Disruption of early embryo-maternal crosstalk *via* peptidoglycan in the infected uterus in the cow

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細菌感染したウシ子宮内におけるペプチドグリカンによる初期胚ー母体のクロストーク阻害

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Table of contents

Abbreviations

BEECs	Bovine endometrial epithelial cells
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
ECM	Embryo culture media
DMEM/F12	Dulbeccos's Modified Eagle Medium: Nutrient Mixture F-12
E2	Estrogen
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
HBSS	Hank's balanced salt solution
IFN	Interferon
IF	Immunofluorescence
IFNAR1	Interferon receptor 1
IFNAR2	Interferon receptor 2
IFNT	Interferon-tau
IL	Interleukin
IL1B	Interleukin 1B
ISGs	Interferon-stimulated genes
ISG15	Interferon-stimulated gene-15
IVM	In vitro maturation
IVF	In vitro fertilization
LE	Luminal epithelium
GE	Glandular epithelium
MRP	Maternal recognition of pregnancy
Nod2	Nucleotide-binding oligomerization domain-containing protein 2
OAS1	2′-5′ oligoadenylate synthetase 1
P4	Progesterone
PBS	Phosphate buffered saline

PGN	Peptidoglycan
PGRPs	PGN recognition proteins
PP	Postpartum
rbIFNT	Recombinant interferon-tau
Real-time PCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute-1640
STAT1	Signal transduction and activator of transcription 1
TLR	Toll like receptor
TNFA	Tumor necrosis factor apha

General introduction

Postpartum (pp) bacterial diseases are considered as one of the main causes that hinders the development of dairy industry. A sterile and healthy uterine environment is thought to be the basis for successful conception and early embryonic development [1]. Inflammation of uterus due to pp infection is one of the most important causes of reproductive disorders that could hamper implantation and eventually development of embryo. After successful fertilization, bovine embryo needs to initiate a bidirectional biomolecular dialogue with maternal immune system to change the immune response towards the semi-allogenic embryo. Maternal immune system plays a very important role for embryo-maternal immune crosstalk during early pregnancy and disruption of this immune crosstalk may lead to the rejection of embryo. Interferon tau (IFNT), a type I interferon secreted from the developing bovine embryo acts as one of the main factors that mediates this immune crosstalk [2]. Presence of bacteria during pp uterine infection may interfere with IFNT mediated embryo-maternal immune communication which could be one of the reasons for early embryonic losses in cows.

1. Postpartum uterine diseases

Microbial infection of female reproductive tract is common and bears economic significance for both human and animals [3]. It disrupts uterine receptivity and ovarian activity that leads to decreased productivity and cause subfertility. At the time of parturition, physical barriers of genital tract such as cervix, vagina, vulva etc. are compromised and provides routes for bacteria to go up the reproductive tract from external environment and also from the skin and feces of animal. Soon after parturition, it is well recognized that for the majority of cows, uterine bacterial contamination is an unavoidable part of natural involution process [4]. Genital tract microbial infections are related to increased implantation failure and infertility [5] and causes about 40 % spontaneous premature births in woman [6]. Bacterial endotoxin induces the release of TNF-a, which may cause embryo resorptions up to 100 % in mice [7].

2. Impact of pp uterine diseases

PP uterine diseases affects the welfare of infected cows by producing uneasiness and thus the cows may need to be culled from the population [8]. It has been reported that due to bacterial contamination of uterus, elevated acute phase protein concentration in the blood of pp cows causes discomfort and pain [9]. Uterine disease negatively affects the reproductive performance of cows (Fig. 1). Cows with endometritis needs 27% more longer time to be pregnant than healthy cows and its conception rate is 17-20% lower than normal cows [10]. The performance of affected cows is reduced and cannot be restored even after giving sufficient and successful treatment [11]. Additional days without no conception cost $3/cow/day and cost for extra artificial insemination also added cost [12]. Prevalence of 20% metritis costs € 1.411 billion/year in the EU and $ 650 million/year in US [13].

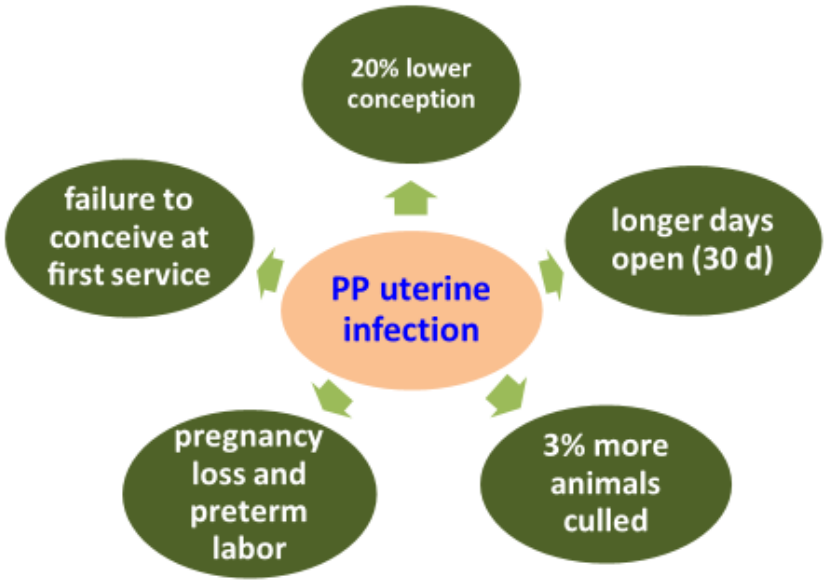

Figure 1. Impact of postpartum uterine disease.

3. Definition of uterine diseases

There are different types of uterine diseases usually found in pp dairy cows such as metritis, endometritis, clinical endometritis, subclinical endometritis and pyometra are most frequently reported. Some researcher [11] classified pp uterine diseases into three classes. They proposed grade I metritis if the uterus is unusually enlarged and contains purulent discharge with no systemic signs. Animals showing added systemic signs of illness like reduced milk yield, dullness and body temperature

39.58°C were categorized in grade II clinical metritis and animals presenting signs of toxemia like inappetence, cold extremities, depression and collapse are categorized in grade III metritis.

When uterine bacterial contagion continues beyond 4 weeks of pp then the infection is stated as endometritis. Clinical endometritis is defined as the presence of a pus-mixed uterine discharge visible in the vagina at 21 days or more pp or mucopurulent fluid seen in the vagina after 26 days pp [14]. Subclinical endometritis may be defined as inflammation of the endometrium generally diagnosed by cytology without presence of purulent discharge in the vagina [15]. In comparison to metritis, in case of subclinical/chronic endometritis, the patient does not show any visible clinical signs.

Pyometra refers to the accumulation of purulent or mucopurulent fluid in uterus and distension of the uterus when an active corpus luteum is present and contains an increased number of pathogenic bacteria [16].

4. Bacteria associated with pp uterine disease

Soon after parturition, uterine bacterial contamination is common (Fig. 2) [4] and most of the contaminating bacteria found in the pp uterus most likely originate from the environment [17]. Bacteriological studies have identified *Trueperella pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum* and *Prevotella* spp. as major uterine pathogens [4, 18]. Moreover, additional flora isolated along with the major uterine pathogens are *Staphylococcus* spp., *Streptococcus* spp. or non-*E. coli* aerobic gram-negative rods [19]. As a consequence, presence of pathogenic microorganisms and its association with clinical and subclinical endometritis may reduce the reproductive performance of dairy cattle [20]. Bacterial presence in the uterine lumen following parturition can delay the physiological regeneration of endometrium [21] and in many cases, early pregnancy failure is thought to be associated with poor uterine receptivity [22]. Uterine receptivity is a physiological condition of the uterus which is firmly regulated by various factors like hormones, cytokines, adhesion molecules and cellular immunity [23].

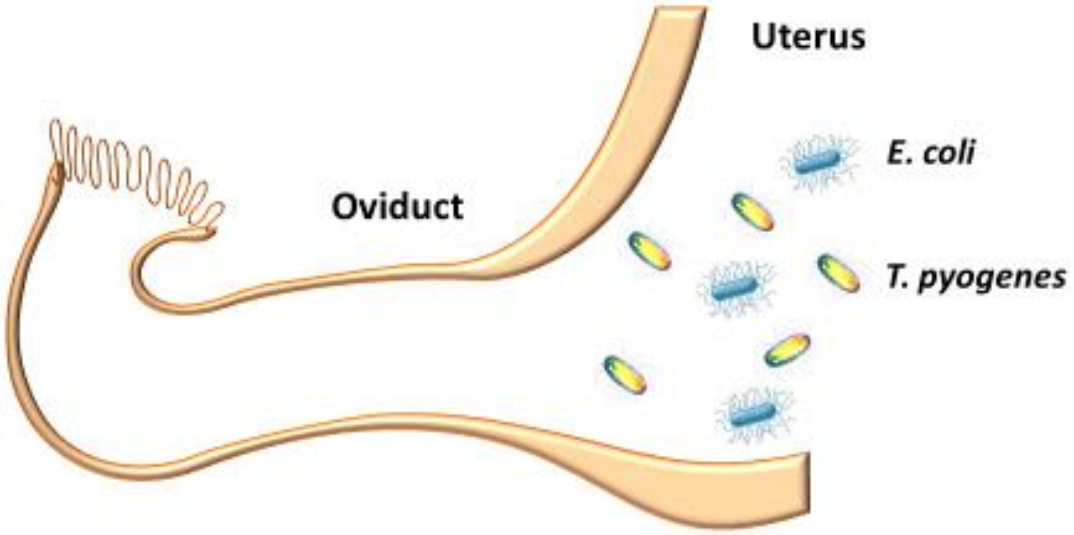

Figure 2. Most common bacteria within uterus.

5. Innate immunity of endometrium

Endometrium is considered as the first line of defense mechanism that forms physical protection towards the infection of uterus. It has toll like receptor (TLR) for recognition of bacteria [24]. The contact between endometrium and invading pathogen leads to an inflammatory process which is essential for the elimination of infection. It is reported that uterine diseases of cattle increase the endometrial gene expression of proinflammatory cytokine and cows with clinical or subclinical endometritis had elevated expression of proinflammatory cytokine (TNFA, IL1B, IL8) at 21-27 days in milk [25].

6. Uterine disease and infertility

Bacterial infection causes injury to the uterine tissues and damage of endometrial wall that leads to the poor reproductive performance [26]. Presence of anaerobic bacteria such *Trueperella pyogenes* also act as risk factor for reduced fertility. At the course of infection, endometritis causes infertility and subsequently, it causes subfertility even after successful recovery from the diseases (Fig. 3) [27]. It has been reported that cows having mucopurulent vaginal discharge has a poor submission rate [28]. Bacterial infection perturbs endocrine signaling in hypothalamic pituitary-gonadal axis and

secretion of gonadotrophin hormones [29]. Microbial infection also hampers ovarian follicular growth and function with reduced and smaller steroidogenic ovarian follicle [30].

