Control Mechanisms of the Bovine Oviduct Function by the Immune System

September 2013

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Doctoral Program in Animal and Food Hygiene

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Agriculture and Veterinary Medicine

ウシ卵管機能の免疫システムによる調節機構

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Dedication

To my parents, my wife Zahra, and my son Taha

Acknowledgement

The work presented in this thesis was carried out at the Department of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine. Supervision has been provided by Professor Akio Miyamoto. Assistance was also provided by my Co-supervisor Associate Professor Takashi Shimizu (Department of Animal and food Hygiene).

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Chapter IV: Function and Regulation of alpha 1-Acid Glycoprotein in the Bovine Oviduct Epithelial Cells *in Vitro*

Abbreviations

α-1-AGP:	Alpha-1-acid glycoprotein
AGPR:	Alpha-1-acid glycoprotein receptor
AIJ:	Ampullary-isthmic junction
APP:	Acute phase protein
BOEC:	Bovine oviduct epithelial cell
CK:	Cytokeratin
CL:	Corpus luteum
COX-2:	Cyclooxygenase-2
cysLTs:	Cysteinyl leukotrienes
DC:	Dendritic cell
E2:	Estradiol
EC:	Epithelial cell
ELISA:	Enzyme-linked immunoabsorbent assay
EOS:	Eosinophils
FCS:	Fetal calf serum
FSH:	Follicle stimulating hormone
GnRH:	Gonadotrophin releasing hormone
IFN-τ:	Interferon-tau
IκBα:	Inhibitor of κB
IL:	Interleukin
ISG-15:	Interferon stimulated gene-15
LH:	luteinizing hormone
LPS:	Lipopolysaccharide

MCP-1:	Monocyte chemotactic protein-1
mPGES-1:	microsomal Prostaglandin E synthase-1
NF-κB:	Nuclear factor-kappa B
NFKBIA:	Nuclear factor-kappa B inhibitor A
NK:	Natural killer
OEC:	Oviductal epithelial cell
P4:	Progesterone
PBMC:	Peripheral blood mononuclear cell
PBS:	Phosphate-buffered saline
PGF2α:	Prostaglandin F-2 alpha
PRR:	Pattern recognition receptor
real-time PCR:	Real-time Polymerase Chain Reaction
SLPI:	Secretory leukocyte protease inhibitor
sTNFR1:	soluble Tumor necrosis factor receptor 1
TGF-β:	Transforming growth factor-β
Th:	T helper
TLR:	Toll-like receptor
TNF-α:	Tumor necrosis factor- α
VEGF:	Vascular endothelial growth factor

Chapter I

General Introduction

The mucosal immune system in the female reproductive tract has been equipped by unique requirements of dealing with bacterial and viral pathogens, allogeneic spermatozoa, and the immunologically semi-allogeneic embryo or fetus. In spite of years of investigation in immunology, the mechanisms that regulate the mucosal immune system in the bovine reproductive tract have received only little attention. This could be due to the complexities of the immune and endocrine system and also to the difficulties of conducting experiments in this field. In this study we were to investigate the immune responses in the bovine oviduct. In this chapter, we explain the anatomy and immunological aspect of female reproductive tract, and, particularly, the oviduct. The oviduct is a narrow tubular organ with very complex functions in which final maturation and transport of the female and male gametes, as well as fertilization, occur [1]. The mechanisms explaining the immune function in the oviduct are still not fully understood [2] and the local immune reactivity in particular needs to be explored.

1. The oviduct in cows: anatomy and histology

In domestic species, the female reproductive tract places beneath the rectum (Fig. 1-1).

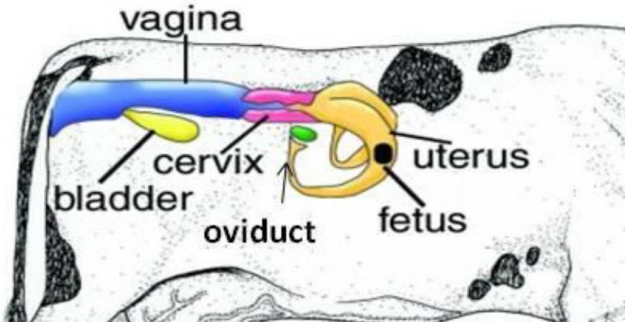


Fig. 1-1 Illustration of the female reproductive tract of the cow (modified from GSNU [3]).

The oviduct in cow is a simple organ (approximately 21-28 cm long) and is composed of 3 parts: the infundibulum, the ampulla and the isthmus (Fig. 1-2) [4]. The junction between the isthmus and the ampulla is barely discriminated, the ampullary-isthmic junction (AIJ). The infundibulum opens by highly vascularised fimbriae into the peritoneal (abdominal) cavity that forms the oviductal funnel. This structure almost completely covers the ovary. The ampulla constitutes about two-thirds of the oviduct with a lot of visible extensible mucosal folds [5]. The isthmus is a thick-wall part of the oviduct with a narrower lumen (Figure 1- 2). Isthmus makes up the remaining third of the oviduct [5].

Similar to other reproductive tract ducts, the oviduct is composed of various layers: outer layers (*tunica muscularis* and *tunica serosa*) and inner layers (*tunica mucosa* and *tunica submucosa*) [6] (Fig. 1-2). A single layer of columnar epithelium including ciliated and secretory cells constitutes the mucosa. The secretory cells release their secretory products into the lumen using exocytose process. The Estrus provides specific histological changes in the secretory cells. The maximum secretory activities with enhanced protein synthesis are shown near the time of ovulation. Cilia are found throughout the bovine oviduct. The number of these cells increases towards the fimbriae [7]. On the other hand, the inner circular smooth muscles of the muscularis layer reduce towards the infundibulum, become thinner and disappeared. These parts of the oviduct are functional compartments that are involved in sperm transport, oocyte picking up, zygotes transportation, fertilization and early embryo development and transport to the uterus [8].

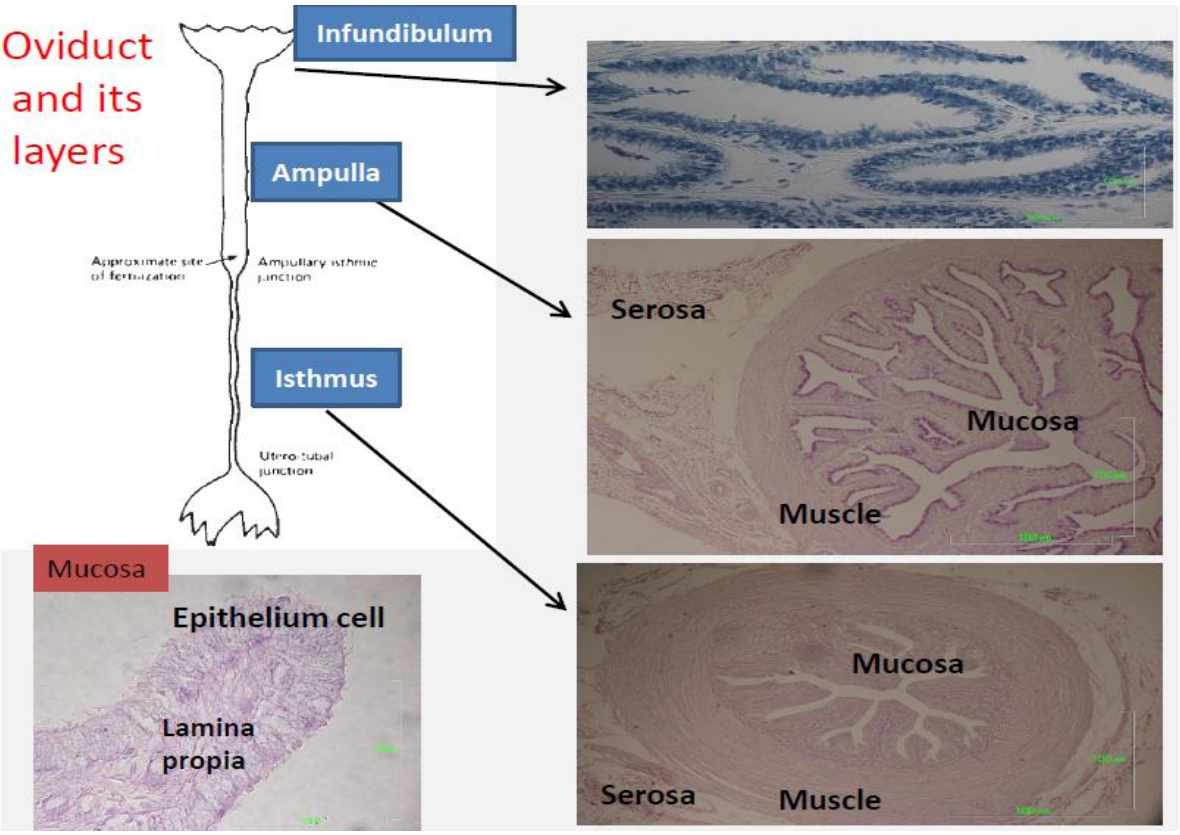


Fig. 1-2 Illustration of the principal layers of oviduct.

2. Oviductal epithelial cells

The epithelium of the oviduct is of the simple columnar type and is composed of two different types of cells, ciliated and secretory cells [6]. The cells are tightly bound together and make epithelia.

Because the plasma membrane of epithelial cells is organized into two distinct poles, the apical and basolateral poles, they are considered as polarized cells [9] (Fig. 1-3). The apical pole espousers the interior of the lumen, having specialized features such as cilia like ciliated cells. The basolateral pole covers the rest of the cell. These domains are separated by tight junctions. The epithelium lies on an extracellular matrix called the basal lamina (basement membrane).

Adjacent cells are interconnected with each other and anchored on the basal lamina by several types of cell junctions.

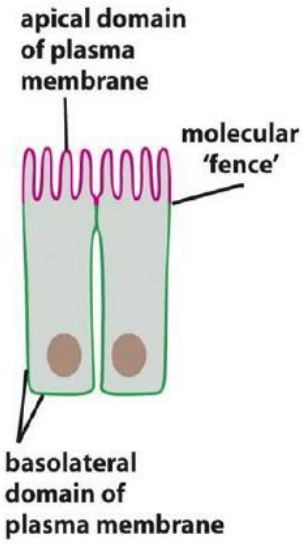


Fig. 1-3 Illustration of the principal domains [apical and basolateral domains] of a polarised epithelial cell. Figure taken from [9].

Intermediate filaments, microtubules and actin filaments constitute the cytoskeleton of animal cells [9]. Intermediate filaments contain cytokeratins (CK). There are two types of cytokeratins: the acidic type I cytokeratins including CK10, CK11, CK12, CK13, CK14, CK15, CK16, CK17, CK18, CK19 and CK20, and the basic or neutral type II cytokeratins including CK1, CK2, CK3, CK4, CK5, CK6, CK7, CK8 and CK9 [9]. Mainly, the expression of cytokeratins is organ or tissue specific. It means that depend on the type of epithelium, the type of cytokeratins expressed by epithelium is varied. For example, tubal epithelium of the genitourinary tract and the gastrointestinal tract express CK7 and CK20 respectively. Therefore, it is a good marker to classification of epithelia on basis of their cytokeratin expression profile. For example CK13 is a

marker to distinguish normal tissues (negative) from epithelial tumours (positive) in the bovine female reproductive tract [10]. Moreover, CK7 and CK20 are helpful in diagnostic differentiation of metastatic lesions from the lungs and colon, and help in determining the origin site of the metastatic lesion [11].

