding affinity to the main binding site of CD44 than TLR2. Moreover, TLR2 interactions with HA oligomer (4- and 8-mers) target a different subdomain (h-bonds) compared to TLR2-agonist (PAM3) which targets a central hydrophobic pocket.

Importantly, the immunohistochemical detection of HA in bovine endometrium at pre-ovulatory stage showed its localization to both endometrial stroma and epithelia. The staining revealed that HA were strongly localized within the stroma and weakly expressed by luminal and glandular epithelium of the endometrium. Similar observations (i.e., intense expression in stroma) were reported in other species such as in ovine and mouse endometrium (35,50). In ovine species, HA was expressed strongly in the follicular stage endometrium compared to luteal stage (50). It is likely that, the lower level of epithelial HA would favor a diffusion from stroma to epithelium which could lead to a dynamic releasing of HA from stroma to the uterine lumen. In support of this, the present ELISA result demonstrated that BEECs have the ability to release detectable levels of HA into the culture media over the co-incubation period.

In the present study, BEECs pretreatment with HA prior to sperm exposure increased the number of attached sperm to BEECs, and upregulated the transcriptional levels of pro-inflammatory genes (TNFA, IL-1B, IL-8, and PGES) in BEECs in response to sperm. However, BEECs treated with HA only (no sperm exposure) did not show any significant effect on the transcript abundance of pro-inflammatory genes when compared to the non-treated BEECs. Besides, HA can form a triple complex with CD44 and TLR2 to promote the invasiveness and pro-inflammatory environment (57). Therefore, it seems that sperm-uterine immune-crosstalk could be regulated by CD44 and TLR2 under the effect of HA. However further investigations are necessary to clarify this phenomenon.

In conclusion, my results revealed that sperm utilize TLR2/1 pathway to induce a weak inflammatory response in bovine endometrium. Activation of TLR2/6 heterodimer could lead to an excessive inflammatory response which assumed to be far from the physiological sperm-triggered

inflammation. Further, in-silico findings revealed that in presence of specific ligand, the ectodomains of bovine TLR2 formed a heterodimer to initiate cell signaling pathway. It is likely that HA can increase the sperm attachment to BEECs via CD44 which in turn activate TLR2 signaling pathway in response to sperm. Concerning the specific ligand(s) on the sperm cell membrane which is required to stabilize TLR2/1 heterodimerization, glycan could be one of possible candidates to fulfill this role. In fact, sperm glycan plays an important role in sperm survival in the FRT. Glycan has several functions for sperm journey in FRT such as cervical mucus transit, immune-modulatory activity in the uterus, sperm attachment to oviduct cells, etc. However, further investigations are required to clarify whether glycan or other possible molecules could regulate the TLR2/1 heterodimerization to induce weak inflammation after sperm attachment. **(Figure 22)**.