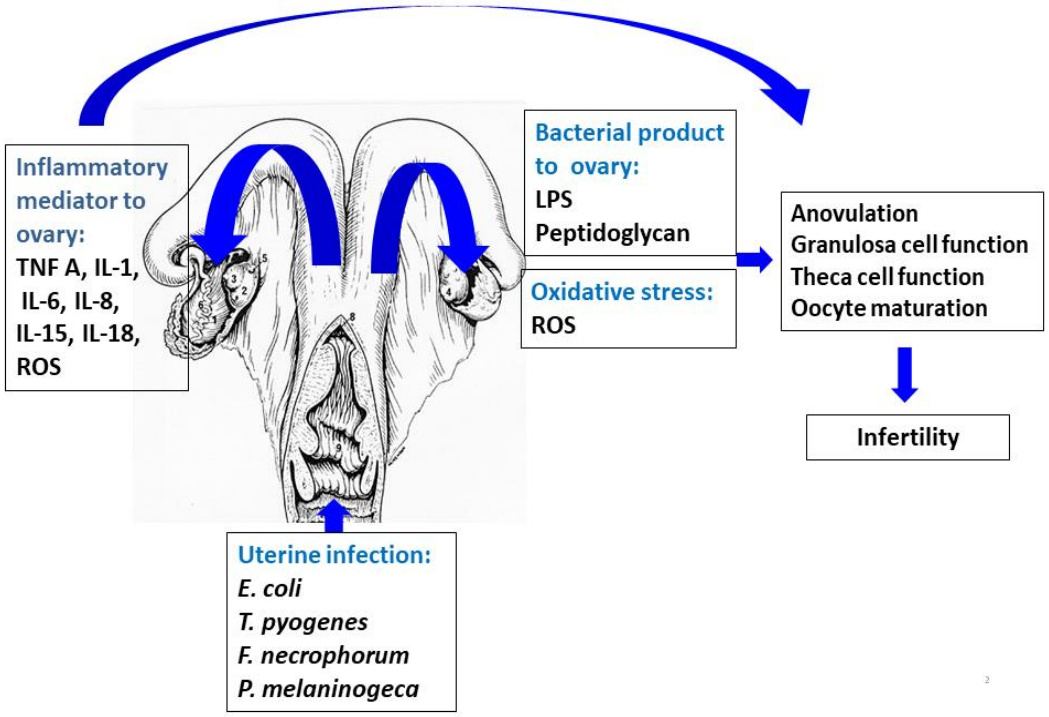

Figure 3. Uterine diseases associated with infertility, adopted from [27].

7. Uterine receptivity in cows

Synchronized alterations in endometrial gene expressions in the trophectoderm are necessary for the attachment and establishment in case of mammals. Interferon tau (IFNT) can work in a paracrine way in that it induces conventional as well as non-conventional expression interferon stimulated genes (ISGs) in various cellular sections of the endometrial epithelial cells to enable the receptivity of uterus for successful grafting and furthermore stimulate the elongation of conceptus and thereby continue IFNT production [31]. There is evidence that chemokine ligand 10 (CXCL10), CXCL9 (monokine induced by IFNG) and interferon-inducible protein-10 kDa (IP-10) have been induced by IFNT in caprine and ovine endometrium [32].

8. Interferon tau and maternal recognition of pregnancy

Before implantation, bovine embryo faces many obstacles to survive and it needs giving signal to mother of its arrival, escaping maternal immune attack, and changing endometrial receptivity for further development. Embryo secretes many bioactive molecules including IFNT and communicate with mother. IFNT is known as the primary molecule which initiates maternal recognition of pregnancy in ruminant (Fig. 4) [33]. Trophoblast cell of the embryo starts to secretes IFNT on around day 8 and continues to go up with the process of elongation of embryo. Peak production and secretion of IFNT is achieved on day 15-17 of ovine pregnancy and then go down and after 22 day of pregnancy, IFNT disappear [34, 35]. It is shown that intrauterine infusion of IFNT from homogenates of elongated embryo extended the lifespan and function of corpus luteum (CL) in cattle and sheep [36]. It is suggested that IFNT can induce ISGs in endometrial epithelium [37], CL [38] and peripheral blood mononuclear cells (PBMCs) during early pregnancy in cows [39].

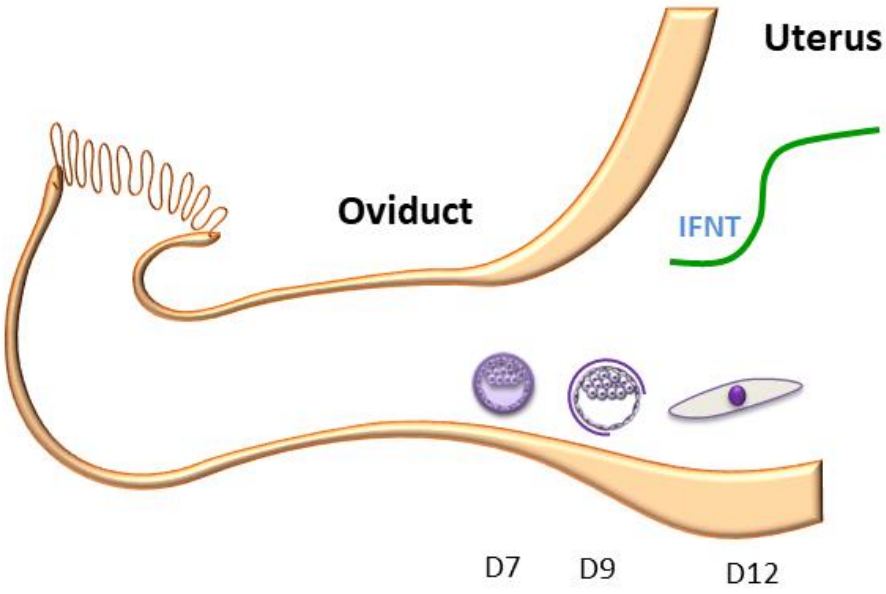

Figure 4. Maternal recognition of pregnancy by IFNT secreted from embryo

9. Interferon-tau and maternal immune regulation

Still it is not clear that how IFNT secreted from developing embryo transmits message to the systemic immune cells in cows. Recently, our group has shown that day 7 bovine embryo start to

communicate with endometrial epithelia cells and immune cells *in vitro* mediated by IFNT (Fig. 5) [40]. It is reported that IFNT suppress lymphocyte proliferation *in vitro* [41] and thus protect semi-allogenic embryo from maternal immune attack. There is evidence that IFNT has protective effects against *Staphylococcus aureus*-induced endometritis in mice and inhibits the proinflammatory cytokines (TNFA, IL1B) production through suppression of the nuclear factor kappa B (NFkB) pathway [42]. In the endometrium of sheep, it activates IP-10 protein and regulates recruitment and distribution of immune cells [43]. It has been stated that IFNT provokes IL10 secretion in dose dependent way and inhibits IL1B secretion in human THP1 macrophages [44].

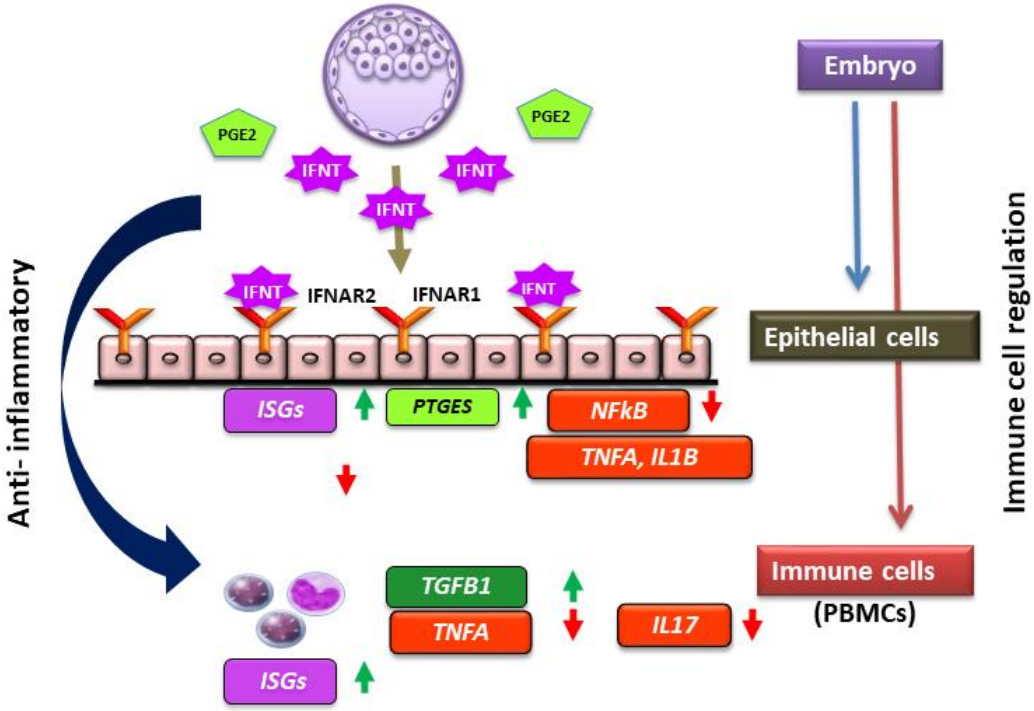

Figure 5. IFNT secreted from embryo regulates maternal immune response, modified from [40].

There is a lack of studies that expose the IFNT-uterine interactions in presence of peptidoglycan. A recent study showed that acute bovine viral diarrhea virus infection inhibits expression of IFNT-stimulated genes in bovine endometrium [45].

10. Objectives of the study

I hypothesized that the presence of bacterial by product, peptidoglycan, affects the action of embryo secreted IFNT-induced interferon stimulated genes in bovine endometrial epithelial cells (Fig. 6).

The general objective of the study was to investigate the interaction of IFNT-bovine endometrial epithelial cells in the presence or absence of peptidoglycan.

The specific objectives of the current study were:

1. To see the effect of embryo culture media on bovine endometrial epithelial cell *in vitro* in presence of peptidoglycan.
2. To see the effect of IFNT on interferon stimulated genes in bovine endometrial epithelial cells *in vitro* and bovine endometrial explant *ex-vivo* in the presence of peptidoglycan.

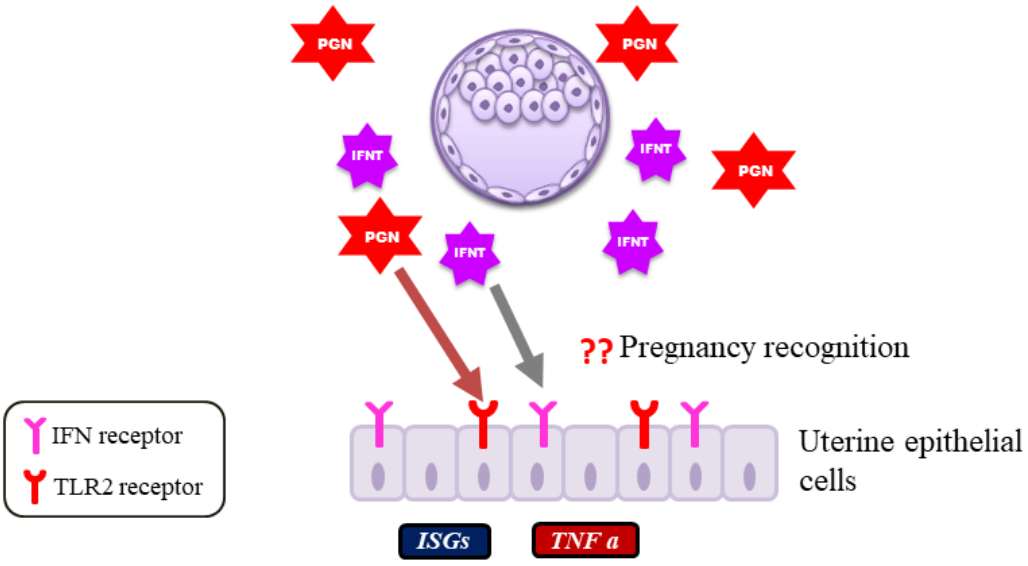

Figure 6. Illustration showing the hypothesis of the study; peptidoglycan disrupts IFNT mediated embryo-maternal communication.