3. Physiology of reproduction in dairy cattle

The estrous cycle in cows normally lasts 21 days (with a range of 17 to 24 days). Duration of estrus is around 15 hours with a range of 6 to 24 hours [6]. The estrous cycle is divided into two endocrine phases i.e. the follicular and luteal phases (Figure 1-4) which are regulated by hormones from the endocrine system. In cows, the follicular phase lasts for about 4 days (including proestrous and estrous stages), whereas the length of the luteal phase is about 17 days [6]. The follicular phase is marked by high levels of estrogen, E2, produced by the ovarian follicles, whereas the luteal phase is characterized by high levels of progesterone, P4, produced by corpus luteum (CL) (Fig. 1-4). The luteal phase (including metestrus and diestrus) starts from the time of ovulation and ends after regression of the CL (luteolysis). The duration of metestrus is about 5 days. This phase is characterized by CL formation (luteinization). Diestrus corresponds to the mid luteal phase, with mature CL producing high levels of P4. During the last 2-3 days of the luteal phase, luteolysis occurs. This results in decreasing amounts of P4 leading to the removal of the negative feedback of P4 on the hypothalamus. Therefore, gonadotrophin releasing hormone (GnRH) is released which then induces increasing amounts of follicle stimulating hormone (FSH) and luteinizing hormone (LH) [7]. These hormone levels are characteristic of proestrus and stimulate follicular development and the production and secretion

of estrogen. There are two or three major phases of growth of large follicles during the estrous cycle.

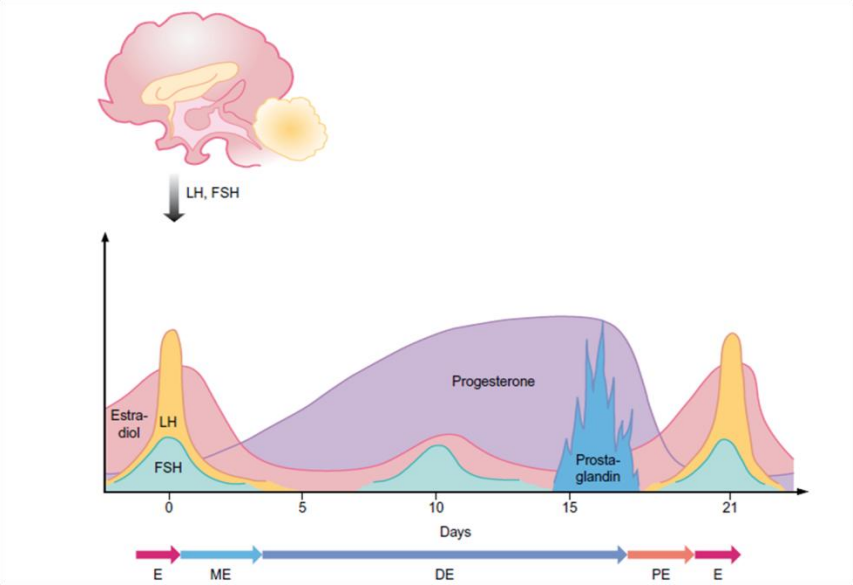


Fig. 1-4 The two phases that constitute the estrous cycle (follicular and luteal phases). Luteolysis followed by LH surge which is responsible for ovulation, marks the principal events at estrus. The luteal phase is known by high levels of P4 and ends with regression of the CL. Developing follicles produce high levels of E2 in the follicular phase. (Figure taken from internet).

When the production and level of E2 in the dominant follicle peaks, the preovulatory LH-surge happens that is responsible for the ovulation. Almost 24 to 30 hours after the surge, the ovulation occurs. After successful fertilization, the endocrine communication between the embryo and the mother is critical to save pregnancy. The blastocyst produces bovine interferon τ, a protein that inhibits the production of oxytocin receptors on the endometrium, such that oxytocin cannot stimulate luteolysis and prostaglandin F-2 alpha (PGF2α) release [13]. Sufficient progesterone production in the corpus luteum is essential for embryonic development [14]. The maintenance of pregnancy in cows is dependent upon progesterone produced by the CL and later on by the placenta during the whole length of the pregnancy.

4. Immunological aspects of the oviduct

An aseptic milieu must be maintained in the oviduct, i.e. it needs to be free from microorganisms such as viruses and bacteria that may sporadically colonize this upper part of the reproductive tract. Spermatozoa are allogeneic [15] and therefore, in the oviduct, successful fertilization and early embryonic development depends on mechanisms that regulate potentially hostile maternal immune reactions to allogeneic spermatozoa and semi-allogeneic embryos without hindering protective immune responses to infectious agents. In fact, mucosal epithelium functions not only as a physical barrier, but also as a regulator of innate and adaptive immune responses against foreign substances and microorganisms. Innate immunity functions as a first-line host defense mechanism through pattern recognition of conserved molecules associated with microorganisms by various families of germ-line-encoded pattern recognition receptors (PRRs), including the toll-like receptors (TLRs) and nod-like receptors. Cells that participate in this first-line defense mechanism include macrophages, dendritic cells (DCs, and epithelial cells (ECs) at mucosal surfaces. These cells are responsible for the immediate, early, and nonspecific immune responses against pathogens. Innate immunity is linked to adaptive immunity through the activation of professional antigen-presenting cells. There has been emerging evidence that ECs have important functions in the initiation of innate immune responses and regulation of adaptive immune responses at mucosal sites. Therefore, the epithelial layer at the mucosal surfaces functions not only as a physical barrier, but also as an innate and adaptive immune regulator [16]. In this context, a research goal should be to identify all molecules used by epithelial cells to instruct immune responses, termed the ‘epimmunome’ [17]. The evidence that epithelial cells can initiate immune cell responses raises some fundamental questions. For example, what are the main epithelial cell pathways implicated in the regulation of immunity, and what are the key

afferent stimuli that feed into those pathways? What molecules do epithelial cells display on their surface or secrete so as to communicate the state of the epithelium to the immune compartment? What types of immune cells do epithelial cells influence? Finally, how diverse are the aggregate immune responses that result from epithelial-immune interactions?

4.1. Leukocyte subsets in the oviduct

Studies on the immune cells in the oviduct have been performed in several species, e.g. humans [18] and rabbits [19]. In the bovine oviduct (the isthmic and ampullary parts), changes in the distribution of lymphocytes [20], mast cells [21] and eosinophils (EOS) [22] during different stages of the estrous cycle have been reported. However, few studies have been made on eosinohils distribution in the bovine oviduct and related to different stages and regions of the estrous cycle.

4.2. Cytokine of interest to study in the oviduct: Proinflammatory vs. anti-inflammatory (Th1 vs. Th2) cytokines

The proposed dual role for immunomodulation in the oviduct (acceptance of allogeneic tissue and immune protection against pathogens) suggests a fine balance in the presence of suppressive and pro-inflammatory cytokines, possibly contrasting in different oviductal parts. Interleukin-4 (IL-4) and IL-10, was chosen as indicators of immune suppression whereas IL-1β and TNF-α were selected to reflect a proinflammatory response. IL-1β and tumor necrosis factor α (TNF-α) are major proinflammatory cytokines which promote inflammation and stimulate an acute phase response. At the cellular level, IL- 1β is critical for neutrophil recruitment by eliciting expression of chemokines and adhesion molecules [23]. IL-10 is considered to be a

potent suppressor of the effector functions of macrophages, T cells and natural killers (NK) cells [24]. It acts to terminate the inflammatory response and limits inflammation-induced tissue changes by de-activating macrophages and inhibiting their synthesis of proinflammatory cytokines and chemokines [25]. A role of IL-10 during pregnancy was observed by Chaouat *et al.* [26], who found that recombinant IL-10 prevented fetal absorption in mice but not after neutralization with anti-IL-10. Robertson *et al* [27] demonstrated, also in mice, that IL-10 modulates resistance to inflammatory stimuli by down-regulating pro-inflammatory cytokines in the uterus and placenta.

4.3. Role of ovarian steroid and LH in immune responses

Importantly, the oviduct, like the other organs of the female reproductive tract, is under hormonal influence from the ovary, i.e. estrogen from the follicles and progesterone from the corpora lutea. In cows, the estrogen levels increase during pro-estrous to reach a peak at the start of standing estrous, the stage when the animals are mated or inseminated. The progesterone levels increase after ovulation at metoestrus and reach a plateau during dioestrus. The bovine oviduct and bovine oviduct epithelial cells (BOECs) express receptors for LH [28], E2, and P4 [29]. Estradiol and P4 have been shown to completely block *Escherichia Coli*-induced inhibitor of κB (IκBα) phosphorylation and nuclear factor-kappa B (NF-κB) nuclear translocation in human cord blood mononuclear cells [30]. Sex hormones exert control over many chemokines/cytokines in the female reproductive tract. For example, P4 withdrawal results in increased expression of monocyte chemotactic protein-1 (MCP-1) and IL-8, leading to chemotaxis and activation of monocytes and neutrophils, which results in the release and activation of matrix metalloproteinases and the initiation of menstruation [31].

5. Aims of the study

It is clear that any changes in the balance between pro-inflammatory and anti-inflammatory responses (Th1vs. Th2) during infection or a particular physiological situation can contribute to low defense against infection, resulting in increasing susceptibility to infection or low tolerance to pathogens. This, in turn, allows a cytotoxic immune response to be generated against antigens present on the normal epithelium cells, allogenic sperm, and semi-allogenic new embryo.

We therefore hypothesized that the oviduct epithelial cells respond to gram-negative bacteria endotoxin, lipopolysaccharide (LPS) and this response is under control of sex steroids and LH. Also, we hypothesized that epithelial cells of the oviduct have a role in balancing Th/Th2 ratio in response to various stimuli such as ovarian steroids, LH, and the new embryo (Fig. 1-5). The general aims of this thesis on the bovine oviduct were to investigate immunological responses of the bovine oviduct epithelial cells, focusing on Th1 vs. Th2 balance in different conditions, as well as to study the effects of ovarian steroids and LH on the cytokine expression in response to LPS stimulation. In addition, we aimed to investigate the regional distribution of immune cells (i.e eosinophils0 in the bovine oviduct throughout the estrous cycle.

Therefore, the specific aims were to investigate:

1- Isthmus, ampulla, and infundibulum of the bovine oviduct regarding:
   - The regional distribution of eosinophils at the different stages of the estrous cycle (in vivo).
2- The bovine oviduct regarding:
   - The gene and protein expression of Toll-like receptor 4 and 2.
   - The gene and protein expression of acute phase protein, alpha-1-acid glycoprotein.

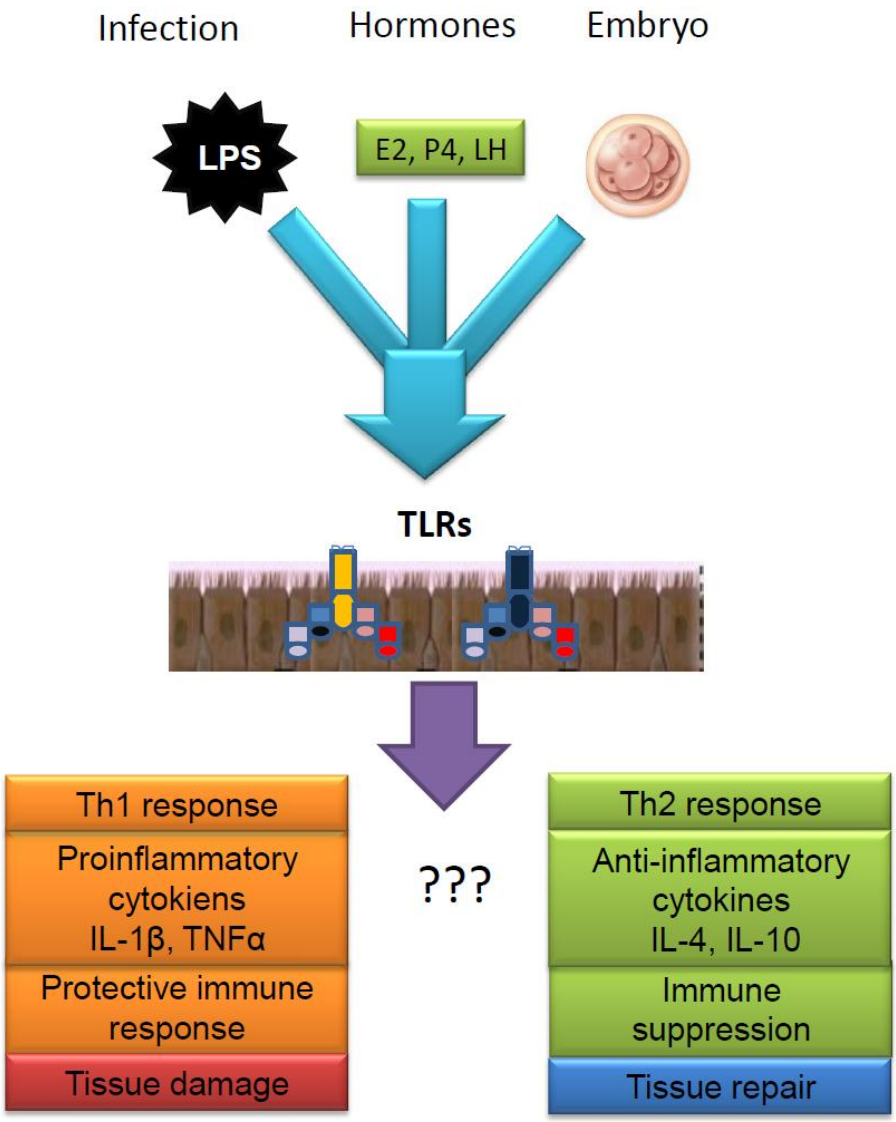


Fig. 1-5 Schematic illustration for a possible regulation of local innate immunity in the bovine oviduct.