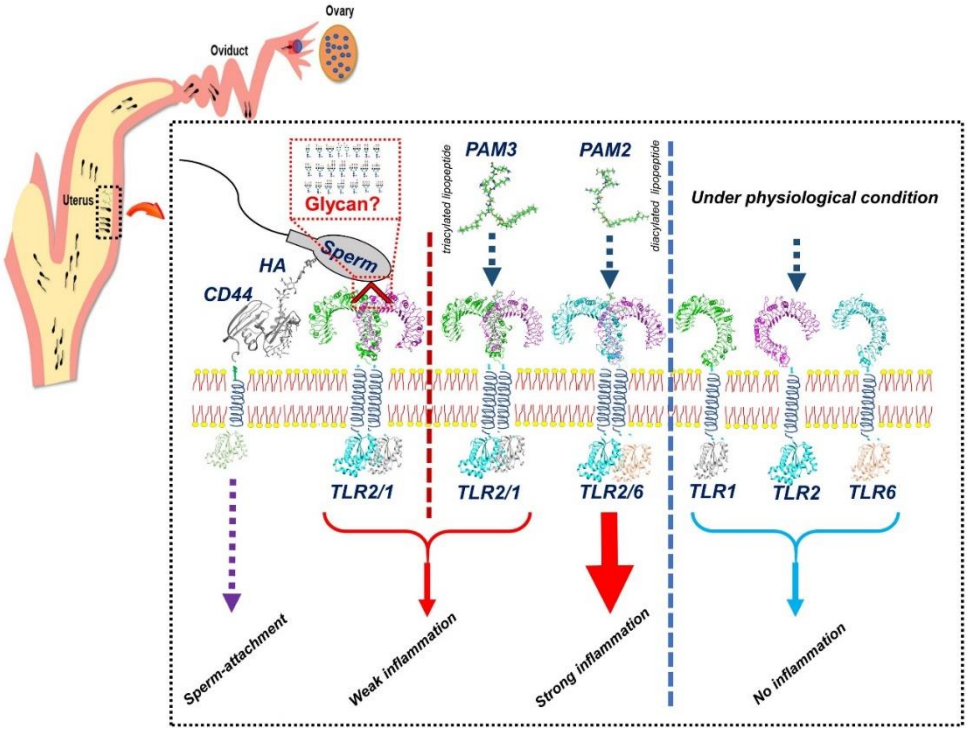

Figure 22. Schematic illustration of the findings of the present study. The experimental findings suggest that the stimulation of endometrial epithelia with PAM3: TLR2/1 and PAM2: TLR2/6 leading to different inflammation response (weaker and stronger intensity, respectively). Additionally, sperm activate TLR2/1 (Not TLR2/6) heterodimerization to induce a weak and transient inflammation. It is still unknown which molecules form sperm surface side could link TLR1 to TLR2. However, glycan of sperm surface can be a possible candidate, acting as a bridging ligand to enhance TLR2/1 dimerization. On the other hand, Hyaluronan (HA) as the most abundant endogenous ligands in uterine lumen could enhance and accelerate sperm attachment (through CD44 in lumen) and subsequent initiate TLR2/1 signaling pathway in response to sperm.

Summary

In cattle, after artificial insemination (AI) or natural mating, a large number of sperm swim up into the female reproductive tract (FRT) toward the site of fertilization. During this journey, sperm interact with different compartments of the immune system of FRT. Bovine uterus has a well-regulated immune response to remove bacterial contamination after parturition, and it tolerates the allogenic sperm and accepts semi-allogenic embryos. Sperm generate transient proinflammatory response in the uterus which is required for the removal of dead/excess sperm with associated contaminants. TLR2 plays a central role in sperm-induced inflammation in the bovine uterus. In general, in immune cells, a dimerization of TLR2 with either TLR1 or TLR6 is required to activate intracellular signaling pathways, thereby inducing the innate immune response, but nothing is known about TLR2 dimerization in the bovine endometrial epithelium in response to sperm attachment. On the other hand, CD44 is a major cell surface receptor for hyaluronan (HA) involved in sperm attachment to the endometrial epithelium. In this study, using multiple approaches based on the computational modelling methods together with the in vitro experimental models, I was able to identify the major part of the molecular mechanism of sperm interaction with the bovine uterine immune system in cattle.

In chapter I, in order to test different TLR2 dimerization pathways in endometrium in an in-vitro model, 100 ng/mL TLR2 agonists (PAM3 as the TLR2/1 agonist, and PAM2 as the TLR2/6 agonist) were used to stimulate bovine endometrial epithelial cells (BEECs). Simultaneously, the expression of TLR1, 2 and 6 protein and gene in BEECs were investigated after exposure to sperm (5 million/mL). Further, sperm induced-inflammation was compared to PAM3 and PAM2 using the uterine explant ex-vivo model. The obtained data indicated that an activation of TLR2/1 signaling pathway in BEECs is involved in a weaker inflammation compared to TLR2/6. Moreover, similar to PAM3, sperm was able to induce TLR2 expression alongside with TLR1 in the uterus (gene and protein), particularly in uterine glands, but not TLR6. In the same way, PAM3 and sperm could induce similar and low gene expression of pro-inflammatory cytokines (TNFA, IL1B and IL8) and TNFA protein to a lesser extent than PAM2 in the bovine endometrium. Thus, it is highly possible that sperm trigger endometrial epithelia to induce a weak inflammatory response through activating TLR2/TLR1 signaling cascade, which is needed to prepare an ideal environment for embryo reception. Afterwards, in-silico approaches were employed to investigate and confirm TLR2 dimerization in bovine species (TLR2/1 or TLR2/6). Homology modeling methods were used to determine the 3D protein structure of bovine TLRs. The in-silico findings suggested that the stability of TLR2 dimerization is heavily depending on the presence of the bridging agonist in bovine, which is similar to human and mouse species.