Chapter 1

Peptidoglycan interferes with early embryo interaction in the bovine endometrium

1. Introduction

Pregnancy is a distinct immunological time period for the mother, where the immune system requires modification so that it favors the growing embryo and at the same time protects mother from microbial threats. Uterine infection causes infertility and subsequently, it causes subfertility even after successful recovery from the diseases [46]. Endometrium is considered as the first line of defense that forms physical barrier against establishment of uterine diseases. There is evidence that endometrium may serve as a 'sensor', through the reflection of its transcriptome profile and capability of developing conceptus [47].

The interferon tau (IFNT) secreted from bovine embryo during implantation modulates the stimulation of interferon stimulated genes (ISGs) such as ISG15, MX1 in peripheral blood mononuclear cells (PBMCs) in pregnant cow [48]. Interferon tau (IFNT) is a novel type I interferon secreted by trophoblast cells in ruminants [49] and is recognized as an early sign of pregnancy [50]. Bidirectional embryo-maternal immune crosstalk is essential for the development, maintenance and progression of a fruitful gestation [51]. Recent studies show that bovine embryos of Day-7 interconnect with the epithelium of uterus and as well with immune cells by IFNT *in vitro* [40]. There is also evidence of existing the immunological dialogue between developing embryo of day 16-17 and maternal endometrium at the time of peri-implantation stage [48, 52]. In addition to these, recent study from our lab demonstrated that anti-inflammatory immunological environment is produced in bovine uterus by IFNT secreted from D-7 embryo *in vivo* [53]. All these results point out that IFNT secreted from growing embryo acts as the main intermediators for early embryo-maternal immune crosstalk for a successful pregnancy.

Peptidoglycan (PGN) is a very important part of cell wall of bacteria [54]. The chemical structure of PGN is very similar among Gram-positive and negative bacteria [55] but it is significantly thicker in case of gram-positive bacteria (20–80 nm) than that of in gram-negative bacteria (7–8 nm). It constitutes about 90% of the dry weight of gram-positive bacteria. PGN originated during lysis of bacteria, partly is taken up and recycled into new peptidoglycan precursors and remaining portions are

released into the extracellular space [56] which provoke an immune response and causes harmful effects on pregnancy maintenance in sheep [57], and humans [58].

Although the effect of PGN on ovarian activity [59] and interruption of early pregnancy [60] has been studied but there is no information about the effect of embryo secreted IFNT on bovine endometrial epithelial cells in presence or absence of peptidoglycan. Although immunosuppressive milieu is essential for an effective gestation, presence of uterine bacterial infection could interrupt embryo-maternal crosstalk [61]. Thus, I hypothesize that a low level of PGN remaining in uterus may have deleterious effect on IFNT mediated embryo-maternal crosstalk.

2. Material and methods

2.1. Ethics of experiment

All animal experiments stated in this manuscript were done following the Guiding Principles for the Care and Use of Research Animals as Promulgated by Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The protocol was duly permitted by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 27-74).

2.2. Experimental design

I used *in vitro* studies (Fig. 7) to investigate the effect of embryo conditioned medium (ECM) on the ISGs expression in the bovine endometrial epithelial cells (BEECs) in the presence or absence of PGN. BEECs after first passage were stimulated with ECM in the presence or absence of PGN (10 pg/ml) for 24 h. Cells without PGN and ECM served as a control. After that cells were analyzed for gene expressions by real-time PCR.

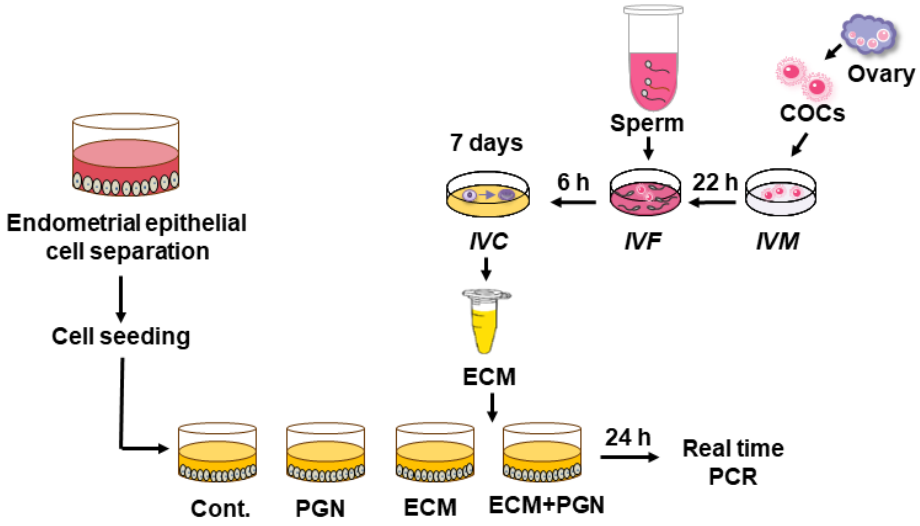

Figure 7. Schematic diagram of experimental design

2.3. Isolation of bovine endometrial epithelial cells (BEECs), culture, and treatment

The female reproductive tracts of cows at luteal phase (D 7-10) were opened (contra-lateral to the corpus luteum) and examined grossly for checking pus, redness, and presence of irregular color in the local abattoir (Hokkaido Livestock, Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan). Only healthy uteri were carefully chosen and brought to the laboratory in ice-cold normal saline comprising 1% penicillin-streptomycin (Gibco, Grand Island, NY) and 1% amphotericin B (Gibco). The horn of uterus, ipsilateral to the corpus luteum, was selected for separation and culture of endometrial epithelial cells ensuring the method as stated earlier [62] with slight alterations. Briefly, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco) media were used to culture the endometrial epithelial cells in complemented by 2.2% $NaHCO_3$ (Sigma–Aldrich, Steinheim, Germany), 0.1% gentamicin (Sigma–Aldrich), 1% amphotericin B, and 10% heat-inactivated fetal bovie serum (Bio Whittaker, Walkersville, MD). The cells were seeded in 25 cm2 culture flasks (Nalge Nunc International, Roskilde, Denmark) and cultured at 38.5ºC in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 48 h until growing cells reached to 70–80% confluence, at which point cells were given a second passage. The cells were trypsinized (0.05% trypsin-ethylene diamine tetraacetic acid (Amresco, Solon, OH), re-plated at a density of 1×10^5 viable cells/ml of 1.5 ml/well in 12well plates (Nalge Nunc International) and cultured until sub confluence. The BEECs from the second passage were supplemented with 5 ng/ml progesterone (P_4) (Sigma-Aldrich) and 3 pg/ml estradiol 17ß (E_2) (Sigma-Aldrich) as described previously [63]. The same concentrations of P_4 and E_2 were maintained throughout all experiments. The purity of the endometrial cells was evaluated by staining of immunofluorescence using a monoclonal antibody against cytokeratin (anti-Cytokeratin 8 + 18; ab53280, Abcam, Tokyo, Japan) as previously described [64] and by their characteristic epithelial morphology. The purity of the cultured endometrial cells was >98% (data not shown). When the BEECs grown to 80-90% confluence in 12-well plate, the cells were stimulated with ECM in presence or absence of PGN 10 pg/ml (Invivogen, USA). Cells without ECM and PGN served as a control.

2.4. Collection of oocyte and *in vitro maturation* (*IVM*)

As stated earlier [40], apparently good quality bovine ovaries were collected from the local abattoir (Hokkaido Livestock Co., Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan) and brought into the research laboratory in warm (temperature 39°C) physiological saline containing 1% penicillin-streptomycinm within 1–2 h of collection.

Follicular fluid was obtained following the procedure stated before [40]. Briefly, follicular fluid from distinct perceptible follicles of 2–8 mm in diameter were collected using 10 ml syringe with 18 G needle and transferred to 60 mm petridishes (Thermo Fisher Scientific, Roskilde, Denmark). After three times washes in oocyte collection medium (OCM; Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan), only cumulus oocyte complexes (COCs) showing a compact cumulus cell layer were selected and used for *in vitro* maturation for 22 h, in a group of 30-45 in 500 µl of 199 medium (HP-M199; Research Institute for the Functional Peptides Co., Ltd.) complemented with 10 µg/ml FSH (Folltropin-V, Bioniche Animal Health Inc., Belleville, Ontario, Canada) and 10% FCS in 4-well plates under mineral oil (Sigma-Aldrich) at 39^{o}C in 5% CO_2 with humidified air.

2.5. *In vitro fertilization* (*IVF*)

Three semen straws (0.5 ml) from the 3 different bulls were thawed at 37°C for 30 sec and washed for two times using fertilization medium IVF100 (Research Institute for the Functional Peptides Co., Ltd.) by centrifugation at 1200 rpm for 5 min. The supernatants were discarded leaving the sperm pellet. IVF100 was used to prepare a final concentration of 5×10^6 sperm/ml.

IFV was carried out following a previously stated procedure [65] with slight alterations. Briefly, droplets of 50 µl from IVF100 medium were prepared in petridish of 60 mm underneath mineral oil. COCs were washed for two times in IVF100 medium after maturation. Lastly, 15 COCs were co-incubated with sperm (5×10^6 /ml) in fertilization droplets for 6 h at 39^{o}C in 5% CO_2 in humidified air.

2.6. Production of embryo culture medium (ECM) *in vitro*

After 6 h of COCs-sperm co-incubation, probable zygotes (n = 40) were shifted to 400 µl droplets of BO-IVC medium (IVF-bioscience, UK) under mineral oil at 38.5ºC in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for *in vitro culture* (*IVC*) until Day 7 (Day 0=day of fertilization). On Day 7, *IVC* medium was collected and kept in -80ºC as embryo culture conditioned medium (ECM).

2.7. Isolation of RNA, cDNA synthesis and analysis of mRNA by real-time PCR

Total RNA from BEECs were extracted using the TRIzol (Invitrogen, Carlsbad, USA) reagent as previously described [66]. NanoDrop Spectrophotometer (2000c, Thermo Scientific, Waltham, MA, USA) was used to measure the concentration and purity of extracted RNA by the absorbance at 260/280 nm ratios. The total collected RNA was kept in RNA storage solution (Ambion, Austin, TX, USA) keeping at –80°C until making of cDNA [66].

cDNA synthesis was performed according to the protocol described previously [66]. First, DNase treatment was completed using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). 1 µg of the harvested RNA was incubated for 30 min at 37ºC in a thermal cycler (Eppendorf, Hamburg, Germany) with mixture of 1 µl RQ1 RNase-free DNase 10X reaction buffer and 2 µl of 1 µg/µl RNase-free DNase (Invitrogen, Carlsbad, CA, USA) to a final volume of 10 µl. To stop the action of DNAse, 1 µl RQ1 DNase Stop solution (20 mM EDTA) was added to the sample, and the mixture was incubated for 10 min at 65ºC. First-strand of cDNA was synthesized according to the commercial protocol described in the SuperScript™ II Reverse Transcriptase kit (Invitrogen). DNase-treated RNA was incubated at 65ºC for 5 min with a second cocktail containing 1.5 µl of 3 µg/µl random primer, 1.5 µl of 10 mM PCR Nucleotide Mix (dNTP) (Roche Diagnostics, Indianapolis, IN, USA) and nuclease-free water to a final volume of 18 µl. A third cocktail containing 6 µl of 5X First-Strand Buffer (5XFS), 3 µl of 0.1M dithiothreitol and 1.5 µl of 40 units/µl Ribonuclease Inhibitor Recombinant (Toyobo, Osaka, Japan), was added in each tube and followed by incubation at 42ºC for 2 min and further followed by the addition of 0.2 µl of 200 units/µl SuperScript™ II Reverse Transcriptase and the thermal cycler was

programmed at 25ºC for 10 min, 42ºC for 50 min and then 70ºC for 15 min [66]. The synthesized cDNA was kept at -30ºC.

mRNA expressions were quantified using synthesized cDNA by real-time PCR [66] with a MiniOpticon (Bio-Rad Laboratories, Tokyo, Japan). A total of 10 µl reaction mixture containing 2 µl/sample synthesized cDNA, 5 µl of QuantiTect SYBR Green PCR Master Mix (QIAGEN, Hilden, Germany), 0.2 µl of the targeted primer pairs (Table 1) and 2.8 µl nuclease-free water (Invitrogen) were prepared. The amplification program was run for 15 min activation at 95ºC as an initial step, followed by 40 cycles of PCR (15 sec denaturation at 95ºC, 15 sec annealing at 55–58ºC and 30 sec extension at 72ºC). A negative control sample using nuclease-free water was set aside with each run. Primer Express® Software v3.0.1 (Thermo Scientific) was used to design the primers pairs. Specificity of amplification was evaluated by melting curve at the end of each run. The calculated cycle threshold (Ct) values were normalized using ß-actin as an internal housekeeping gene by applying the Delta-Delta comparative threshold method [67]. The expression of ß-actin was stable in all experiments without significant difference between treatments.