3- Bovine oviduct epithelial cell culture regarding:

- The expression of TLR4 and TLR4-related proinflammatory cytokines (IL-1β and TNF-α), and the expression of anti-inflammatory cytokines (IL-10 and IL-4) in response to the different doses of LPS (Th1/Th2 response, patho-physiological mode).
- The effect of ovarian steroids and LH on the LPS-derived immune responses.

- Immune function and regulation of alpha-1-acid glycoprotein, a novel player in the regulation of immunity in the oviduct epithelial cell in vitro.
- Possible cross-talk between interferon-tau and Th1/Th2 balance.

Chapter II

Accumulation of Eosinophils in the Infundibulum of the Bovine Oviduct just after Ovulation

1. Introduction

The mammalian oviduct provides an optimal environment for sperm capacitation, fertilization, and transport of gametes and the new embryo. Sperm and the new embryo, which are allogenic and semi-allogenic agents, respectively, directly contact the oviduct epithelial cells, that could induce the immune responses in female [32, 33].

During the estrous cycle in the cow, the oviduct is exposed to different concentrations of the sex hormones estradiol (E2, during the preovulatory phase) and progesterone (P4, during the luteal phase). Moreover, the oviduct ipsilateral to the dominant follicle or corpus luteum (CL) is exposed to much higher levels of E2 and P4, respectively [34]. Importantly, the oviduct ipsilateral to the functional ovary is the site of sperm capacitation and the development of the new embryo. Therefore, immunological discrimination between the ipsilateral and contralateral oviducts could help to explain local reproductive immunity, at least in part.

Eosinophils are involved in both innate and acquired immunity [35], and they are able to regulate local immunity through their secretion of cytokines and enzymes [36, 37]. The EOS also produces cysteinyl leukotrienes (cysLTs), which increases vascular permeability, mucus secretion, and smooth muscle constriction [38]. Since these local phenomena are known to be the feature of the bovine oviduct around the time of ovulation [39], EOS may play a role in the oviduct physiology around this period.

So far, no information is available on the distribution of EOS in the different layers and regions of the oviduct in relation to the functional ovary throughout the estrous cycle. It is hypothesized that the number of EOS in the oviduct changes 1) according to the position of the oviduct relative to the functional ovary bearing the preovulatory dominant follicle, ovulated site,

or CL (i.e., ipsilateral vs. contralateral), 2) in response to fluctuations in the levels of sex steroid hormones during the estrous cycle, and 3) also regarding the different layers of the oviduct, the outer layers (*tunica muscularis* and *tunica serosa*) than the inner layers (epithelial layer, *tunica mucosa* and *tunica submucosa*).Thus, we aimed to investigate in detail the regional distribution of Eos in the bovine oviduct during the estrous cycle.

2. Materials and Methods

2.1. Collection and classification of oviducts

Forty-two oviducts from 21 Holstein cows were transported from the local slaughterhouse to the laboratory immersed in 0.9 % saline solution in an ice box, and were classified based on the stage of the estrous cycle as preovulatory (days 19-20), postovulatory (days 1-2), and mid-luteal (days 10-12). The stage of the estrous cycle was identified by macroscopic observation, based on the size, color, consistency, and connective tissue of the ovaries (CL and follicles) as previously described [40, 41]. Briefly, the preovulatory phase (days 19-20) was estimated as the ovary contained at least one large follicle > 10 mm in diameter and a regressed corpus luteum (< 1 cm in diameter, firm in consistency) with no vasculature visible on its surface. The external appearance of CL was light yellow to white in color. The postovulatory phase (days 1-2) was estimated as the CL recently ovulated with point of rupture that was not covered over by epithelium. The External and internal part of CL (0.5-1.5 cm in diameter) was red in color and cells loosely were organized. There was not any follicle > 8 mm in diameter on the ovary. The mid-luteal phase (days 10-12) was estimated as CL (1.5-2.5 cm in diameter) was mature and its external and internal part was tan or orange.

Oviducts were also classified as ipsilateral to the preovulatory dominant follicle (IDF, *n*=7), contralateral to the dominant follicle (CDF, *n*=7), ipsilateral to the ovulated ovary (IOV, *n*=7), contralateral to the ovulated ovary (COV, *n*=7), ipsilateral to the CL (ICL, *n*=7), and contralateral to the CL (CCL, *n*=7) depending on the side and the stage of the estrous cycle. The connective tissues were removed and the oviducts divided into the isthmus, ampulla, and infundibulum; the ambiguous area between the isthmus and ampulla was discarded. Also, both ovaries and oviducts were macroscopically examined to be healthy.

2.2. Staining and histology

Three paraffin-embedded tissue sections (5-µm thick) of the samples were serially mounted on gelatin-coated slides. The EOS were stained across 3 serial slides using the Luna protocol [42], and positive- stained cells were photographed using a light microscope (Microphot-FX, Nikon, Tokyo, Japan) equipped with a micro digital camera (FX-35DX, DS-5M, Nikon). The number of EOS in each region (i.e. *tunica muscularis*, *tunica serosa*, *tunica mucosa*, and *tunica submucosa*) was counted in 3 serial sections per each oviduct and 3 different 0.01 mm^2 –areas (100 × 100 µm = 0.01 mm^2) in a section per each region. Thus, 3 sections × 3 squares × 0.01 mm^2 = 0.09 mm^2 area of each region were counted and averaged.

2.3. Statistical analysis

Data on EOS number in the different regions, layers, and in ipsilateral vs contralateral oviducts were statistically analyzed with One-way ANOVA followed by Fisher's multiple comparison tests (for 3 groups) and t-test (for 2 groups) using Stat View 5.0 (SAS Institute Inc.).

Data are presented as the mean ± SEM and all results were considered to be statistically significant at $P<0.05$.

3. Results

3.1. Number of EOS in the different regions of the oviduct during the estrous cycle

In the IDF and IOV oviducts, EOS accumulated to higher numbers in the infundibulum than in the isthmus and ampulla (Fig. 2-1A - B, $P<0.05$). The number of EOS was higher in the infundibula of the IOV oviducts than those of the COV oviducts (Fig. 2-1B, $P<0.05$). The numbers of EOS in the different regions (infundibulum, isthmus, and ampulla) of CDF, COV, and CCL oviducts were not affected by the estrous cycle. The numbers of EOS changed during the estrous cycle only in the infundibulum, being higher in the IDF than in the ICL oviducts (Fig. 2-3, $P<0.05$).

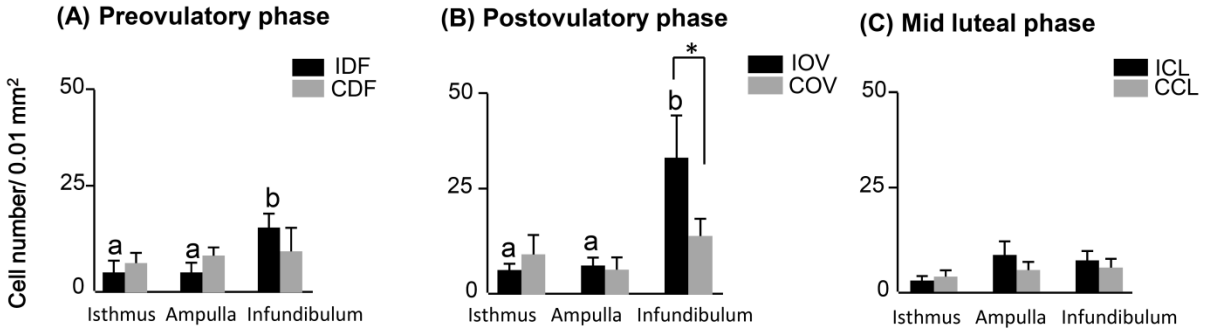


Fig. 2-1 Number of eosinophils in the different regions of the bovine oviduct during the estrous cycle. IDF: oviducts ipsilateral to the preovulatory dominant follicle; CDF: oviducts contralateral to the preovulatory dominant follicle; IOV: oviducts ipsilateral to the ovulated ovary; COV: oviducts contralateral to the ovulated ovary; ICL: oviducts ipsilateral to the corpus luteum; CCL: oviducts contralateral to the corpus luteum. Data are presented as the mean ± SEM. Different letters (a, b) or * indicate significant differences ($P<0.05$).

3.2. Number of EOS in the different layers of the oviduct during the estrous cycle

The number of EOS in the isthmus (both ipsilateral and contralateral) was higher in the outer layers (i.e., *the tunica muscularis* and *tunica serosa*) than in the inner layers (*tunica mucosa* and *tunica submucosa*) at different stages of the estrous cycle (Fig. 2-2 and Fig. 2-4, $P<0.05$). Moreover, the number of EOS in the different layers of the ampulla (inner vs. outer layers) was not different during the estrous cycle (data not shown).

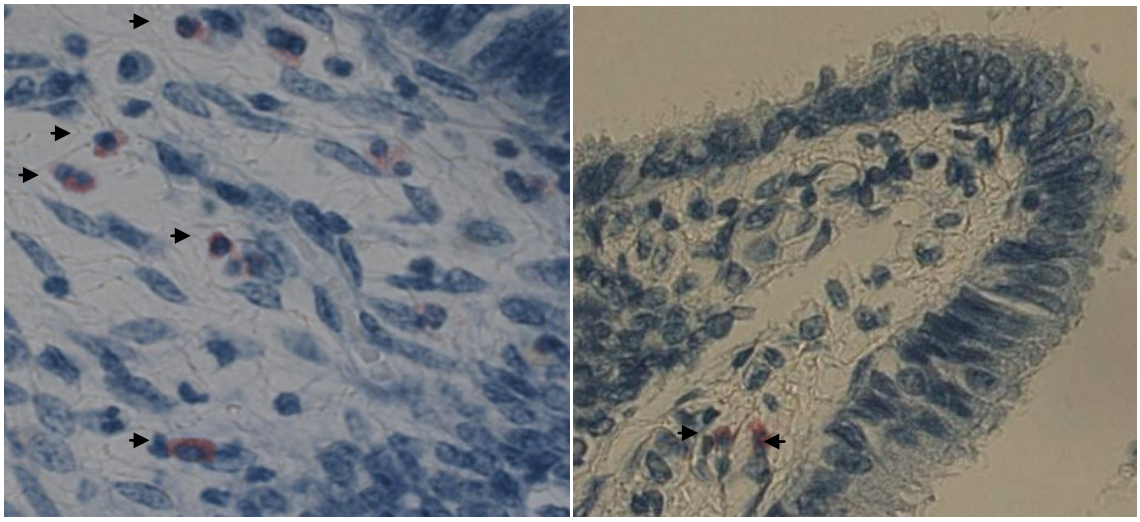


Fig. 2-2 Eosinophils in the bovine oviduct. Eosinophils accumulated to greater numbers in the outer layers than in the inner layers. A: *tunica muscularis* and *tunica serosa*, B: *tunica mucosa* and *tunica submucosa*. The eosinophils were stained using the Luna protocol [42]. Scale bars indicate 50 μm.