In chapter II, I hypothesized that HA may act as a bridging ligand between sperm and CD44/TLR2 of BEECs. To test the above hypothesis, I first determined the binding affinity of HA to

CD44 and TLR2 molecules. my in-silico model revealed that low molecular weight HA molecules have a higher affinity to CD44- than TLR2 interaction. Next, HA existence in bovine endometrium was investigated via immunostaining using a biotinylated HA-binding protein. Notably, HA is localized in the luminal and glandular endometrial epithelia. Moreover, ELISA showed detectable levels of HA (16.05 ± 2.33 ng/ml) in BEECs-conditioned medium. As a result, BEECs were treated with different concentrations of low molecular weight HA (at 0, 0.1, 1, or 10 µg/mL) for 2 h prior to the co-culture with 106 sperm/mL for additional 3 h. Importantly, HA dose-dependently increased the number of sperm attached to BEECs. Besides, the quantitative real-time PCR data illustrated that supplementation of BEECs with HA (at 1 µg/mL) upregulate mRNA expressions of TLR2, pro-inflammatory- cytokines (TNFA and IL1B) and chemokines (IL8) as well as prostaglandins E synthesis (PGES) in BEECs in response to sperm. However, BEECs treatment with HA only (no sperm exposure) did not show any significant difference in transcriptional levels of the selected genes when compared to the non-treated BEECs. Collectively, the findings provide evidence that HA, primarily through CD44 interaction, has the capacity to facilitate sperm attachment to the endometrial epithelia with a subsequent TLR2-mediated immune response.

Overall, my findings in chapter I revealed that sperm activate TLR2/1 heterodimerization, but not TLR2/6, to trigger a weak physiological inflammatory response in bovine endometrium. The data of chapter II suggested that sperm keep a higher affinity for attaching to the BEECs in presence of HA through interaction with CD44, consequently inducing proinflammatory response through TLR2 signaling pathway. Collectively, this weak inflammation triggered by sperm with the molecular network of TLR2/1, CD44 and hyaluronan must be the specific way to remove excess/dead sperm remaining in the bovine uterine lumen without tissue damage for providing the ideal environment for embryo implantation.

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和 文 要 約

ウシでは、人工授精（AI）または自然交配の後、多数の精子が雌の生殖器官（FRT）内を受精部位に向かって泳ぎ上がってゆく。この旅の間、精子は FRT の免疫系のさまざまな部分と相互作用する。ウシの子宮は、出産後に細菌汚染を除去するためによく調節された免疫応答を備えており、同種異系精子を一部は許容し、半同種異系胚を受け入れる。精子は、関連する汚染物質を伴う死んだ、あるいは過剰な精子の除去のために子宮内で一時的な炎症誘発反応を引き起こす。TLR2 は、ウシ子宮における精子誘発性炎症において中心的な役割を果たす。一般に、免疫細胞では、細胞内シグナル伝達経路を活性化し、それによって自然免疫応答を誘導するには、TLR1 または TLR6 による TLR2 の二量体化が必要であるが、精子の付着に応答したウシ子宮内膜上皮における TLR2 の二量体化については何もわかっていない。一方、CD44 は、子宮内膜上皮への精子の付着に関与するヒアルロン酸（HA）の主要な細胞表面受容体である。この研究では、コンピュータモデリング法と in vitro 実験モデルに基づく複数のアプローチを使用して、牛の精子とウシ子宮免疫系の相互作用の分子機構の主要部分を特定することができた。