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

Gene		Sequence of nucleotide (5'→3')	Accession no.	Fragment size (bp)
B-actin	Forward	TCACCAACTGGGACGACATG	AY141970.1	51
	Reverse	CGTTGTAGAAGGTGTGGTGCC		
ISG15	Forward	TCTGAGGGACTCCATGACGG	NM_174366	51
	Reverse	TTCTGGGCGATGAACTGCTT		
OAS1	Forward	TAGGCCTGGAACATCAGGTC	NM_178108	51
	Reverse	TTTGGTCTGGCTGGATTACC		
STAT1	Forward	CTCATTAGTTCTGGCACCAGC	AW289395	51
	Reverse	CACACGAAGGTGATGAACATG		
TNFA	Forward	CAAAAGCATGATCCGGGATG	NM_173966.3/	51
	Reverse	TTCTCGGAGAGCACCTCCTC		
IL1B	Forward	AATCGAAGAAAGGCCCGTCT	NM_174093.1	51
	Reverse	ATATCCTGGCCACCTCGAAA		
TLR2	Forward	CATGGGTCTGGGCTGTCATC	NM_174197.2	51
	Reverse	CCTGGTCAGAGGCTCCTTCC		

2.8. Statistical analysis

Data are presented as the mean ± SEM of 3 independent experiments. Statistical analyses were done using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). One-way ANOVA followed by Bonferroni's test was used to compare the mean differences among the groups. All values are presented as mean ± standard error of the mean (SEM). Data were considered to be statistically significant at $P<0.05$.

3. Results

3.1. PGN suppressed embryo culture media (ECM)-induced *ISGs* mRNA expression in BEECs *in vitro*

In the recently, no differences were observed between the impact of day 7 embryos and ECM on the upregulation of endometrial ISGs expression which suggest that these effects could be mediated by IFNT [68]. To study the effects of PGN on embryo-uterine communication, endometrial epithelial cells were cultured in ECM in the presence or absence of PGN (10 pg/ml). Real-time PCR analysis revealed that ECM stimulated gene expressions of *ISGs (OAS1* and *ISG15)* and *STAT1* in BEECs while, addition of PGN (10 pg/ml) to ECM significantly inhibited these effects (Fig. 8). Gene analysis result also showed that PGN (10 pg/ml) failed to produce clear inflammation and interestingly, no modulatory effects on pro-inflammatory genes (*TNFA* and *IL1B*), or *TLR2* mRNA expression were observed in all groups (Fig. 8).

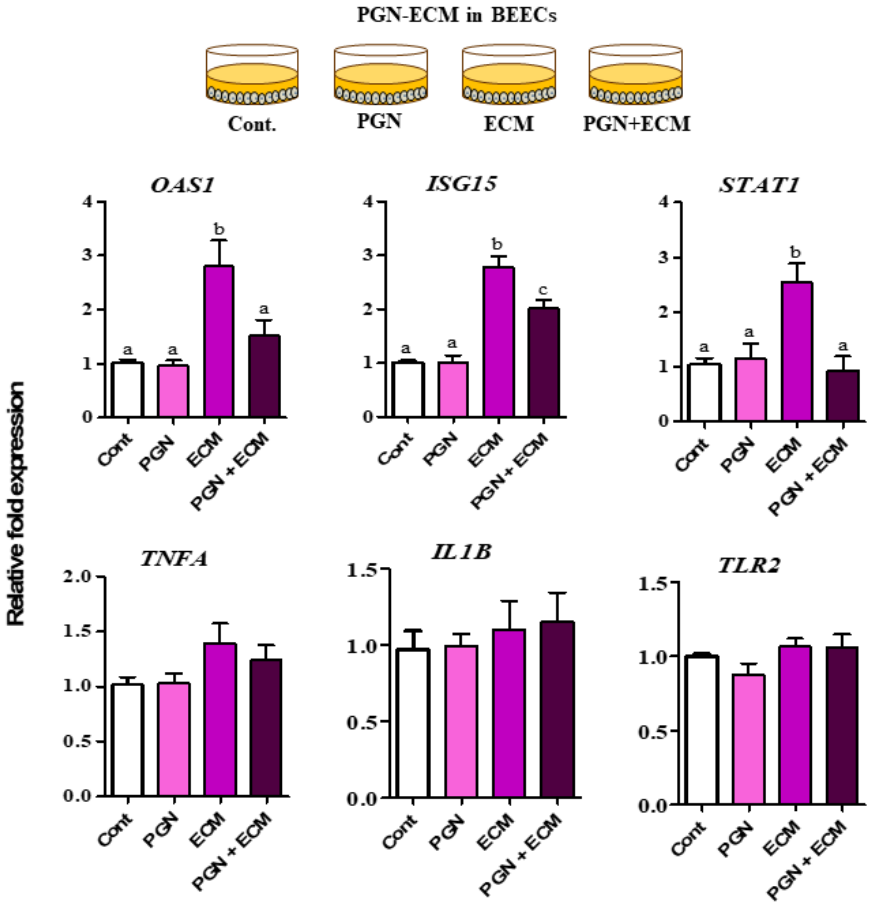

Figure 8. PGN suppressed ECM-induced *ISGs* and *STAT1* mRNA expression in BEECs *in vitro*.

Relative mRNA expressions of IFN-stimulated genes (*ISGs: OAS1* and *ISG15*), *STAT1*, pro-inflammatory

cytokine (TNFA and *IL1B*) and *TLR2* in BEECs. Data are presented as mean ± SEM of three independent experiments. Different letters denote a significant variance ($P< 0.05$) between the different groups.

4. Discussion

The present study shows an evidence that the bacterial cell wall component, PGN, interrupts the early embryo-maternal crosstalk through disrupting ISGs expressions induced by embryo conditioned medium in the bovine endometrium. Particularly, the results demonstrated that very low concentration of PGN (10 pg/ml) which is unable to induce clear inflammatory responses completely reduced the effects of embryo conditioned medium on the upregulation of ISGs in the bovine endometrial epithelium. The results proved that the presence of PGN residues with the bacteria in the bovine endometrium severely disturbs early MRP processes which may lead to pregnancy failure in cattle.

Recognition of embryo for establishment of pregnancy in cows starts very early when the external monolayer of trophectoderm of day 7 blastocyst commences to secrete a very little amount of IFNT into the lumen of uterine horn with production increases dramatically during conceptus elongation period [53]. In ruminants, embryo stimulates transcription of ISGs within the endometrium that are implicated for the creation of uterine receptivity and conceptus elongation and implantation [69]. Gene analysis results exhibited that embryo conditioned medium stimulated the upregulations of *ISGs* (*OAS1* and *ISG15*) expression in the endometrium epithelial cells but the presence of PGN at a minute concentration obviously suppressed this effect.

PGN possesses inflammatory properties [72] similar to LPS which suggest that PGN both from gram-positive and gram-negative bacteria is involved in the pathogenesis of inflammatory diseases and induces release of proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1 β (IL-1β) and IL-6 [73]. In my research work, I stretched mainly on very low concentrations of PGN that may be present in the uterine luminal environment after eliminating the uterine bacterial infection. Interestingly, qRT-PCR results demonstrated that a very low concentrations of PGN could not induce clear pro-inflammatory responses in endometrial epithelium, but their presence suppressed IFNT-stimulated ISGs expression in the BEECs. The bovine endometrium epithelial cells express TLR2 to recognize PGN [62]. However, in my study, I could not find any change of *TLR2* expression among different groups. One possible reason might be that PGN could be digested and transferred intracellularly by intracellular receptors like PGN-recognition proteins (PGRPs) [74] and Nod2 [75].

But this was not examined in my study, however, it would be interesting to investigate in future whether PGRPs is involved in the suppressive effect of PGN on ISGs expression in the endometrial epithelium.

It can be concluded that the presence of extremely low level of bacterial PGN could interfere embryo induced ISGs upregulation which might be one the reason for early pregnancy failure in cattle.

Chapter 2

Peptidoglycan suppresses endometrial expression of ISGs induced by interferon tau secreted from early embryo

1. Introduction

Early pregnancy losses are highly prevalent in lactating cows as evidenced that almost 40% embryos are missing within the first 2 weeks of pregnancy [76], and in many cases, it is thought to be associated with poor uterine receptivity [22]. A proper embryo-maternal communication is required for conditioning uterine endometrium and establishment of pregnancy [77]. Early embryonic death has been attributed to poor communication between the conceptus and uterine endometrium in cattle [50]. One major factor which can interrupt the uterine environment and therefore, the signals between the embryo and maternal immune system is the presence of infectious agents and inflammation of the uterus [78]. Endometrial epithelial cells express Toll-like receptors (TLRs) to recognize antigenic cell wall components of bacteria such as lipopolysaccharides (LPS) and peptidoglycan (PGN) [24]. Consequently, these cells secrete inflammatory cytokines including interleukins (IL), tumor necrosis factor-A (TNFA) and other inflammatory mediators which impair embryo development and implantation [79] and cause embryo resorptions [80].

Peptidoglycan (PGN) is an essential cell wall component of virtually all bacteria and constitutes about 90% of the dry weight of gram-positive bacteria [54]. PGN released during lysis of bacteria, partly is taken up and recycled into new PGN precursors and remaining portions are released into the extracellular space [81]. It has been reported that PGN could interrupt early pregnancy recognition [60] and pregnancy maintenance in sheep [57], and humans [58]. However, the detailed molecular mechanism explaining the deleterious effects of PGN at the embryo-maternal interface on early embryo recognition and establishment of pregnancy has not been well characterized.

Pregnancy recognition in cows is initiated by interferon tau (IFNT), which is a type I interferon and acts as a potential intermediator for maternal recognition of pregnancy (MRP) in ruminants and their interruption may potentially lead to pregnancy failure [50]. The vast majority of genes induced by IFNT in the bovine endometrium are classical *ISGs* which have crucial roles in modulation of uterine innate immunity, stromal remodeling, stimulating hyperplasia of the endometrial glands, and development of the uterine vasculature [82]. Other type I interferons such as IFNA and IFNB possess antiviral activities and also induce upregulation of ISGs [83]. Importantly, secondary infection by

bacteria or bacterial byproducts, LPS, can disrupt the interferon-induced upregulation of ISGs expressions during viral infections [83, 84]. Therefore, I hypothesized that PGN can interfere with early MRP in cows through disrupting IFNT-induced ISGs expression in the bovine endometrium. To this end, I investigated the impact of PGN on the upregulation of ISGs expressions in response to IFNT in bovine endometrial epithelial cells. However, embryo interactions *in vivo* with the bovine endometrium could be mediated by the luminal epithelium and/or glandular epithelium. Therefore, I used an *ex-vivo* bovine explant culture model to identify the location at which PGN may interferes with the embryo-maternal communications under conditions that more closely resemble those *in vivo*.