4. Discussion

The present study indicates the higher numbers of EOS in the infundibula of the oviducts ipsilateral to the preovulatory dominant follicle than in the corresponding isthmus and ampulla regions. Valle et al., [22] also reported higher number of EOS in the infundibulum than isthmus and ampulla during the estrous. The reason for the higher number of EOS in the infundibula than isthmus and ampulla during preovulatory phase is not clear; further studies are required to

determine the exact reason of this phenomenon. However, the smaller numbers of EOS in the isthmus (sperm reservoir) and ampulla (fertilization site) may reduce the capacity for unwanted immune responses, especially during the preovulatory and postovulatory phases.

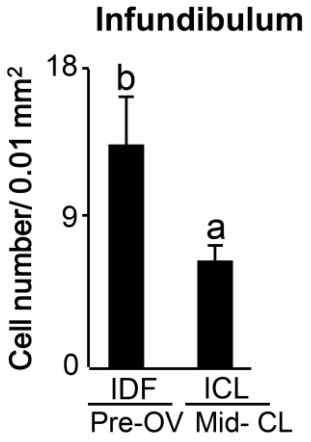


Fig. 2-3 Number of eosinophils in the infundibula of the bovine oviduct during preovulatory and mid luteal phases. The data in this figure are derived from Fig. 1, showing higher number of eosinophils in the infundibula of the IDF oviducts than those of ICL oviducts. Pre-OV: preovulatory phase; Mid-CL: mid-cycle corpus luteum. IDF: oviducts ipsilateral to the preovulatory dominant follicle; ICL: oviducts ipsilateral to the corpus luteum. Data are presented as the mean ± SEM. a, b indicates significantly different values (P<0.05).

Interestingly, in the postovulatory oviducts, the numbers of EOS were significantly higher in the infundibula of the oviducts ipsilateral to the ovulated ovary in comparison with those contralateral to the ovulated ovary. There may be two possibilities for this phenomenon. First, ovulation, along with the entry into the oviduct of ovulated follicular fluid containing eosinophilic chemokines such as eotaxin, RANTES [43], and IL-8 [44, 45], could induce migration of EOS [46] toward the oviduct ipsilateral to the ovulated ovary. Second, the

infundibulum is anatomically in a direct contact with the ovary and is exposed to the ovulation. Thus, ovulation, itself an inflammatory event with bleeding, may induce the infiltration of EOS into the infundibulum. It is reported that EOS infiltrates into the inflamed tissues rapidly [47]. Therefore, the present data suggest that ovulation has a profound effect on increase in number of EOS in the infundibulum ipsilateral to the ovulated ovary.

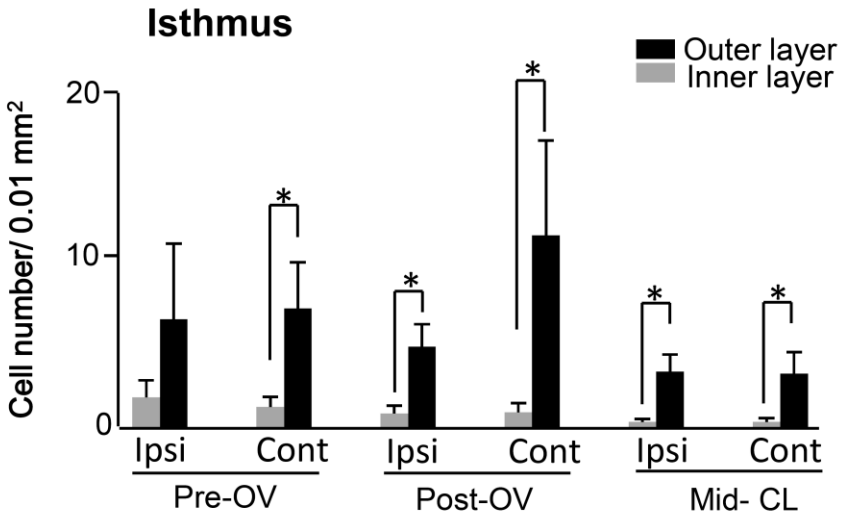


Fig. 2.4 Number of eosinophils in the different layers of the isthmus during the estrous cycle. Pre-OV: preovulatory phase; Post-OV: postovulatory phase; Mid-CL: mid-cycle corpus luteum; Ipsi: ipsilateral; Cont: contralateral. Inner layer: *tunica mucosa* and *tunica submucosa*, outer layer: *tunica musc*ularis and *tunica serosa*. Data are presented as the mean ± SEM. * indicates significantly different values (P<0.05).

It has been shown that circulating E2 enhances the migration of EOS to the rat uterus [48] and P4 decreases estrogen-induced migration to the rat uterus [49] and cervix [50]. Therefore, it was hypothesized that ovarian steroids may regulate the number of EOS in the oviduct. To examine this possibility, we compared the oviducts from preovulatory phase (high E2/low P4, ipsilateral to the dominant follicle) and oviducts from mid luteal phase (high P4/low

E2, ipsilateral to the CL). The data showed that number of EOS changed only in the ipsilateral infundibulum, but not in the ampulla and isthmus, with higher number of EOS in the preovulatory phase than in the mid luteal phase. This suggests that E2 from dominant follicle and P4 from CL may regulate the number of EOS in the ipsilateral infundibula. Although we previously reported the concentrations of ovarian steroids in the oviduct throughout estrous cycle [34], we did not measure the concentrations of the ovarian steroids in the infundibula in that study. Further investigations are necessary to confirm the effect of E2 and P4 on the migration and number of EOS in the bovine oviduct.

The present data showed the higher accumulation of EOS in the outer layers (*tunica muscularis* and *tunica serosa)* than in the inner layers (*tunica mucosa* and *tunica submucosa*) of the isthmus at all examined stages of the estrous cycle. However, this phenomenon was not observed in the ampulla. Since isthmus is a site for sperm reservoir, the lower number of EOS in isthmus may reduce the direct contact of EOS with sperm in healthy oviducts.

The exact reason for the presence of EOS in the healthy oviduct remains unclear; however, it is suggestive that EOS may enhance luminal secretion *via* their secretions and also increase vascular permeability, mucus secretion, and smooth muscle constriction [38], which could affect oviduct function and thus fertility.

The results of this study show a specific regional distribution of EOS in the healthy bovine oviduct throughout the estrous cycle. The number of EOS in the oviduct is profoundly affected by ovulation and may be regulated, in part, by ovarian steroids from the preovulatory follicle and mid-cycle corpus luteum.

Chapter III

Regulation of Innate Immune Function in the Bovine Oviduct Epithelial Cells in Culture: The Homeostatic Role of Epithelial Cells in Balancing Th1/Th2 Response

1. Introduction

The mammalian oviduct provides an optimal microenvironment for the activation and transportation of gametes, sperm capacitation, fertilization, and early embryonic development, which is critical for the establishment of a successful pregnancy [51]. The oviduct is classically described as a sterile milieu even though pathogens and endotoxins can invade the mucosal surfaces of the oviduct via the uterus, peritoneal cavity, and follicular fluid [52]. Therefore, the oviduct should be equipped with an efficient and strictly controlled immune system that would maintain optimal conditions for fertilization and early embryo development. Local immune responses, regulated by the secretions of epithelial cells, form a part of the mucosal innate immunity. In recent years, these responses, termed epimmunome [17], have been recognized as critically important defense mechanisms, and their significance seems far greater than that of the systemic immune responses. The direct response of epithelial cells to microbial (i.e., pathogens) or non-microbial (i.e., stress or hormones) stimuli includes activation of the innate immune responses and regulation of subsequent adaptive immune responses [53]. This process is mediated by the secretion of different molecules such as prostaglandins, chemokines, and cytokines, which affect the conditioning of mucosal dendritic cells (DCs) [16]. Therefore, mucosal epithelial cells can orchestrate and provide early signals to drive immunity toward tolerance or inflammation.

The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) using pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) [54, 55]. At least 10 bovine TLRs have been identified [56], and these PRRs bind to a range of microbial products and endogenous ligands. TLR signals are involved in the primary induction of inflammation as well as in the secondary activation of anti-inflammatory mechanisms [57]. For example, TLR-4

is a signal transducer of LPS, a component of the outer membrane of gram-negative bacteria. The ligation of TLR-4 by LPS leads to the activation of nuclear factor-kappa B (NF-κB), and consequently transcription of pro-inflammatory cytokines and chemokines [58]. Another member of the TLR family, TLR-2, recognizes PAMPs associated with both gram-negative and gram-positive bacteria, including lipopeptides/lipoproteins, lipoteichoic acid, zymosan, and components of peptidoglycan [59]. *E. coli* LPS enhances *TLR-2* mRNA and cell surface TLR-2 in the mice [60], human [61, 62], and bovine [63] in an NF-κB-dependent manner [64, 65]. Several investigations have shown the involvement of TLR-2 in LPS signaling [66, 67]. Engagement of TLR-2 in DCs by the bacterial lipopeptide Pam3Cys enhances extracellular signal-regulated kinase activation, resulting in the stabilization of the transcription factor C-FOS, a suppressor of Th1 cytokines [68].

The oviduct is exposed to the female sex hormones, and these hormones clearly influence the immune system [69]. The bovine oviduct and BOEC express receptors for LH [28], E2, and P4 [29]. Estradiol and P4 have been shown to completely block *E. coli*-induced IκBα phosphorylation and NF-κB nuclear translocation in human cord blood mononuclear cells [30]. Sex hormones exert control over many chemokines/cytokines in the female reproductive tract. For example, P4 withdrawal results in increased expression of MCP-1 and IL-8, leading to chemotaxis and activation of monocytes and neutrophils, which results in the release and activation of matrix metalloproteinases and the initiation of menstruation [31].

It is clear that any changes in the balance between pro-inflammatory and anti-inflammatory responses (Th1vs. Th2) during infection or a particular physiological situation can contribute to low defense against infection, resulting in increasing susceptibility to infection or low tolerance to pathogens. This, in turn, allows a cytotoxic immune response to be generated

against antigens present on the normal epithelium cells, allogeneic sperm, and semi-allogeneic new embryo. It is therefore hypothesized that BOEC can differentially respond to pathophysiological (i.e., LPS) and physiological (i.e., sex hormones) stimuli through the secretion of different cytokines and chemokines and the use of specific receptors, TLR-4 and TLR-2. We therefore investigated the regulation of local immune responses, Th1- or Th2- type responses, induced by *E. coli* LPS, ovarian steroids and LH at the levels around ovulation, and their interactions in BOEC *in vitro*.

2. Materials and Methods

2.1. Primary bovine oviduct epithelial cell (BOEC) isolation

Epithelial cells were isolated as previously described [70, 71]. Briefly, fifteen oviducts were transported in an ice box from the local slaughterhouse to the laboratory, with the oviducts immersed in phosphate-buffered saline (PBS) solution without Ca^{2+}/Mg^{2+} ($PBS^{-/-}$) (Sigma, St. Louis, MO. USA) but with 0.3% gentamicin (Sigma) and amphotericin B (Illkirch, France). They were cut and separated from the connective tissue and pathologically examined to be healthy, and washed twice with PBS. The lumen of oviducts was flushed with 15 ml PBS. The BOEC was mechanically dislodged while being flushed with the same volume of PBS. Over a period of 15 min, the pooled sheets of BOEC from 2-3 cows settled at the bottom of the tube, and the cells were then washed with PBS followed by a medium consisting of D-MEM/F12, 0.1% gentamicin, 1% amphotericin, and 2.2% $NaHCO_3$. Thereafter, the cells were harvested by centrifugation at 300*g* for 10 min at 4°C. The resultant cell pellet was suspended in 10 ml PBS, layered over 10 ml Percol, and centrifuged at 900*g* for 20 min at 4°C. Finally, the cell pellet was washed once with the abovementioned medium, harvested by centrifugation at 300*g* for 10 min

at 4°C, and cultured in culture medium (D-MEM/F12, 0.1% gentamicin, 1% amphotericin, and 2.2% $NaHCO_3$ supplemented with 10% fetal calf serum [FCS; Bio Whittaker, Walkersville, MD]) in 6-well culture dishes (Nalge Nunc International, DK-4000 Roskilde, Denmark) at 38.5°C in 5% CO_2 and 95% air. The following day, the BOEC culture was washed twice with PBS and incubated with culture medium supplemented with 5% FCS. After monolayer formation, cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH) until single cells appeared, and these cells were again plated in 6-well culture dishes at a density of 3×10^4/ml and incubated at 38.5°C in 5% CO_2 and 95% air in culture medium supplemented with 5% FCS and the medium was renewed every 48 hours, until the growing BOEC monolayer covered up to 70%–80% of bottom of the culture plate. The purity of epithelial cell preparations was evaluated by reacting the cultured cells with monoclonal antibodies to cytokeratin (anti-cytokeratin-CK1) and immunostaining. The cells in culture medium showed characteristic epithelial morphology. Approximately 98% of the cells were positive for anti-cytokeratin (CK1) antibodies.