第 I 章では、in vitro モデルで子宮内膜の様々な TLR2 二量体化経路をテストするために、100 ng/mL の TLR2 アゴニスト(TLR2/1 アゴニストとしての PAM3、および TLR2/6 アゴニストとしての PAM2）を使用してウシ子宮内膜上皮細胞（BEEC)を刺激した。 同時に、精子（500 万/mL）への曝露後の BEEC における TLR1、2、および 6 タンパク質および遺伝子の発現を調査した。さらに、子宮外植片の ex vivo モデルを使用して、精子による誘発性炎症を PAM3 および PAM によるものと比較した。 得られたデータは、BEEC における TLR2/1 シグナル伝達経路の活性化が、TLR2/6 と比較して弱い炎症に関与していることを示した。 さらに、PAM3 と同様に、精子は子宮（遺伝子およびタンパク質)、特に子宮腺で TLR1 とともに TLR2 発現を誘導したが、TLR6 は誘導しなかった。加えて、PAM3 と精子はウシ子宮内膜において、炎症誘発性サイトカイン（TNFA、IL1B、および IL8）および TNFA タンパク質と遺伝子発現を、PAM2 よりも低い程度で同様に誘導していた。したがって、精子が子宮内膜上皮を刺激し、胚の受容に理想的な環境を準備するために必要な TLR2/TLR1 シグナル伝達カスケードの活性化を通じて弱い炎症反応を誘発する可能性が非常に高い。これらの実験系に引き続き、in silico アプローチを使用してウシにおける TLR2 二量体化（TLR2/1 または TLR2/6）を調べた。相同性モデリング法を使用して、ウシ TLR の 3D タンパク質構造を決定した。In silico 法での発見は、TLR 二量体化の安定性が、ヒトやマウス種と同様にウシにおける架橋アゴニストの存在に大きく依存していることを示唆した。

第 II 章では、HA が精子と BEEC の CD44/TLR2 の間の架橋リガンドとして機能する可能性があるという仮説を立てた。この仮説を検証するために、私はまず、CD44 および TLR2 分子に対する HA の結合親和性を決定した。 私の in silico モデルでは、低分子量 HA 分子が TLR2 との相互作用よりも、むしろ CD44 との相互作用に対して高い親和性を持っていることが明らかになった。 次に、ビオチン化 HA 結合タンパク質を用いた免疫染色により、ウシ子宮内膜における HA の存在を調べた。注目すべきことに、HA は管腔および腺の子宮内膜上皮に局在していた。さらに ELISA によって、BEEC 培養上清中に検出可能なレベルの HA（16.05 ± 2.33 ng/ml）が検出できた。さらに、BEEC を様々な濃度の低分子量 HA（0、0.1、1、または 10 μg/mL）で 2 時間処理した後、10^6 精子/mL とさらに 3 時間共培養した。 重要なことに、HA は BEEC に付着する精子の数を用量依存的に増加させた。さらに、定量的リアルタイム PCR データから、BEEC に HA（1 μg/mL）を添加すると、TLR2、炎症誘発性サイトカイン（TNFA および IL1B）、ケモカイン（IL8）の mRNA 発現、およびプロスタグランジン E 合成酵素が増加することがわかった。しかし、HA 添加だけでは BEEC（精子曝露なし）の焦点とした遺伝子の転写レベルに有意な差は示されなかった。まとめると、これらの発見は、HA が主に CD44 との相互作用を通じて、その後の TLR2 媒介による免疫応答を伴う子宮内膜上皮への精子の付着を促進する能力があるという証拠を提供した。

全体として、第 1 章での私の発見は、精子が TLR2/1 ヘテロ二量体化を活性化するが、TLR2/6 は活性化せず、この経路がウシ子宮内膜における弱い生理的炎症反応を引き起こすことを明らかにした。 第 2 章のデータは、精子が HA の存在下で CD44 との相互作用を通じて BEEC への結合に対してより高い親和性を維持し、その結果、TLR2 シグナル伝達経路を通じて炎症誘発性反応を誘導することを示唆した。以上をまとめると、TLR2/1、CD44、およびヒアルロン酸の分子ネットワークに支えられた精子によって引き起こされる弱い炎症は、胚の着床に理想的な環境を提供するために、ウシの子宮内腔に残っている過剰または死滅した精子を組織損傷なしに除去するための特別なシステムであると考えられた。