2. Materials and methods

2.1. Ethics statement

All animal experiments described in this manuscript were conducted following the Guiding Principles for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. The protocol was duly approved by the Committee on the Ethics of Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Permit number 27-74).

2.2. Experimental model

I used both *in vitro* and *ex-vivo* studies (Fig. 9) to investigate the effect of IFNT on the embryo-maternal immune crosstalk in the presence or absence of PGN. In the *in vitro* study (Fig. 9a), BEECs after first passage were stimulated with IFNT (1 ng/ml) in the presence or absence of PGN (10 pg/ml) for 24 h. In the *ex-vivo* study (Fig. 9b), endometrial explants were treated with IFNT (1 ng/ml) in the presence or absence of PGN (10 pg/ml) for 12 h. Cells/explant without PGN and IFNT served as a control. BEECs and endometrial tissues were analyzed for gene expressions by real-time PCR. OAS1 protein was detected by immunofluorescence in endometrial tissues.

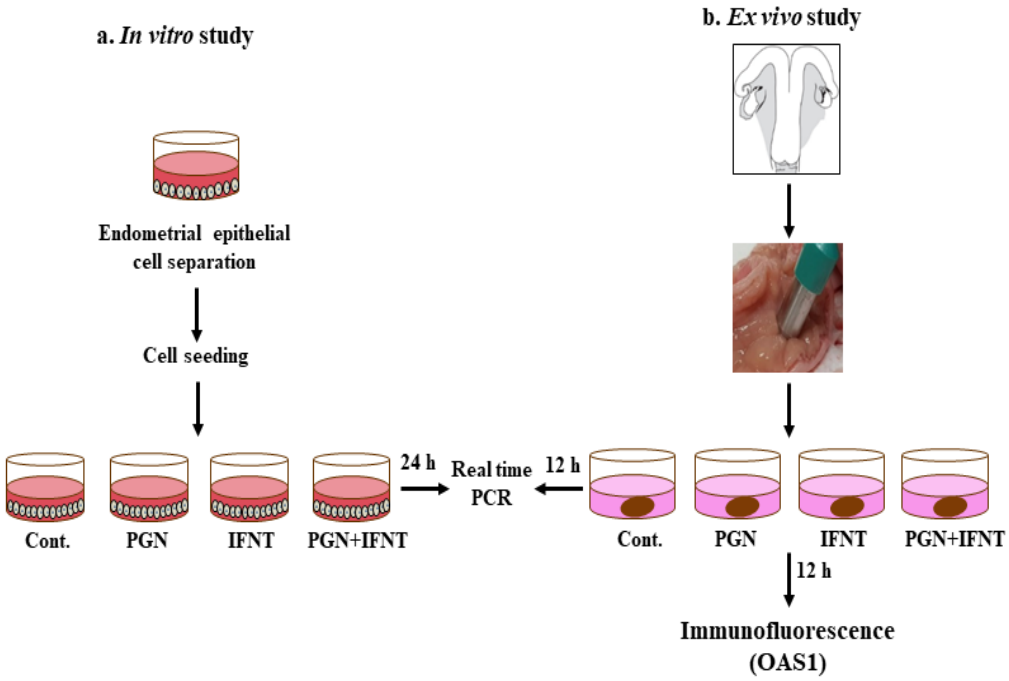

Figure 9. Schematic representation of the experimental design

2.3. Isolation and culture of bovine endometrial epithelial cells (BEECs)

Isolation and culture of BEECs were conducted as previously described [62] with minor modifications. Briefly, the bovine healthy uteri from the luteal phase (D 7-10) were collected and macroscopically examined to be free from pus, inflammation, and abnormal color. BEECs were isolated from the uterine horn ipsilateral to the corpus luteum, and cultured until cells reached 70–80% confluence, at which point cells were given a first passage. The cells were re-plated and cultured until sub-confluence. BEECs were supplemented with 5 ng/ml progesterone (P_4) (Sigma-Aldrich) and 3 pg/ml estradiol 17ß (E_2) (Sigma-Aldrich) as reported previously during early luteal phase [63] and were maintained throughout all experiments. The purity of the endometrial cells was >98% as evaluated by immunofluorescence staining using a monoclonal antibody against cytokeratin (anti-Cytokeratin 8 + 18; ab53280, Abcam, Tokyo, Japan) and by their characteristic epithelial morphology. When BEECs grown to 80-90% confluence, the cells were stimulated with IFNT at 1 ng/ml (recombinant bovine IFNT (rbIFNT) 2B, specific activity = $4.15X10^7$ U/mg; Zenoaq, Koriyama, Japan), in the presence or absence of PGN 10 pg/ml (Thermo Fisher Scientific, USA), and cultured for 24 h.

2.4. Preparation of bovine endometrial explants

The explants were collected and prepared according to the protocol previously described [85] with minor modifications. Briefly, uteri of the luteal phase (Day 7-10) were obtained and explants were collected from the intercaruncular areas of the endometrium using sterile 8 mm biopsy punches (Kai industries, Oyana, Japan), and cultured in Roswell Park Memorial Institute medium (RPMI 1640; Sigma-Aldrich), supplemented with 10% heat-inactivated, endotoxin-free, fetal bovine serum (Bio Whittaker, Walkersville, MD). The explants were treated with IFNT (1 ng/ml) in the presence or absence of PGN (10 pg/ml) and cultured for 12 h.

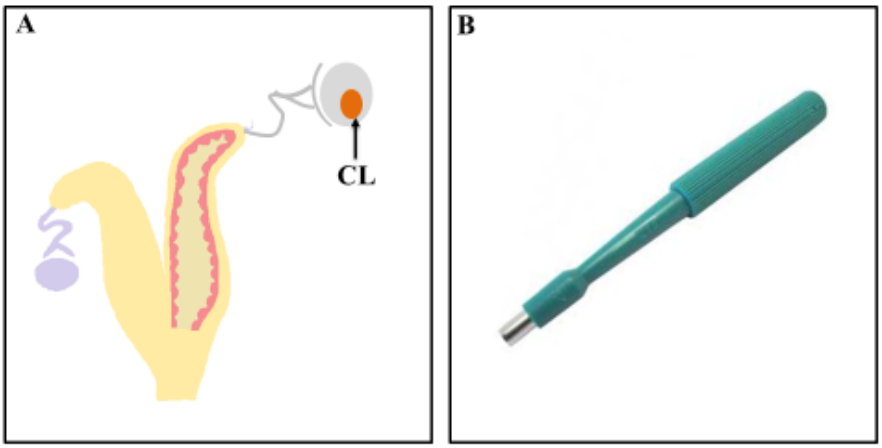

Figure 10. A. Diagram showing the ipsilateral horn of uterus containing ovary with corpus luteum (CL) of D 7-8 of bovine reproductive tract. **B.** Biopsy punch (8 mm diameter).

2.5. Immunofluorescence (IF) analysis of OAS1 in endometrial explants

Endometrial explants were incubated with IFNT (1 ng/ml) in the presence or absence of PGN (10 pg/ml) for 12 h. Then, immunofluorescence was performed as previously described [85]. Polyclonal anti-OAS1 primary antibody (1:50; ab86343, abcam, UK) was used with all tissue sections except for negative control. The sections were stained with DAPI (1:50, 340-07971, Dojindo Laboratories, Japan) followed by labeling with the secondary antibody (1:200, goat anti-rabbit IgG conjugated with Alexa Fluor, Invitrogen, Thermo Fisher Scientific). The fluorescence signal was captured using an all-in-one fluorescence microscope (Keyence, BZ-X800, Osaka, Japan) using the BZ-X TexasRed (OP-87765) and BZ-X DAPI (OP-87762) filters set for red and blue wavelengths

respectively. Mean fluorescence intensity of OAS1 protein expressions were quantified by the corrected total cell fluorescence (CTCF) using ImageJ software (Version 1.51j8) and arbitrary fluorescence units (AU) were calculated.

2.6. Quantitative real-time PCR

RNA extraction, cDNA synthesis and quantitative real-time PCR were performed following the previously described protocol [66]. Briefly, total RNA was extracted using the Trizol reagent (Thermo Fisher Scientific). Total RNA concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany), and stored in RNA storage solution (Ambion, Austin, TX, USA) at −80°C until cDNA conversion. The cDNA synthesis was carried out as previously described [66] and the synthesized cDNA was stored at −30°C. Quantitative real-time PCR of target genes (Table 2) was performed using QuantiTect SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) by an iCycler iQ (Bio-Rad Laboratories, Tokyo, Japan). The amplification program was set up according to a previously described protocol [66]. The calculated cycle threshold values were normalized using B-actin as the internal housekeeping gene by applying the Delta-Delta comparative threshold method to quantify the fold change between samples.

2.7. Statistical analysis

Data are presented as the mean ± SEM of 3 independent experiments. Statistical analyses were done using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). One-way ANOVA followed by Bonferroni's test was used to compare the mean differences among the groups. All values are presented as mean ± standard error of the mean (SEM). Data were considered to be statistically significant at $P<0.05$.

Table 2. List of primers used in real-time PCR

Gene		Sequence of nucleotide (5'→3')	Accession no.	Fragment size (bp)
B-actin	Forward	TCACCAACTGGGACGACATG	AY141970.1/	51
	Reverse	CGTTGTAGAAGGTGTGGTGCC		
ISG15	Forward	TCTGAGGGACTCCATGACGG	NM_174366	51
	Reverse	TTCTGGGCGATGAACTGCTT		
OAS1	Forward	TAGGCCTGGAACATCAGGTC	NM_178108	51
	Reverse	TTTGGTCTGGCTGGATTACC		
STAT1	Forward	CTCATTAGTTCTGGCACCAGC	AW289395	51
	Reverse	CACACGAAGGTGATGAACATG		
TNFA	Forward	CAAAAGCATGATCCGGGATG	NM_173966.3/	51
	Reverse	TTCTCGGAGAGCACCTCCTC		
IL1B	Forward	AATCGAAGAAAGGCCCGTCT	NM_174093.1	51
	Reverse	ATATCCTGGCCACCTCGAAA		
PGES	Forward	AAAATGTACGTGGTGGCCGT	NM_174443.2	51
	Reverse	CTTCTTCCGCAGCCTCACTT		
TLR2	Forward	CATGGGTCTGGGCTGTCATC	NM_174197.2	51
	Reverse	CCTGGTCAGAGGCTCCTTCC		
IFNAR1	Forward	GCGAAGAGTTTCCGCAACAG	NM_174552.2	51
	Reverse	TCCAAGGCAGGTCCAATGAC		
IFNAR2	Forward	TCGTATGTTGCGCCTGTTCT	NM_174553.2	51
	Reverse	GTCCGTCGTGTTTACCCACA		

3. Results

3.1. PGN suppressed IFNT-induced *ISGs* and *STAT1* mRNA expression in BEECs

To further study the detailed suppressive effect of PGN on IFNT-triggered ISGs gene expressions, BEECs were stimulated either with exogenous recombinant bovine IFNT (rbIFNT) (1 ng/ml), PGN (10 pg/ml), or combinations of both for 24 h. The results showed that IFNT stimulated *ISGs (OAS1* and *ISG15)* and *STAT1* mRNA expression while PGN suppressed this effect (Fig. 11). Additionally, no changes in gene expression of pro-inflammatory cytokines (*TNFA* and *IL1B*), *or TLR2* were found (Fig. 11).