2.2. LPS and hormone treatments

After the first passage and until the growing BOEC monolayer covered up to 70%–80% of bottom of the culture plate, in a preliminary study, BOEC monolayer was washed twice with culture medium supplemented with 0.1% FCS and incubated for 24 hours with 4 doses (1, 10, 100, and 1000 ng/ml) of LPS (serotype *E. coli* 055:B5; Sigma). In this dose-response study, 1000 ng/ml of LPS had lethal effects on cell viability, and 1 ng/ml of LPS showed a lack of significant responses (Fig. 2). Therefore, for further experimentation, doses of 10 and 100 ng/ml of LPS were used. BOEC was stimulated with one of the following: (1) LPS (10 or 100 ng/ml); (2) LPS (10 or 100 ng/ml) + LH (10 ng/ml, USDA-bLH-B6, USDA Animal Hormone Program, Bethesda,

MD, USA); (3) LPS (10 or 100 ng/ml) + P4 (1 ng/ml, Sigma); (4) LPS (10 or 100 ng/ml) + E2(1 ng/ml, Sigma). As a control, culture medium without any LPS or hormones was added to the BOEC. The concentrations of hormones in this study were maintained similar to their physiological level in the bovine oviduct during preovulatory period *in situ* [34]. This was done to mimic the local hormonal conditions around the time of ovulation in the oviduct when allogeneic sperm enters the oviduct and a semi-allogeneic embryo is about to start its development. Finally, the medium was collected and cells in the plates were trypsinized, washed twice with $PBS^{-/-}$ and re-suspended in 300 µl $PBS^{-/-}$. A 10-µl aliquot of the cell suspension was used to evaluate the cell viability. Cell viability was estimated using Trypan-blue staining and was confirmed to be more than 90% at each time of plating as well as at the end of the experiment. The remaining cells were again separated by centrifugation at 300*g* for 10 min at 4°C, and lysed by TRIzol (Invitrogen Corporation) and stored at -80°C until RNA extraction.

2.3. Extraction of RNA, production of cDNA, and real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from the BOEC using TRIzol (Invitrogen Corporation) as described in the protocol of Chomczynski and Sacchi [72]. The yield of extracted RNA for each sample was determined by ultraviolet (UV) spectroscopy (optical density, 260). The RNA concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at absorbances of 260 and 280 nm.

The extracted total RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at –80°C until it was used for cDNA production. DNase treatment was carried out using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) as described before [73]. The

synthesized cDNA was stored at –30°C. We analyzed the following genes: *TLR-4*, *TLR-2*, *COX-2*, *mPGES-1*, *TNF-α*, *IL1-β*, *IL-10*, *IL-4*, *NFKBIA*, *C-FOS*, and *β-actin* (Table 3-1 lists primer sequences).

The quantifications of mRNA expression were performed using synthesized cDNA via real-time PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany) using a QuantiTect™ SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany). The primers were designed using Primer3 based on bovine sequences. The amplification program consisted of 15 min activation at 95°C, followed by 40 cycles of PCR (15 sec denaturation at 95°C, 30 sec annealing at 54–58°C and 20 sec extension at 72°C). The values of mRNA expression were assayed by normalization to β-actin as the internal standard. The expression of *β-actin* was stable in all experiments and no significant difference was detected in the levels of *β-actin* expression between treatments.

2.4. Measurement of PGE2

The prostaglandin E2 measurements in the medium were performed without further preparation of medium, using second antibody enzyme immunoassay (EIA) according to a previous study [34]. Briefly, the assay was performed using 96-well immuno plates (Corning, NY, USA) coated with anti-rabbit lgG (Seikagaku Co., Tokyo, Japan). Basically, plates were incubated with 100 μl polyclonal antibody solution (1:600,000) for 24 hours at 4°C. The following day, plates were decanted, and 15 μl of standards or samples were incubated with 100 μl of PGE2-HRP (1:40,000) for 24 hours at 4°C.

Table 3-1 Bovine primers were used in real-time PCR.

Gene		Sequence of nucleotide (5'-3') *	Accession No.	Tm (℃)	Product size (bp)
TLR-4	F	CTTGCGTACAGGTTGTTCCTAA	NM_174198.6	56	153
	R	CTGGGAAGCTGGAGAAGTTATG			
TLR-2	F	GCTCCTGTGACTTCCTGTCC	NM_174197	54	501
	R	CCGAAAGCACAAAGATGGTT			
NFKBIA	F	AAGTGGTCCGCCAAGTGAAG	NM_001045868	58	105
	R	CGATTTCTGGCTGGTTAGTGATC			
C-FOS	F	GAACGGAATAAGATGGCTGC	NM_001001162	58	220
	R	CCACAGACATCTCCTCTGGG			
COX-2	F	TCCTGAAACCCACTCCCAACA	AF031698	54	241
	R	TGGGCAGTCATCAGGCACAG			
mPGES1	F	AGGACGCTCAGAGACATGGA	NM174443	58	142
	R	TTCGGTCCGAGGAAAGAGTA			
*IL-1*β	F	ATGAAGAGCTGCATCCAACA	NM_174093	56	196
	R	ATGGAAGACATGTGCGTAGG			
IL-4	F	GCCACACGTGCTTGAACAAA	NM_173921	56	63
	R	TGCTTGCCAAGCTGTTGAGA			
IL-10	F	TTCTGCCCTGCGAAAACAA	NM_174088	58	85
	R	TCTCTTGGAGCTCACTGAAGACTCT			
TNF-α	F	TGACGGGCTTTACCTCATCT	NM_173966	56	221
	R	TGATGGCAGACAGGATGTTG			
β-actin	F	CCAAGGCCAACCGTGAGAAAAT	K00622	58	256
	R	CCACATTCCGTGAGGATCTTCA			

* F, forward; R, reverse

2.5. Immunohistochemistry

The oviducts were transported from the local slaughterhouse to the laboratory immersed in 0.9 % saline solution in an ice box. The oviducts were used from preovulatory phase (days 19-20 of the cycle). The phase of the estrous cycle was identified as previously reported [34] based on the appearance of the corpus luteum, weight and color of the corpus luteum, and follicular diameter. Paraffin-embedded tissue sections (4-μm thick) of bovine ampulla or isthmus from preovulatory phase were mounted on silane-treated glass slides (Histobond Superior; Paul Marienfeld Laboratory Glassware, Laud-Königshofen, Germany) and dried at 37°C for 24 h.

After drying, they were deparaffinized in xylene and rehydrated in a series of solutions of graded alcohol concentrations. To block endogenous peroxidase activity, the sections were incubated for 30 min in 80% alcohol solution containing 2% hydrogen peroxide. After rinsing the sections thrice for 5 min in PBS (pH 7.2), antigen retrieval was performed by boiling the sections in 10mM Tris Base and 1mM EDTA solution (pH 9) for 30 min. The sections were then incubated for 20 min in 20% normal goat serum (in PBS) at room temperature to saturate any sites for non-specific binding of proteins. Antibodies used for immunohistochemistry were rabbit anti-TLR-2 (1:100; orb11487, Biorbyt, Cambridge, UK) and rabbit anti-TLR-4 (1:200; 251111, Abbiotec, San Diego, USA). The antibodies were diluted in PBS containing 1.5% bovine serum albumin and incubated in a humidifier over night at 4°C. For detection, the EnVision™ anti-rabbit immunoglobulin conjugated to peroxidase labeled dextran polymer system (DAKO, Glostrup, Denmark) was used in accordance with the manufacturer's protocol. Finally, sections were washed with PBS, and peroxidase activity was detected using DAB (Sigma, Steinheim Germany) as a substrate for 5 min at room temperature. Sections were counterstained with hemalum, dehydrated, and mounted with DPX (Fluka, Buchs, Switzerland). To analyze unspecific binding, primary antibodies were replaced with rabbit IgG (Sigma) at the same concentration as that used for the primary antibody.

2.6. Statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed with Stat View 5.0 (SAS Institute Inc.). One-way ANOVA followed by multiple comparison tests, Fisher (3 groups) and Bonferroni (more than 3 groups), was performed and all results were considered to be statistically significant at $P< 0.05$.

3. Results

3.1. The bovine oviduct expresses proteins for TLR-4 and TLR-2

We investigated the expression of TLR-4 and TLR-2 in the bovine oviduct because it is hypothesized that the oviduct controls immune responses through variation in TLRs signaling, which, in turn, results in the production of different cytokines and chemokines. Fig. 3-1 shows, by means of immunohistochemistry and intensive staining of epithelial cells, the expression of TLR-4 and TLR-2 in the bovine oviduct.

3.2. Dose-dependent stimulation of BOEC by LPS results in different expressions of TLR-4 and TLR-2

A dose-response study was performed, in which epithelial cells were incubated in 0.1% FCS medium alone or in the increasing doses of LPS (1, 10, 100, and 1000 ng/ml) for 24 hours. LPS at a dose of 1000 ng/ml was toxic and significantly reduced the cell viability of BOEC (Fig. 3-2A, $P<0.05$). The results also showed that BOEC is sensitive to LPS. Elevated expression of *TLR-4* indicated early-immune responses after administration of a low dose of LPS (10 ng/ml) (Fig. 3-2B, $P<0.05$).

Higher doses of LPS (100 and 1000 ng/ml) did not induce *TLR-4* expression. However, administration of LPS 100 ng/ml upregulated TLR-*2* expression while LPS 1000 ng/ml downregulated *TLR-2* expression (Fig. 3-2C, $P<0.05$).

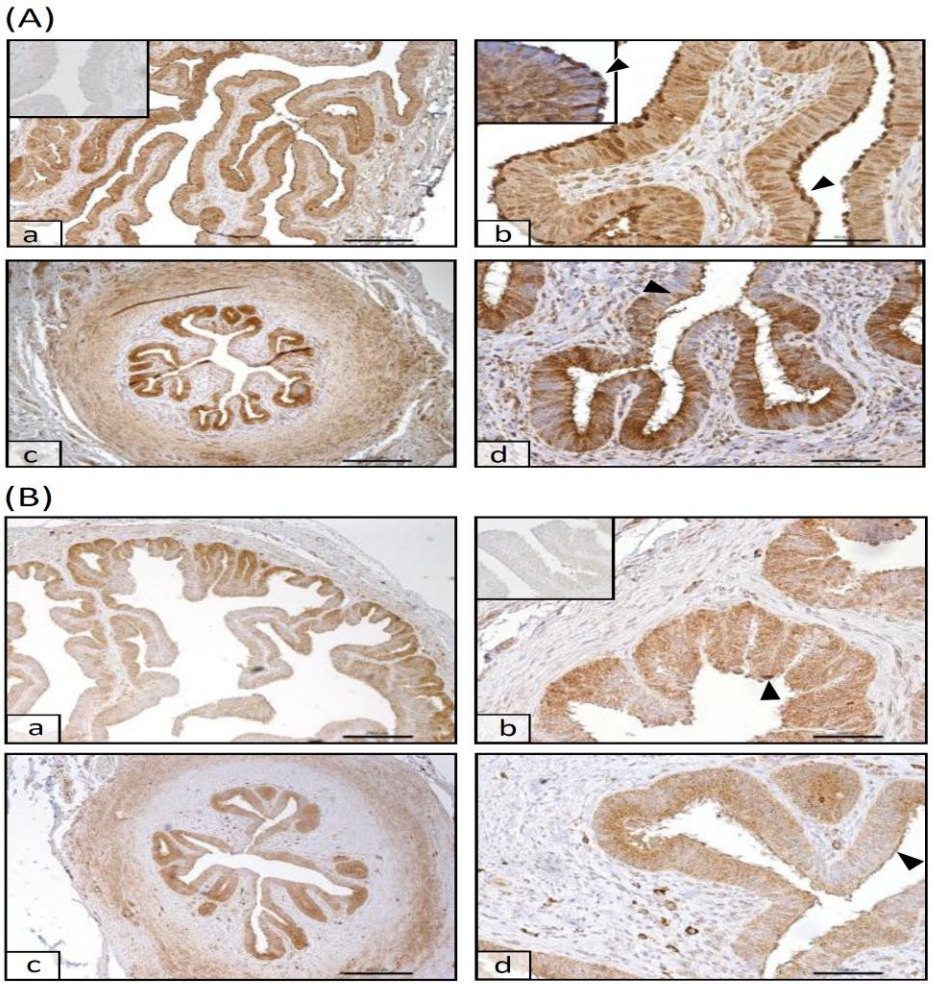


Fig. 3-1 (A) Immunohistochemical detection of TLR-2 in paraffin wax sections of the ampulla (a, b) and isthmus (c, d) of the bovine oviduct. Arrowheads (b, d) indicate apical staining as well as staining of cells with apical cilia in the detailed picture in b. Insert in a: the control. Insert in b: detail of staining of cells with apical cilia. (B) Immunohistochemical detection of TLR-4 in paraffin wax sections of the ampulla (a, b) and isthmus (c, d) of the bovine oviduct. Arrowhead indicates apical staining in d. Insert in b: the control. Scale bars represent 50 µm.