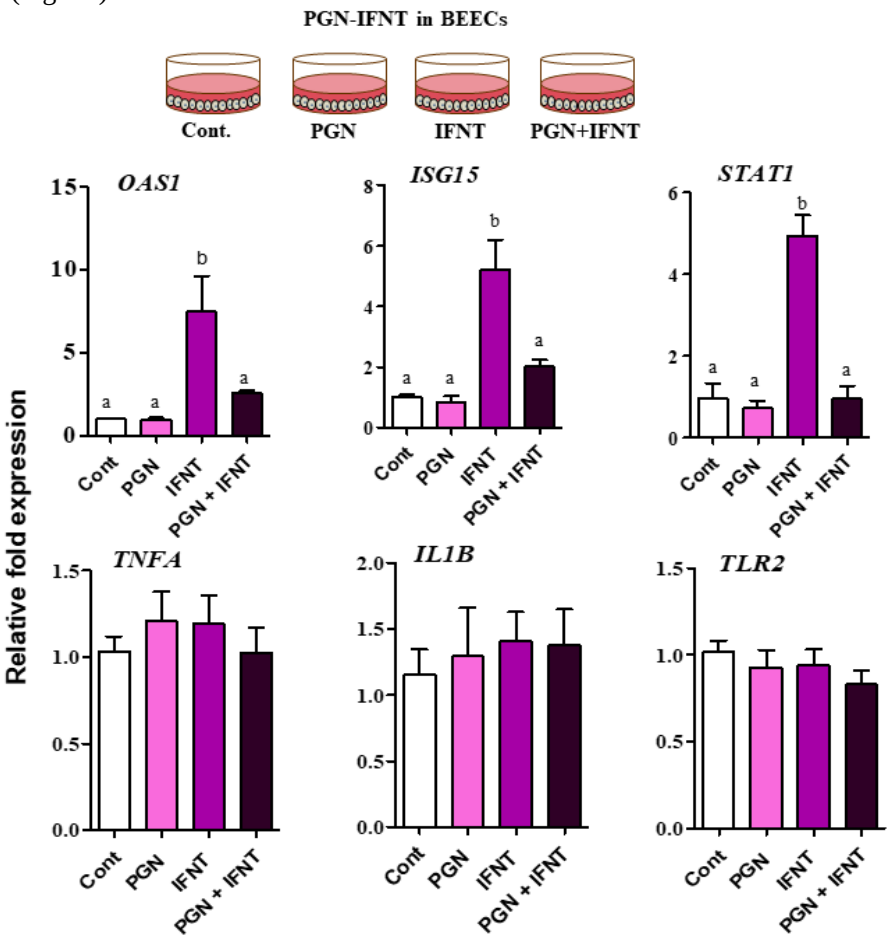

Figure 11. PGN suppressed IFNT-induced *ISGs* and *STAT1* mRNA expression in BEECs. Relative mRNA expressions of IFN-stimulated genes (*ISGs: OAS1* and *ISG15*), a *STAT1*, pro-inflammatory cytokine (*TNFA* and *IL1B*) and *TLR2* in BEECs. Data are presented as mean ± SEM of three independent experiments. Different letters denote a significant variance ($P< 0.05$) between the different groups.

3.2. PGN inhibited IFNT-induced *ISGs* and *STAT1* mRNA expressions in endometrial explants

Results from real-time PCR analysis revealed similar response pattern in endometrial explants. As anticipated, PGN significantly suppressed the IFNT-triggered *ISGs (OAS1* and *ISG15)* as well as *STAT1* gene expression (Fig. 12). However, no changes in pro-inflammatory cytokines (*TNFA* and *IL1B*), *TLR2, or IFNA receptor type1 (IFNAR1) or type2 (IFNAR2)* mRNA transcripts were observed (Fig. 12).

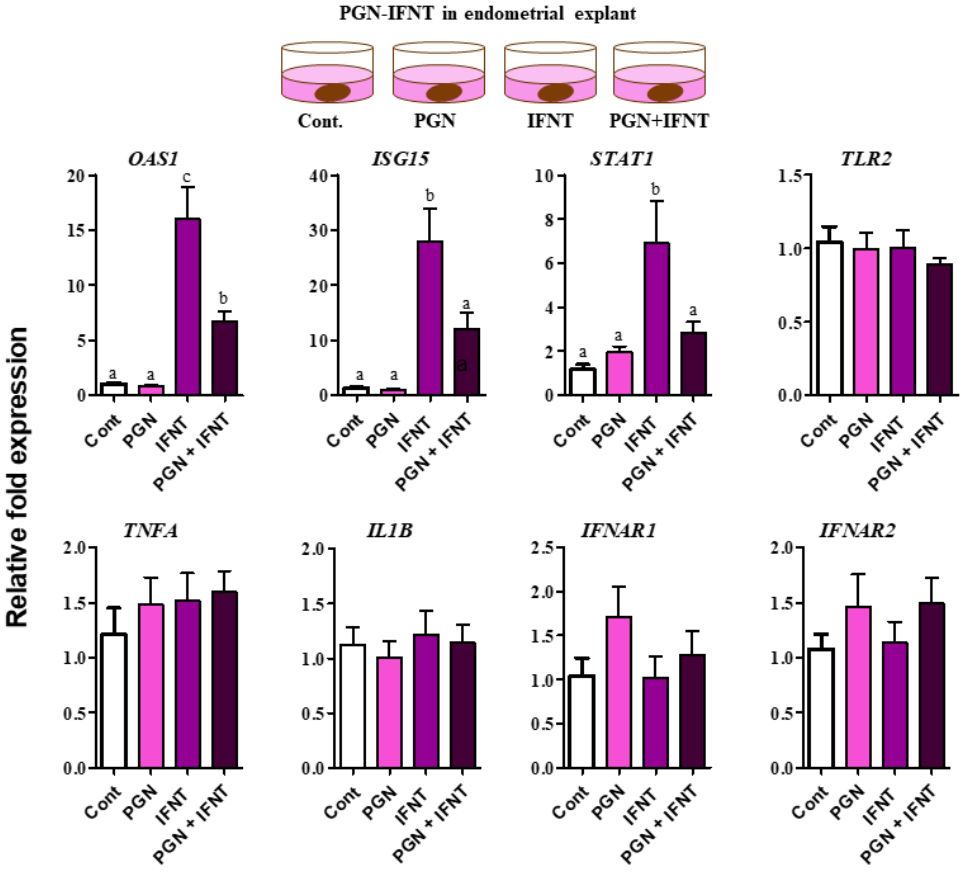

Figure 12. PGN inhibited IFNT-induced *ISGs* and *STAT1* mRNA expressions in endometrial explants. Relative mRNA expressions of IFN-stimulated genes (*ISGs: OAS1* and *ISG15*), *STAT1*, pro-inflammatory cytokine (*TNFA* and *IL1B*), *TLR2, IFNA receptor type1 (IFNAR1)* or *type2 (IFNAR2)*. Data are presented as mean ± SEM of four independent experiments. Different letters denote a significant variance (P< 0.05) between the different groups.

3.3. Immunofluorescence analysis of OAS1 protein in endometrial explants

As illustrated in Fig. 13, OAS1 protein was detected in paraffine-embedded endometrial tissues. Basically, the bovine endometrial tissue expresses OAS1 protein. Additionally, IFNT induced intensive OAS1 protein expression throughout the whole luminal epithelium (LE) and some resident immune cells with weak expressions in the deeper glandular epithelium (GE) and stroma of the endometrial tissue. While, PGN clearly suppressed OAS1 protein expression in endometrial tissues.

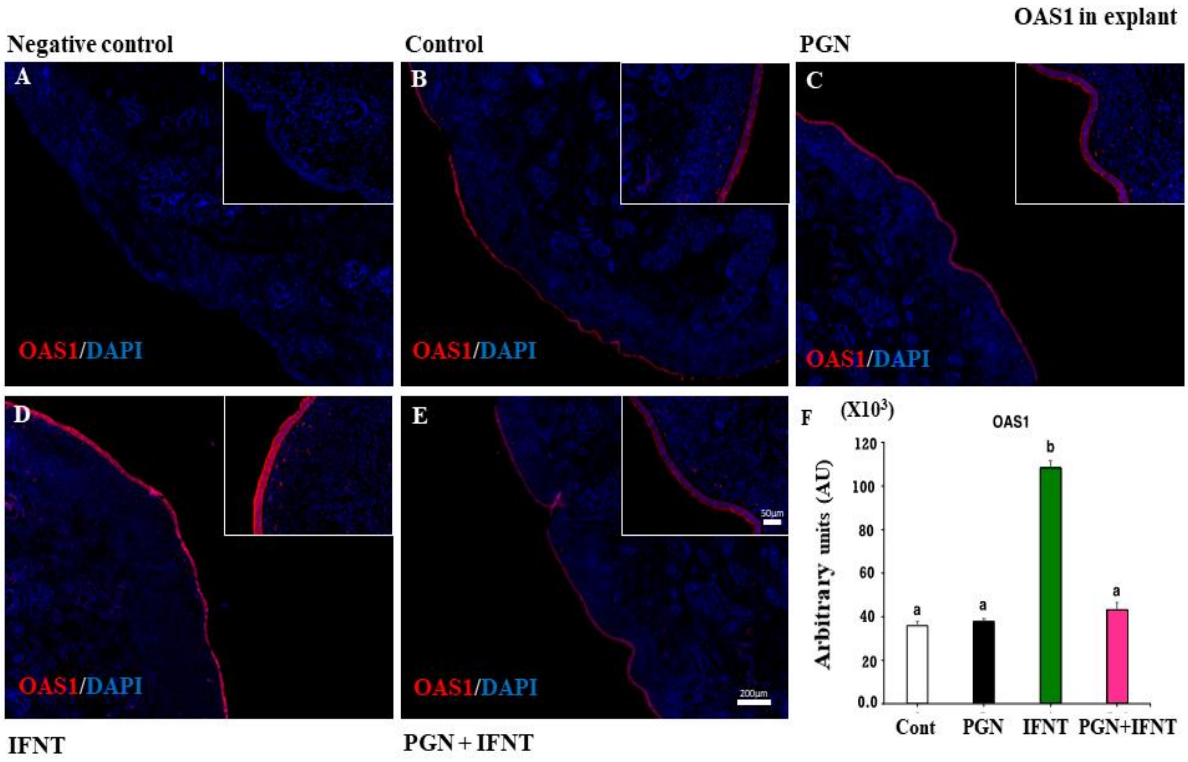

Figure 13. Immunofluorescence activity of OAS1 protein localizes in the luminal epithelium of the bovine endometrium, at which IFNT clearly increases its expression but PGN completely blocks IFNT effect. Endometrial explants were incubated with IFNT (1 ng/ml) in the presence or absence of PGN (10 pg/ml) for 12 h. Overlay images of OAS1 localization and nucleus in explant after incubation (A) negative control, (B) without PGN or IFNT (control), (C) with PGN, (D) IFNT, (E) PGN + IFNT by immunofluorescence labeling with Alexa-Fluor-conjugated anti-OAS1 antibody. DAPI stains the nucleus. The primary antibody was omitted in the negative control sections. Insert images show higher magnification of the sections. Bars, 50µm, 200µm. (F) Mean fluorescence intensity of OAS1 protein expression as quantified by the corrected total cell fluorescence (CTCF) using ImageJ software (Version 1.51j8) and

expressed in arbitrary fluorescence units (AU). Data are presented as mean ± SEM of three independent experiments. Different letters denote a significant variance ($P < 0.05$) between the different groups.

4. Discussion

The present study provides the evidence that the bacterial byproduct, PGN, disrupts ISGs expressions induced by IFNT in the bovine endometrium. In particular, the results showed that low concentration of PGN (10 pg/ml), unable to raise some clear inflammatory responses, completely suppressed the effects of IFNT on the upregulation of ISGs in the bovine endometrial epithelium *in vitro* and *ex-vivo*. The results demonstrated that the presence of PGN residues with the bacteria in the bovine endometrium severely disrupts early MRP processes which may lead to pregnancy failure in cattle.

Embryo recognition for establishment of pregnancy in cows begins very early when the outer monolayer of trophectoderm of day 7 blastocyst starts to secrete a minute amount of IFNT into the uterine horn lumen, with production increasing dramatically during conceptus elongation period [53]. IFNT, the signal for MRP in ruminants, stimulates transcription of ISGs within the endometrium that are implicated in the establishment of uterine receptivity and conceptus elongation and implantation [69]. Gene analysis results showed that recombinant IFNT induced upregulations of ISGs expression in the endometrial epithelium, while PGN at a low concentration clearly suppressed this effect. Of note, IFNT induced intensive OAS1 protein expression throughout the whole luminal epithelium (LE) with faint expressions in the deeper glandular epithelium (GE) and stroma of the uterine tissue. The pattern of OAS1 expression in the LE may be associated with the presence of IFNAR1 and IFNAR2 receptors in the endometrial epithelium during early luteal phase (Day 7-10) [86] Real time-PCR results showed that PGN did not change mRNA expressions of *IFNAR1* and *IFNAR2* receptors for IFNT in endometrial tissues. While, PGN clearly suppressed IFNT-induced OAS1 expression in the *ex-vivo* model of endometrial explants. These findings suggest that PGN interferes with embryo-uterine crosstalk mainly *via* disrupting IFNT-induced ISGs expression in the LE of bovine endometrium.

All type I IFNs, including IFNT, activate the canonical Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway [87] to stimulate the classical ISGs [69]. Most recently, it was shown that the infection with bovine viral diarrhea virus interferes with early pregnancy recognition through disrupting STAT pathway for IFNT and consequently perturbs ISGs

expressions in BEECs [71]. I, focused on STAT1 mRNA expressions known to be initiated following activation of the type I IFNs receptors in the endometrium. Gene analysis results showed that IFNT stimulated the gene expression of *STAT1* in BEECs and explants, while PGN perturbed IFNT-induced *STAT1* gene expressions in the endometrial epithelium. The data suggest that PGN suppresses IFNT-induced ISGs expression in the bovine endometrium mainly via disrupting JAK-STAT pathway which consequently interferes with embryo-maternal recognition in the uterus. Further detailed investigations are needed to investigate the impact of PGN on INFT-triggered STAT1 phosphorylation as an upstream regulatory pathway for ISGs expressions in the bovine endometrium.

The detrimental impact of the presence of high levels of bacterial byproducts in the uterus and consequently released inflammatory mediators on the embryo competence and pregnancy establishment have been well-recognized [79]. The *in vivo* administration of high levels of PGN (30-60 µg/kg) on day 5 after mating disrupted early pregnancy mainly *via* the release of inflammatory mediators, such as prostaglandin PGF (2alpha) in ewes [60]. Moreover, it has been shown that the bacterial infection of the uterus persists to reduce conception rates after the resolution of infection as observed when cows are inseminated several weeks after the infection was resolved [88]. One reason is that the uterine infection causes a persistent change in the transcriptome of granulosa cells, for 6 weeks after resolution of the uterine infection [89]. In our model, we focused mainly on extremely low concentrations of PGN that may reside in the uterine milieu after eliminating the uterine infection. Nevertheless, my research results showed that very low concentrations of PGN were unable to induce clear pro-inflammatory responses in endometrial epithelium, but they suppressed IFNT-induced ISGs expression in the bovine endometrium. The bovine endometrium epithelial cells express TLR2 to recognize PGN [63]. Earlier study from our group showed that TLR2 immunoreactivity in histochemistry was observed in both LE and GE of bovine endometrium during luteal phases [66], while, the current study showed that OAS1 protein was expressed mainly in the LE. Therefore, I suggest that the LE of the bovine endometrium is the main site at the embryo-maternal interface where PGN disrupts embryo-derived IFNT signals.

Collectively, the research findings demonstrate that presence of extremely low levels of PGN interferes with IFNT signals for ISGs expression in the LE of bovine endometrium, possibly leading to pregnancy failure. This study may help in the better understanding of the molecular mechanism of the

early embryonic losses caused by of the presence of traces of PGN at the embryo-maternal interface and their deleterious effects on the early embryo recognition and pregnancy establishment in cows.

General discussion and conclusion

Acceptance of semi-allogenic embryo by maternal immune system is considered as the prerequisite for successful pregnancy which is achieved by a complex and orchestrated bidirectional communication between mother and embryo. The uterine environment needs to be in a favorable condition so that the developing embryo can continue towards a successful pregnancy. IFNT is secreted from the trophoectoderm of embryo and acts as a signaling molecule for embryo-maternal crosstalk during early pregnancy. IFNT is recognized as a biomolecule which is known as primary agent for maternal recognition of pregnancy and acts to continue the pregnancy [49]. Early embryonic losses in cattle on D 8-17 is thought to be due to interrupted communication between the embryo and mother [90]. Presence of microbial agents and or inflammatory condition of the uterus could be one of the main factors that can affect the maternal environment to be more furious for growing embryo and ultimately disruption of signals between fetal side and maternal side [78].

Bacterial contamination of uterus soon after parturition is inevitable. Postpartum uterine environment supports growth of a variety of aerobic and anaerobic bacteria. The process with contamination, elimination and subsequent re-contamination is complex and is not well understood [48]. This concept becomes more complex with the fact that the gravid uterus is not sterile, as thought before for many years, and may be colonized with numerous bacterial species including *Trueperella pyogenes* [91]. Statistically, the most prevalent pathogens are *E. coli* (37%) and *Actinomyces pyogenes* (49%) [4]. It is reported that persistent *A. pyogenes* (now *Trueperella pyogenes*) infection after 21 days postpartum reduces conception rates at the first postpartum service [92]. All these findings support my hypothesis in that PGN produced during the lysis of bacteria [56] might have deleterious effect on IFNT mediated immunological crosstalk.

Until now, there is little or no information concerning the effects of IFNT on bovine endometrial epithelial cells in presence of peptidoglycan during early pregnancy in the cow. Therefore, this is the first report to see the interaction of IFNT with bovine endometrial epithelial cells in the presence of peptidoglycan. The study demonstrates that bacterial cell wall byproduct, PGN, hampers the early embryo-maternal communication *via* disturbing embryo secreted IFNT-induced ISGs expression in the bovine endometrial epithelial cell *in vitro* and endometria explant *ex-vivo*. The data proved that very low concentration of PGN (10 pg/ml) could not produce clear inflammatory response

but disturbed the upregulation of ISGs stimulated by IFNT in the bovine endometrium *in vitro* as well as in *ex-vivo*. The results indicated that bacterial presence, in particular, bacterial byproduct, PGN in the bovine endometrium negatively affect MRP which may be one of the many reasons for early pregnancy losses in the cow.

Process of embryo recognition for successful pregnancy begin at a very early when the outer monolayer of trophectoderm of D-7 blastocyst starts to produce a little amount of IFNT within the uterine environment and the production goes to higher when the conceptus become elongated [53]. IFNT, the key molecule for MRP in cattle, induces ISGs transcription in bovine endometrium which thought to be responsible for creation of uterine receptivity, conceptus elongation, and finally implantation [69]. Gene analysis results indicated that ISGs expression were stimulated by embryo conditioned medium induced in the endometrial epithelium whereas, presence of PGN at a low dose obviously curbed this effect. In addition to that, Real time PCR results demonstrates that ISGs were upregulated by rbIFNT in the endometrium but presence of PGN at minute concentration completely disturbed these effects. Pattern of OAS1 protein was intensely higher and clear in luminal epithelium (LE) while very scanty expressions was observed in the stromal cell and glandular epithelium (GE) of endometrial explant. IFNAR1 and IFNAR2 receptor in endometrial epithelium may be involved in expression of OAS1 protein during early luteal stage (D 7-10) [87]. The result showed that mRNA expression of both *IFNAR1* and *IFNAR2*, the receptor of IFNT, were not affected by PGN. Whereas, OAS1 protein expressions was evidently suppressed by PGN in endometrial explant *ex-vivo*. These findings demonstrate that very low concentration of PGN interrupts embryo-maternal communication through suppression of IFNT-stimulated ISGs expression in the luminal epithelial cells of endometrium.

IFNT, including all type I IFNs activate Janus-kinase signal transducer and activator of transcription (JAK-STAT) signaling pathway [88] and induce ISGs expression [70]. Very recently, it was demonstrated that bovine viral diarrhea infection interrupts early pregnancy *via* disturbing STAT signaling pathway for IFNT and eventually inhibits expression of ISGs in bovine endometrium [71]. In my research, qRT-PCR result showed that IFNT induced *STAT1* expression both in BEECs *in vitro* and endometrial explant in *ex- vivo* but presence of PGN suppressed IFNT-provoked *STAT1* expression in

both models. The results indicate that PGN disturbed ISGs expression by IFNT via interfering JAK-STAT pathway.

It is well recognized that bacterial byproducts in a high concentration have harmful effects on uterus and induces release of inflammatory mediators that causes detrimental effect on the embryo acceptance and establishment of pregnancy [79]. It has been reported that PGN (30-60 µg/kg) administered after 5 days of mating induces release of inflammatory mediators such as prostaglandin PGF (2 alpha) and interrupted early pregnancy in ewes *in vivo* [60]. In my model, I focused very low level of PGN which may be present within the uterine environment after recovery from uterine bacterial infection. Moreover, my results showed that very low level of PGN could not produce any detectable inflammatory responses in endometrial epithelium but they were able to suppress ISGs expression induced by IFNT in the bovine endometrium. Bovine endometrial epithelium express TLR2 to recognize PGN [63]. Previous work from our group demonstrated that TLR2 was localized in LE and GE by immunohistochemistry [66]. In my study, OAS1 protein was found mainly on LE. Therefore, I propose that LE is the main site where embryo-maternal crosstalk mediated through embryo secreted IFNT is disrupted by PGN.

Taken all together, my results showed that the presence of a very low level of PGN suppressed the expression of ISGs induced by embryo derived IFNT through the STAT1 signaling pathway in the LE of bovine endometrium *in vitro* as well as in *ex-vivo* model which might be one of the main causes for early pregnancy failure in cattle as summarized in Fig. 14. These finding may help to reveal the molecular mechanism of early embryonic losses in cow caused by the presence of extremely low concentration of PGN at the embryo-maternal interface and their detrimental effects on MRP process and establishment of pregnancy in cows.