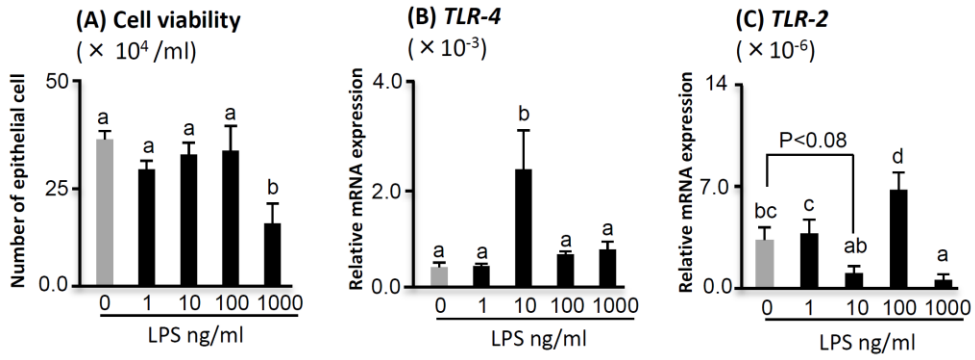


Fig. 3-2 Effect of O55:B5 *E. coli* lipopolysaccharide (0, 10, 100, and 1000 ng/ml) on (A) cell viability estimated with the use of trypan blue, (B) relative mRNA expression of *TLR-4*, and (C) relative mRNA expression of *TLR-2* in a dose-dependent study in the bovine oviduct epithelial cell culture. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P<0.05$ as determined by ANOVA followed by Bonferroni's multiple comparison test.

3.3. Immunological responses of BOEC to the different doses of LPS

Stimulation of BOEC with 10 ng/ml LPS significantly increased the expression of *TLR-4*, *NFKBIA*, *COX-2*, *IL1-β*, and *TNF-α* (Fig. 3-3A-E, $P<0.05$). However, 100 ng/ml LPS did not induce the expression of *TLR-4*, *NFKBIA*, *COX-2*, *IL1-β*, and *TNF-α*, but increased the expression of *TLR-2*, *IL-10*, and *IL-4* (Fig. 3-3F, H, and I, $P<0.05$).

3.4. Ovarian steroids and LH completely block LPS-induced responses in the BOEC

The effect of ovarian steroids, E2 and P4, and LH on the pro-inflammatory response of BOEC to the LPS was investigated because the oviduct microenvironment is constantly exposed to sex hormones during the estrous cycle. BOEC was stimulated with LH (10 ng/ml), P4 (1

ng/ml), and E2 (1 ng/ml) at concentrations observed during the preovulatory period *in situ*. Ovarian steroids and LH completely suppressed LPS (10 ng/ml)-induced expression of *TLR-4*, *IL-1β*, and *TNF-α* (Fig. 3-4A, C, and D, P<0.05). Stimulation of BOEC with 10 ng/ml of LPS together with either P4 or LH also suppressed the stimulatory effect of LPS (10 ng/ml) on *COX-2* expression (Fig. 3-4E, P<0.05). The stimulation of BOEC by 10 ng/ml LPS together with either P4 or E2 reduced LPS (10 ng/ml)-induced *NFKBIA* expression (Fig. 3-4B, P<0.05).

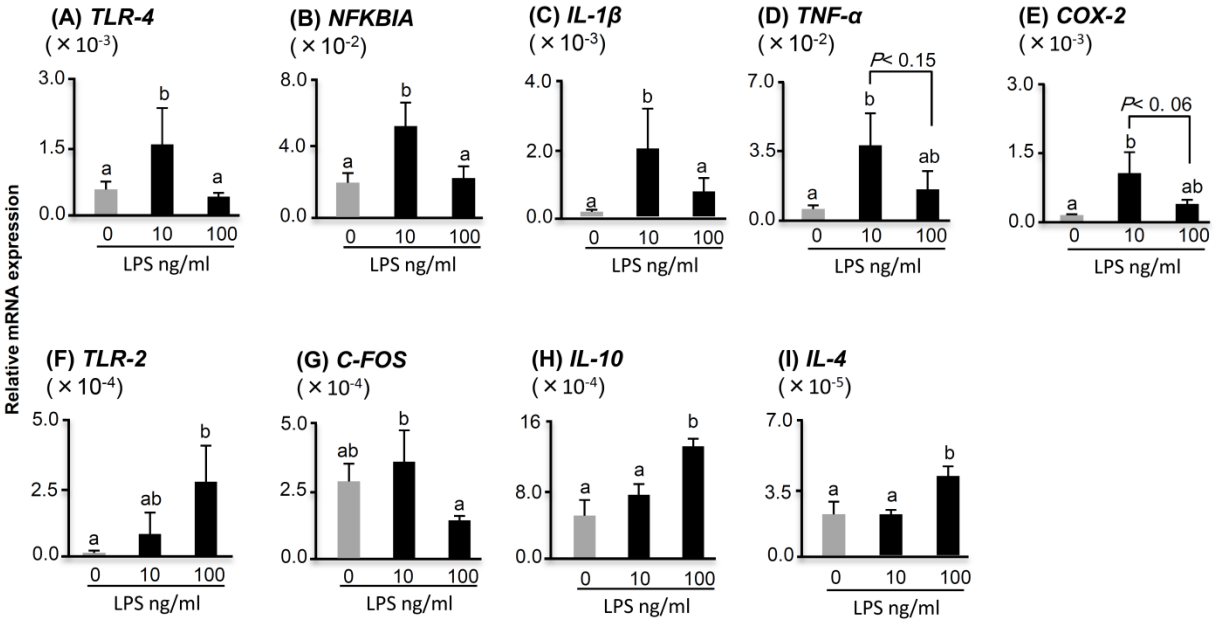


Fig. 3-3 Relative mRNA expression of *TLR-4*, *COX-2*, *IL-1β*, *TNF-a*, *TLR-2*, *C-FOS*, *IL-10*, *IL-4*, and *NFKBIA* in the bovine oviduct epithelial cell culture stimulated with different doses of O55:B5 *E. coli* lipopolysaccharide (10 and 100 ng/ml) and harvested after 24 hours. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at P<0.05 as determined by ANOVA followed by Fisher's multiple comparison test.

Ovarian steroids and LH also inhibited stimulatory effect of 100 ng/ml LPS on *TLR-2* expression (Fig. 3-4F, P<0.05). Moreover, E2 inhibited stimulatory effects of 100 ng/ml LPS on *IL-10*

expression (Fig. 3-4H, P<0.05). Stimulation of BOEC with LPS 100 ng/ml together with E2 induced *C-FOS* expression compared with LPS (100 ng/ml) - stimulated cells (Fig. 3-4G, P<0.05).

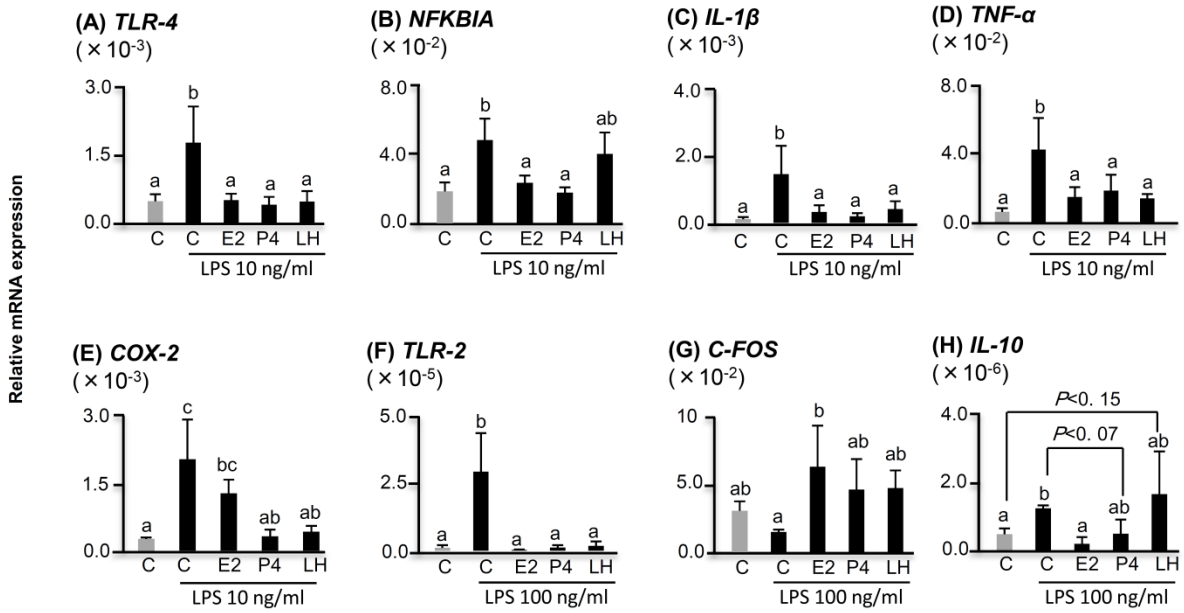


Fig. 3-4 Relative mRNA expression of *TLR-4*, *COX-2*, *IL-1β, TNF-α, TLR-2*, *C-FOS*, *IL-10*, *IL-4*, and *NFKBIA* in the bovine oviduct epithelial cell culture stimulated with different doses of O55:B5 *E. coli* lipopolysaccharide (10 and 100 ng/ml) together with E2 (1 ng/ml), P4 (1 ng/ml), and LH (10 ng/ml) at concentrations observed during preovulatory *in situ* and harvested after 24 hours. C (gray bar): control without any stimulant. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at P<0.05 as determined by ANOVA followed by Bonferroni's multiple comparison test.

3.5. Regulation of prostaglandin E2 secretion by LPS, E2, and LH in the BOEC

A dose of 100 ng/ml of LPS significantly stimulated m*PGES-1* expression and PGE2 secretion in BOEC culture (Fig. 3-5A-B, P<0.05).

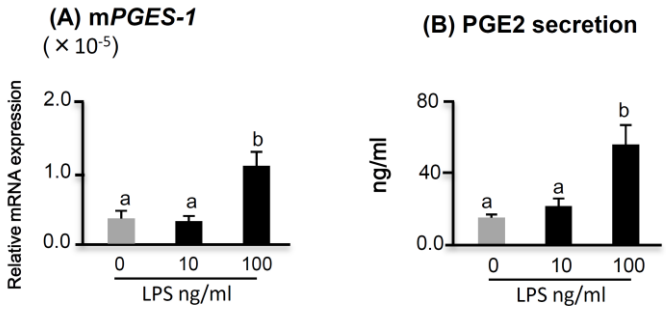


Fig. 3-5 Effect of different doses of O55:B5 *E. coli* lipopolysaccharide (0, 10, and 100 ng/ml) on (A) mRNA expression of m*PGES-1* and (B) PGE2 secretion in the bovine oviduct epithelial cell culture. C (gray bar): control without any stimulant. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P<0.05$ as determined by ANOVA followed by Fisher's multiple comparison test.

4. Discussion

The results of this study provide the evidence that the bovine oviduct expresses genes and proteins for both TLR-4 and TLR-2. The intensive expression of these TLRs was found within the epithelial cells. Interestingly, TLR-2 and TLR-4 were highly expressed in the apical cilia rather than basolateral pole of the bovine oviduct epithelial cells. This suggests the higher sensitivity of the oviduct epithelial cells to the possible TLRs ligands in the oviduct fluid. Previous studies showed that TLR-2, but not TLR-4, is expressed in the murine oviduct [74]. Human oviduct is shown to express TLR-2, and TLR-4 is expressed within oviductal stromal fibroblasts but not oviductal epithelial cells [75, 76]. These findings indicate the existence of a species-specific difference in the oviduct mucosal immunity.

In this study, 10 ng/ml LPS induced the expression of *TLR-4* but not *TLR-2*, and a 10-fold higher concentration of LPS increased the expression of *TLR-2* but not *TLR-4*. Hearth *et al*. [77] reported that 1000 ng/ml *E. coli* LPS induced *TLR-4* expression in the endometrial cells of bovine uterus, and Ibeagha-Awemu *et al*. [63] found that 0.01–10 μg/ml LPS stimulates the expression of both *TLR-4* and *TLR-2* in the bovine mammary epithelial cells. The absence of effect of LPS at dose of 100 ng/ml on the expression of *TLR-4* could be due to the concomitant up-regulated Th2 cytokines such as *IL-4*. It has been shown that IL-4 reduces LPS responsiveness and TLR-4 protein surface expression, *TLR-4* mRNA expression and transcriptional activity of the upstream region of TLR-4 in intestinal epithelial cells and peripheral blood mononuclear cells (PBMCs) [78, 79]. This effect is tyrosine kinase and STAT6 dependent. A STAT6 binding site has been determined in an area of the TLR-4 gene necessary to mediate the inhibitory effects of IL-4 on TLR-4 transcription [79]. The present data may imply a novel autonomous innate immunity in the bovine oviduct.

Exposure of epithelial cells to 10 ng/ml LPS induced the expression of *TLR-4*, *COX-2*, and Th1 cytokines (i.e., *IL-1β* and *TNF-α*), demonstrating a high sensitivity of oviduct epithelial cells to low-dose LPS. Interleukin-1β and TNF-α are known to be involved in the inflammatory responses of human oviduct to *Chlamydia trachomatis* infection [80, 81]. The induction of *COX-2*, an important downstream target of TLR-4 signaling [82], increases epithelial production of PGE2 and causes proliferation in infected epithelial cells [83]. The present results imply that the bovine oviduct epithelial cell may employ an early and sensitive innate surveillance (manifested by elevated *TLR-4* expression) to preclude infection. This could be sufficient to provide a sterile microenvironment, and at the same time, protection from infection.

Interestingly, low-dose LPS also stimulated the expression of *NFKBIA*, which is NF-κB-dependent. NFKBIA strongly sequesters NF-κB in the cytoplasm, thereby generating auto-regulatory feedback loops in the NF-κB response [84]. Rodrigues *et al*. [85] found increased *NFKBIA* expression in the rat epididymis challenged by *E. coli* LPS after 2 hours of exposure *in vivo*. Thus, in the present study, a low-dose LPS induced *NFKBIA* expression, possibly providing a compensatory mechanism to balance activation of NF-κB.

Importantly, 100 ng/ml LPS did not induce *TLR-4* and Th1 cytokines, but stimulated the expression of *TLR-2* and Th2 cytokines (i.e., *IL-4* and *IL-10*). We propose two possibilities to explain this phenomenon (Fig. 3-6). First, the high levels of IL-1β and TNF-α during infection are clearly detrimental to the human oviduct [80, 81], leading to barrier disruption, subsequent translocation of bacteria to the lamina propria [86], and disturbance of the natural tolerance mediated by regulatory T cells and immunosuppressive cytokines [87]. Because of this, it is hypothesized that the BOEC responds to 100 ng/ml LPS through suppression of *TLR-4* and Th1 cytokines to prevent severe tubal injury. Moreover, TLR-2 critically participates in protection of the intestinal epithelial barrier and controls mucosal inflammation by directly preserving tight junctions [59]. Thus, upregulation of *TLR-2* by 100 ng/ml LPS may also involve in preserving oviductal epithelial barrier. In addition, according to the theory of Th2-mediated repair, many of the proteins produced in response to IL-4 are associated with injury and have well-known roles in tissue repair [88].

Second, although TLRs are crucial for an efficient immune response, certain pathogens use TLR-based strategies to evade the host defense, preventing further elimination of the microorganisms [57]. TLR-2, which is being strong mediator of anti-inflammatory effects [57], and TLR-4 are involved in *IL-10* expression [89]. *E. coli* LPS induces *TLR-2* expression and

sensitivity of TLR-2 to stimulations [60-62]. LPS is known to signal through TLR-2 [90]. *E. coli* LPS *in vitro* activates spleen cells from TLR-$2^{+/+}$ mice to induce production of the Th2-polarizing cytokine IL-10 [91]. Moreover, in the presence of myeloid differentiation protein 2 (MD-2, a protein associated with TLR-4 on the cell surface and needed for TLR-4 to respond to LPS), both TLR-2 and TLR-4 are highly and equally sensitive to all endotoxic LPS, including protein-free LPS [92]. MD-2 enhances TLR-2-mediated responsiveness to both gram-negative and gram positive bacteria [92]. The present results suggest that a dose of 100 ng/ml of LPS increases the expression of TLR-2, which is accompanied by elevated expression of Th2 cytokines, and corresponding decrease in the expressions of *TLR-4* and Th1 cytokines. It is proposed that this arrangement is related to mucosal homeostasis *via* mucosal barrier protection and the prevention of expanding inflammation. It may also serve as a mechanism of escape from the oviduct defense for gram-negative bacteria.

A dose of 100 ng/ml LPS stimulated the expression of m*PGES-1* and PGE2 secretion, but not *COX-2* expression. It is documented that both TLR-4 and TLR-2 are involved in PGE2 secretion [93, 94]. LPS stimulates PGE2 production in bovine endometrial cells [77]. TLR-2 is also the major receptor that mediates PGE2 production in response to *C. albicans* in murine peritoneal macrophages and splenocytes [94]. The present results suggest that elevated expression of *TLR-2* in cells treated with 100 ng/ml could induce m*PGES-1* expression and PGE2 secretion. Interestingly, despite the well known regulatory coupling of the two enzymes, COX-2 and mPGES-1, LPS at dose of 10 ng/ml induced *COX-2* expression, but not m*PGES-1* expression and resultant PGE2 secretion. Arosh *et al.* [95] also reported that m*PGES-1* expression is not coupled to the expression of *COX-2* in endometrium stimulated with interferon-tau. It seems that the expression of *COX-2* and m*PGES-1* in response to LPS may also be

uncoupled in the BOEC. Therefore, PGE2 secretion could be controlled by mPGES-1, but not COX-2 in BOEC culture in response to LPS.

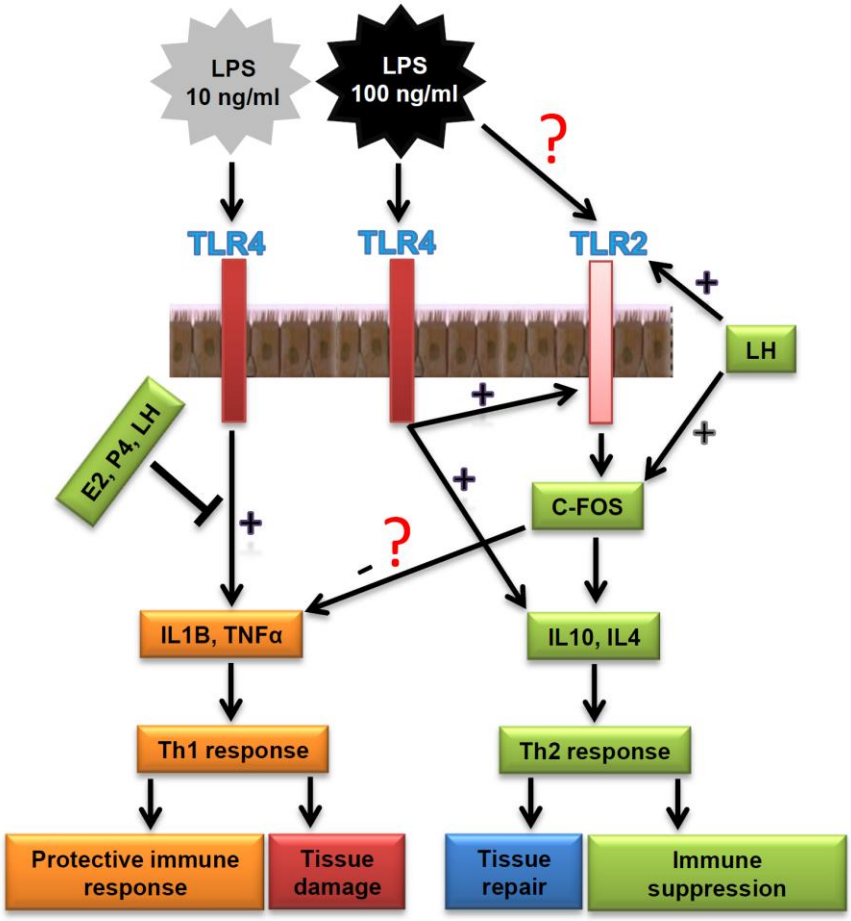


Fig. 3-6 Schematic illustration for a novel regulation of local innate immunity in the bovine oviduct. Differential TLR-mediated pathways can induce specificity to innate immunity. Recent literature suggests that certain microorganisms not only are recognized by TLRs for the activation of host defense but also activate alternative TLR pathways with inhibitory effects on innate immunity. Interaction of 10 ng/ml *E. coli* LPS with TLR4 induces expression of Th1 cytokines toward protective immune response, while activation of TLR-4, together with possible TLR-2 signaling by 100 ng/ml *E. coli* LPS, induces the expression of Th2 cytokines toward immune suppression. This results in inhibition of host defense and increased susceptibility to infection. Similar mechanisms have been suggested for other microorganisms, such as *Y. enterocolitica* and *A. fumigates* [68]. On the other hand, Th2 cytokines and TLR-2 play important roles in preserving tight junction and tissue repair. Moreover, ovarian steroids and LH markedly influence innate immune protection by epithelial cells in the oviduct through inhibition of pro-inflammatory responses.

An important finding of our study is that E2, P4, and LH, at concentration around ovulation in situ, completely reversed the stimulatory effect of 10 ng/ml LPS on *TLR-4* expression and TLR4-related genes. Moreover, E2, P4 and LH suppressed *TLR-2* expression in response to 100 ng/ml LPS and E2 reversed LPS induction of *IL-10* expression. Fahey *et al*. [96] reported that E2 can reverse the stimulatory effects of IL-1β on the expression of *TNF-a*, *IL-8*, and *NF-κB* in human uterine epithelial cells. It has been shown that the inhibitory effects of E2 and P4 on LPS-induced immune responses are receptor-mediated in human uterine epithelial cells and murine bone marrow stem cell [96, 97]. Since the bovine oviduct and BOEC express receptors for LH [28], E2, and P4 [29], possibly, the inhibition of LPS-induced TLR-4 and TLR-4 related genes by these hormones may be mediated through receptors in BOEC. The present data imply that the periovulatory levels of E2, P4 and LH accomplish downregulation of both Th1 and Th2 responses during infection to prevent harmful changes in the oviduct microenvironment, particularly, when allogeneic sperm enters the oviduct and a semi-allogeneic embryo is about to start its development. However, this may lead to cancel the immune responses, resulting in tissue damage by bacteria or toxin. It has been reported that despite suppression of LPS-induced NF-κB and proinflammatory responses by E2 in human uterine epithelial cells, E2 induces antimicrobial protein secretory leukocyte protease inhibitor (SLPI) [96]. Therefore, on the one hand, these hormones may enhance protection against pathogens by increasing antimicrobial activity. On the other, it may ensure an environment for successful fertilization by suppressing proinflammatory responses detrimental to allogeneic sperm and a semi-allogeneic embryo. However, if the invasion of infection overwhelms the immune capacity, the affected cells or tissue appear to be endangered by bacteria or toxin.