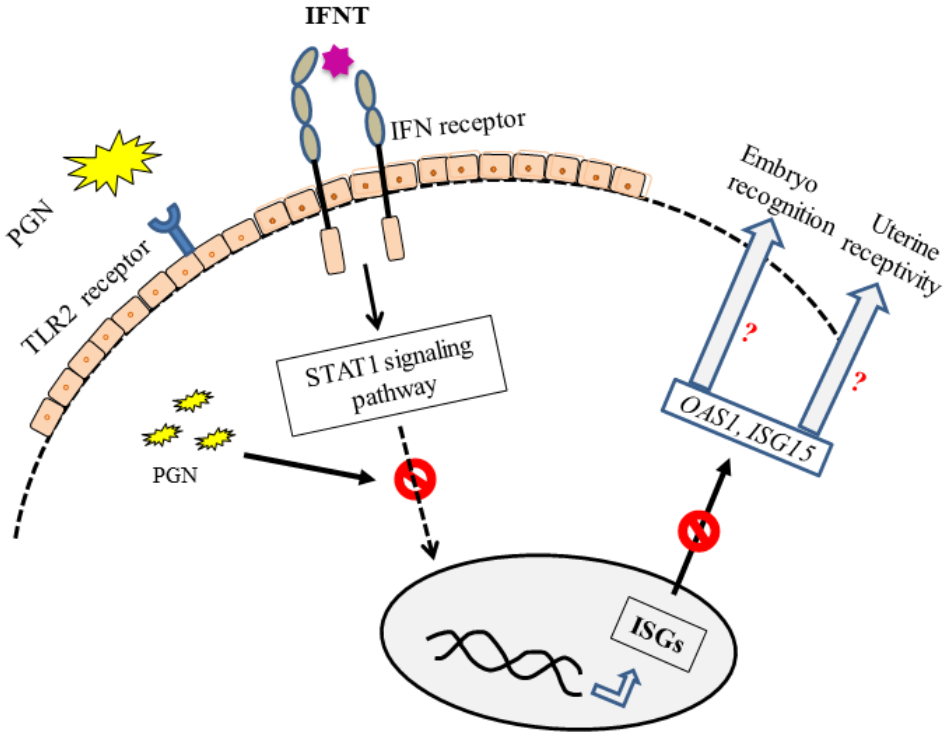

Figure 14. Summarized illustration of possible outcome of effects of PGN on IFNT signaling during early maternal recognition of pregnancy. IFNT binding with IFNT receptor stimulates ISGs expression through STAT1 signaling pathway. Presence of PGN interrupts STAT1 signaling leading to decreased transcription of ISGs which causes failure of maternal embryo recognition and poor uterine receptivity.

Summary

Bidirectional embryo-maternal communication is critically important for appropriate maternal recognition of pregnancy and thereby establishing a successful gestation. Any alteration in maternal recognition of pregnancy may result in early embryonic loss. Bio-molecular dialogue between the embryo and mother should be started by developing bovine embryo in order to establish a suitable uterine environment that could support the growth of developing embryo leading to successful pregnancy. In case of ruminant, interferon tau (IFNT) is secreted from the trophoectoderm and acts as a signaling molecule for recognition of pregnancy.

After parturition, natural orifice such as vaginal opening remains open. Soon after parturition, bacteria present in feces, urine, and bedding materials can enter into the uterus and may cause infection. Moreover, biodegradable materials present within the uterus after partition serve as a good media for the growth of a variety of aerobic and anaerobic bacteria. These perceptions become more complicated with the fact that the gravid uterus is not germ-free as it has been thought for many years, and it may be colonized with numerous bacteria including *Trueperella pyogenes.* These bacteria persistently remain within the uterus and continue to grow. As a result, peptidoglycan (PGN), the important cell component of bacteria is continuously producing as byproduct which remains within the uterine environment. It has been reported that PGN administered after 5 days of mating interrupts early pregnancy in ewes *in vivo*. Therefore, I hypothesized that presence of PGN within the uterine environment has deleterious effects on embryo-derived IFNT mediated embryo-maternal immune crosstalk.

In chapter 1, I used embryo conditioned media (ECM) to see the effect of embryo culture media on bovine endometrial epithelial cells (BEECs) in presence or absence of PGN. I used this model to mimic *in vivo* real situation where the uterus contains persistent bacterial infection and the embryo is coming and/or transferring to that uterus. Healthy bovine uteri of luteal phage (D 7-8) were collected from local slaughterhouse for isolation and culture of epithelial cells. When the cells from second passage reached at 80-90% confluence, then cells were cultured in ECM in presence or absence of PGN for 24 h in a 38.5^{o}C incubator with 5% CO_2 in humidified air. Cells without ECM or PGN served as control. BEECs were analyzed to see the *mRNA* expressions of *ISGs* (*OAS1* and *ISG15*) and *STAT1*, a

key factor for IFNT signaling by real-time PCR. Real-time PCR result demonstrated that ECM induced *mRNA* expression of *ISGs* (*OAS1* and *ISG15*) and *STAT1*, a crucial molecule for IFNT signaling. On the other hand, presence of PGN significantly suppressed the upregulation of *ISGs* (*OAS1* and *ISG15*) and *STAT1* in BEECs. Surprisingly, no modulatory effects on the *mRNA* expression of pro-inflammatory cytokine (*TNFA* and *IL1B*) or *TLR2* were observed by PGN. The findings strongly suggest that ECM contains IFNT secreted from cultured embryos, and that PGN blocks IFNT signaling in BEECs.

In chapter 2, I investigated the direct effect of IFNT, secreted from embryos, on BEECs *in vitro* and endometrial explants *ex-vivo* in presence or absence of PGN. Similar to chapter 1, when the cells from second passage reached 80-90% confluence, cells were stimulated with IFNT in the presence or absence of PGN for 24 h. Healthy uteri of the same stage were obtained and using an 8 mm biopsy punch, disks of endometrial tissue (2 mm thick) were separated from the intercaruncular regions ipsilateral *to* the corpus luteum. Then explants were cultivated in Roswell Park Memorial Institute (RPMI)-1640 media supplemented with 10% fetal bovine serum (FBS) with IFNT in the presence or absence of PGN for 12 h in a 38.5^{o}C incubator with 5% CO_2 in humidified air. Cells/explants without IFNT and PGN served as control. BEECs and endometrial tissues were analyzed for *mRNA* expressions of *ISGs* (*OAS1* and *ISG15*) and *STAT1*, a key factor for IFNT signaling, by real-time PCR. OAS1 protein was identified by immunofluorescence in LE of endometrial explants. Gene analysis results showed that IFNT stimulated the expression of interferon stimulated genes (*ISGs: OAS1, ISG15*) and *STAT1* in BEECs *in vitro* and explants *ex-vivo*. While the presence of PGN significantly suppressed the upregulation of *ISGs* and *STAT1* in both models. Interestingly, PGN did not stimulate *mRNA* expressions of the pro-inflammatory cytokines (*TNFA* and *IL1B*) or *TLR2* in BEECs or explants. I also analyzed *interferon receptor 1 (IFNAR1)* and *IFNAR2* to see the any fold expression changes in explant. Real-time-PCR result showed no changes of *mRNA* expression of *IFNAR1* and *IFNAR2* among all groups. Further, OAS1 protein expression was detected using immunofluorescence (IF) staining in the luminal epithelia endometrial tissue, and PGN suppressed the IFNT-triggered intensive OAS1 protein expression throughout the whole luminal epithelium.

It can be concluded that the findings of this study demonstrate that the presence of low levels of PGN suppresses early embryo-derived IFNT signals for *ISGs* expression in the bovine endometrium and thereby, interferes with endometrial epithelium receptivity and early embryo-maternal crosstalk in cows, which leads to pregnancy failure.

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和 文 要 約

初期胚と母体の双方向のコミュニケーションは、妊娠を適切に母体で認識し、妊娠を成功させるために非常に重要である。母体の妊娠認識に何らかの変化があると、初期胚が失われる可能性がある。初期胚と母体の間の生体分子による対話は、発育中の初期胚の成長をサポートして妊娠の成功に向けて適切な子宮環境を確立するために必要となる。反すう動物の場合、インターフェロンタウ（IFNT）が栄養外胚葉から分泌され、妊娠を認識するためのシグナル伝達分子として機能している。

ウシでは分娩後、膣の開口部は開いたままである。分娩後すぐに、糞便、尿、寝わらなどに存在する細菌が子宮に侵入し、感染症を引き起こす可能性がある。さらに、分娩後の子宮内に存在する生体性の残渣は、さまざまな好気性細菌および嫌気性細菌の増殖のための優れた媒体として機能する。重要なことは、妊娠子宮が無菌ではなく、*Trueperella pyogenes* を含む多数の細菌がコロニーを形成する可能性があることで、とても複雑な環境となることである。これらの細菌は子宮内に持続的に残り、増殖し続ける。その結果、細菌の重要な細胞主成分であるペプチドグリカン（PGN）は、子宮内に残る副産物として継続的に生成される。交配の 5 日後に投与された PGN は、*in vivo* で雌羊の妊娠初期を中断させることが報告されている。したがって、私は子宮内環境の PGN の存在が初期胚由来の IFNT を介した胚–母体の免疫クロストークに有害な影響を与えると仮定した。

第 1 章では、初期胚を培養した上清（ECM）を使用して、PGN の存在下または非存在下での子宮内膜上皮細胞（BEEC）に対する ECM の影響を確認した。上皮細胞の分離と培養のために、黄体開花期の健常なウシ子宮（D 7-8）を食肉処理場から採取した。2 回目の継代細胞が 80〜90％のコンフルエントに達したとき、5％CO2、38.5℃下で、PGN の存在下または非存在下で細胞を ECM 添加して 24 時間培養した。ECM または PGN を含まない細胞群を対照区とした。BEEC をリアルタイム PCR により、IFNT シグナル伝達の主要因子である *ISG*（*OAS1* および *ISG15*）および *STAT1* の mRNA 発現を分析した。その結果、ECM が *ISG*（*OAS1* および *ISG15*）および *STAT1* の mRNA 発現を誘導することが示された。一方、PGN の存在は、BEEC における *ISG* と *STAT1* の誘導を大幅に抑制した。驚くべきことに、低濃度の PGN によって炎症性サイトカイン（*TNFA* および *IL1B*）および *TLR2* の mRNA 発現は誘導されなかった。以上の結果より、ECM には初期胚から分泌された IFNT が含まれており、IFNT シグナル伝達が PGN の存在で遮断されることが強く示唆された。

第 2 章では、初期胚から分泌されたと考えられる IFNT について、PGN の存在下または非存在下で、*in vitro* での BEEC 細胞培養系、そして *ex vivo* での子宮内膜小片の器官培養系の 2 つの実験モデルを用いて直接的な影響を検証した。第 1 章と同様の条件で、PGN の存在下または非存在下で、BEEC を IFNT で刺激した。同じ段階の健康な子宮から 8 mm の生検パンチを使用して、子宮内膜組織のディスク（厚さ 2 mm）を、黄体と同側の子宮内膜から分離した。次に、子宮内膜小片を IFNT を含む 10％ウシ胎児血清（FBS）を補充した Roswell Park Memorial Institute（RPMI）-1640 培地で、PGN の存在下ま

たは非存在下で 12 時間培養した。IFNT および PGN を含まない子宮内膜小片を対照区とした。BEEC と子宮内膜小片は、リアルタイム PCR によって、*ISG*（*OAS1* および *ISG15*）および *STAT1*（IFNT シグナル伝達の重要な因子）の mRNA 発現を分析した。分析結果は、細胞培養系と期間培養系の双方で、IFNT がインターフェロン刺激遺伝子（*ISG*：*OAS1*、*ISG15*）および *STAT1* の発現を刺激したことを示した。PGN の存在は両方のモデルで *ISG* と *STAT1* の発現増加を大幅に抑制した。興味深いことに、PGN は炎症性サイトカイン（*TNFA* および *IL1B*）または *TLR2* の mRNA 発現を全く刺激しなかった。加えて、IFNAR1 と IFNAR2 の mRNA 発現も変化しなかった。OAS1 タンパク質は、免疫蛍光法により子宮内膜小片の内腔側の上皮で局在が確認された。さらに、PGN が IFNT によって引き起こされるこの強い OAS1 タンパク質発現を完全に抑制した。

本研究における一連の結果は、低レベルの PGN の存在が、初期胚由来 IFNT シグナルを子宮内膜での ISG 発現レベルで完全に抑制し、それによってウシの子宮内膜上皮の初期胚受容性および初期胚–母性クロストークを妨げることを示しており、このことが妊娠の失敗につながると考えられた。