Taken together, these findings shed a light on a novel immune function of BOEC, which initiates a biphasic and sensitive pro-inflammatory response to the infection. Low dose of *E. coli* LPS initiate a Th1-type response, but at higher LPS dose, the immune response switches over toward Th2-type response. In addition, ovarian steroids and LH suppress LPS-induced pro-inflammatory responses. Therefore, epithelial cells employ ovarian steroids and LH in maintaining microenvironmental homeostasis of the oviduct, guaranteeing an optimal microenvironment to achieve successful fertilization via inhibition of Th1 responses detrimental to allogeneic sperm and the semi-allogeneic embryo in the bovine oviduct. Since the responses to LPS and hormones could be modulated by other cells and factors as well as epithelial cells in the oviduct, further investigation will focus on the possible interactions among epithelial cells, immune cells and other cell types.

Chapter IV

Function and Regulation of alpha-1-Acid Glycoprotein in the Bovine Oviduct Epithelial Cells *In Vitro*

1. Introduction

Epithelial cells are among the first cells exposed to pathogens, and at the mucosal surfaces function not only as a physical barrier, but also as an innate and adaptive immune regulator (16). In female reproductive tract, oviductal epithelial cells, OECs, are critical for establishment of a successful pregnancy (96, 98). The OECs is exposed to pathogens and endotoxins from uterus and peritoneal cavity, resulting in the oviduct infection. Thus, the mucosal OECs should sense and respond to the potential pathogens as well as provide an environment for fetal survival (99).

The epithelial cells clearly have been known to play roles in the innate immunity (53). To recognize pathogens, the innate immunity uses pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), which recognize microbial antigens, pathogen-associated molecular patterns (PAMPs) (54)(Kumar *et al*. 2009). We have shown that TLR4 and 2 are expressed in oviduct epithelia cells (chapter 3)**.** TLR4 pathway is known to induce the expression of acute phase proteins (APPs) in liver (100). It is likely that nuclear factor-kappa B (NFKB), a key intracellular component of the lipopolysaccharide (LPS) signaling pathway, participates in the subsequent downstream transcriptional induction of genes encoding APPs (101). Moreover, Cheng et al. (102) reported that TLR2 is a functional receptor for acute-phase serum amyloid A. The serum concentration of APPs dramatically increases in response to systemic inflammation or infection. APPs such as Alpha-1-acid glycoprotein (AGP) and haptoglobin are primarily synthesized by the liver but can be produced also in the extra-hepatic organs (103, 104). Thus; the acute phase response may take place in the extra-hepatic cell types. The antimicrobial peptides such as β-defensins and secretory leukocyte protease inhibitor (SLPI) (99), and APPs have been shown to resolve the local inflammation, repair the injured tissue, re-establish

homeostasis (105), and provide non-specific protection against microbes (106). AGP or orosomucoid is one of the main APPs in humans, rats, mice and other species (107, 108), and expression is regulated by sex steroids (106). Although AGP expression has not been evaluated in the bovine oviduct, it is expressed in the bovine ovary and uterus (104), and rat and human alveolar epithelial cells (109). The exact biological and local function of AGP remains unknown; but several lines of evidence support a modulatory action on the immune response. For example, AGP induces release of proinflammtory cytokines by mononuclear leukocytes induced by LPS (110). On the other hand, AGP has in fact the capacity to act as an anti-inflammatory molecule (111, 112). AGP has been demonstrated to increase secretion of the IL1 receptor antagonist (113), inhibit chemotaxis and activation of neutrophils (114), and inhibit the pro-angiogenic effect of TNFα (115).

It is well known that epithelial cells play the key role in the defense against microbial infection. The present study tested the hypothesis that BOEC expresses the non-specific effector molecule, AGP, for defense against bacteria. The first objective was to establish if bovine oviduct expresses AGP. The next questions were the regulation and local function of AGP in the BOEC challenged by LPS and sex hormones.

2. Materials and Methods

2.1. Immunohistochemistry

Paraffin-embedded tissue sections (4 μm thick) of bovine Ampulla or Isthmus were mounted on silane-treated glass slides (Histobond Superior; Paul Marienfeld Laboratory Glassware, Laud-Königshofen, Germany) and dried at 37 °C for 24 h. Afterward, they were

deparaffinized in xylene and rehydrated in a series of solutions of graded alcohol concentrations. To block endogenous peroxidase activity the sections were incubated for 30 min in 80% alcohol solution containing 2% hydrogen peroxide. After rinsing the sections three times for 5 min in PBS (pH 7.2), antigen retrieval was performed by boiling the sections in 10mM Citrate Acid (pH 6) for 15 min. Subsequently they were incubated for 20 min in 20% normal goat serum (in PBS) at room temperature to saturate any sites for non-specific binding of proteins. Antibody used for immunohistochemistry was rabbit anti-Alpha-1-Acid Glycoprotein (a1AGP) (1:200; PAA816Bo0; Uscn Life Science Inc.; China). The antibody was diluted in PBS containing 1% bovine serum albumin and incubated in a humidified chamber over night at 4 °C. For detection the EnVision™ anti-rabbit immunoglobulin conjugated to peroxidase labelled dextran polymer system (DAKO, Glostrup, Denmark) was used in accordance with the manufacturer's protocol. Finally, sections were washed with PBS and peroxidase activity was detected with DAB (Sigma, Steinheim Germany) as substrate for 5 min at room temperature. Sections were counterstained with hemalum, dehydrated and mounted with DPX (Fluka, Buchs, Switzerland). To analyse unspecific binding, primary antibody was replaced by rabbit IgG (Sigma) at the same concentration of the primary antibody.

2.2. In vitro study

Bovine oviduct epithelial cell isolation and culture

Epithelial cells were isolated as previously described (70, 71). Briefly, the oviducts were transported in an ice box from the local slaughterhouse to the laboratory, with the oviducts immersed in PBS solution without Ca^{2+}/Mg^{2+} ($PBS^{-/-}$) (Sigma, St. Louis, MO. USA) but with

0.3% gentamicin (Sigma) and amphotericin B (Illkirch, France). They were cut and separated from the connective tissue and washed twice with PBS. The oviductal lumen was flushed with 15 ml PBS, and the BOEC were mechanically dislodged while being flushed with the same volume of PBS. Over a period of 15 min, the pooled sheets of BOEC from several cows settled at the bottom of the tube, and the cells were then washed with PBS followed by a medium containing D-MEM/F12, 0.1% gentamicin, 1% amphotericin, and 2.2% $NaHCO_3$. Thereafter, the cells were harvested by centrifugation at 300*g* for 10 min at 4°C. The resultant cell pellet was suspended in 10 ml PBS, layered over 10 ml Percol, and centrifuged at 900*g* for 20 min at 4°C. Finally, the cell pellet was washed once with the abovementioned medium, harvested by centrifugation at 300*g* for 10 min at 4°C, and cultured in culture medium (D-MEM/F12, 0.1% gentamicin, 1% amphotericin, and 2.2% $NaHCO_3$ supplemented with 10% fetal calf serum [FCS; Bio Whittaker, Walkersville, MD]) in 6-well culture dishes (Nalge Nunc International, DK-4000 Roskilde, Denmark) at 38°C in 5% CO_2 and 95% air. The following day, the BOEC culture was washed twice with PBS and incubated with culture medium supplemented with 5% FCS. After monolayer formation, cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH) until single cells appeared, and these cells were again plated in 6-well culture dishes at a density of 3×10^4/ml and incubated at 38°C in 5% CO_2 and 95% air in culture medium supplemented with 5% FCS, until the growing BOEC monolayer covered up to 70%–80% of bottom of the culture plate.

LPS and hormone treatments

BOEC were washed twice with culture medium supplemented with 0.1% FCS and incubated for 24 hours with one of the following: (1) AGP (1, 10, 100, and 1000 ng/ml,); (2) LPS (10 or 100 ng/ml, serotype *E. coli* 055:B5; Sigma); (3) LPS (10-100 ng/ml) + AGP (10-100

ng/ml); (4) LH (10 ng/ml, USDA-bLH-B-6, USDA Animal Hormone Program, Bethesda, MD, USA); (5) E2 (1 ng/ml, Sigma); (6) P4 (1 ng/ml, Sigma); (7) a combination of E2+P4+LH; (8) LPS (10 or 100 ng/ml) + LH; (9) LPS (10 or 100 ng/ml) + P4; (10) LPS (10 or 100ng/ml) + E2. Hormonal concentrations were the same for all these experiments. As a control, culture medium without any stimulant was added to the BOEC. The concentrations of hormones in this study were maintained similar to their physiological level in the bovine oviduct during preovulatory period *in situ* (34). This was done to mimic the local hormonal conditions around the time of ovulation in the oviduct. Cell viability was estimated using Trypan-blue staining and was confirmed to be more than 90% at each time of plating as well as at the end of the experiment. The purity of epithelial cell preparations was evaluated by reacting the cultured cells with monoclonal antibodies to cytokeratin (anti-cytokeratin-CK1) and immunostaining. The cells in culture medium showed characteristic epithelial morphology. Approximately 98% of the cells were positive for anti-cytokeratin (CK1) antibodies. AGP was assessed by ELISA (USCN Life Science Inc. Wuhan, China) with sensitivity of 5.5 ng/ml.

2.3. Gene expression

Extraction of RNA

Total RNA was extracted from the oviduct tissue and BOEC using TRIzol reagent (Invitrogen Corporation) as described in the protocol of Chomczynski and Sacchi (72). The yield of extracted RNA for each sample was determined by ultraviolet (UV) spectroscopy (optical density, 260). The RNA concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at absorbances of 260 and 280 nm. The extracted total RNA was stored in

RNA storage solution (Ambion, Austin, TX, USA) at –80°C until it was used for cDNA production.

Production of cDNA

DNase treatment was carried out using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). Two micro-liters of the extracted RNA was incubated for 30 min at 37°C with 1 μL RQ1 RNase-free DNase 10× reaction buffer and 2 μL of 1 μg/μL RNase-free DNase. To halt the reaction, 1 μL RQ1 DNase Stop solution (20 mM EDTA) was added to the sample, and the mixture was incubated for 10 min at 65°C. First-strand cDNA synthesis was conducted according to the commercial protocol described in the SuperScript™ II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The first cocktail was prepared using 2 μL total RNA extracted from the BOEC sample, 1.5 μL of 50 ng/μL random primer (Invitrogen), 1.5 μL of 10 mM PCR Nucleotide Mix (dNTP; Roche Diagnostics, Indianapolis, IN, USA) and 12 μL H_2O which yield a final volume of 18 μL per tube. This cocktail was then incubated at 65°C for 5 min in a thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice while the second cocktail copmrising 3 μL of 0.1 M DTT (Invitrogen), 1.5 μL of 40 units/μL RNasin™ Ribonuclease Inhibitor (Promega, Madison, WI, USA), and 6 μL of 5× First-Strand Buffer (Invitrogen), was added to each tube. The samples were incubated for 2 min at 42°C, and 0.2 μL of 200 units/μL SuperScript™ II Reverse Transcriptase was added to each tube. 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.

Real-time polymerase chain reaction (real-time PCR)

We analyzed the following genes: *AGP*, *AGPR*, *TLR4*, *TLR2*, *TNFα*, *IL1B*, and *β-actin*. Quantifications of mRNA expression were performed using synthesized cDNA via real-time PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany) using a QuantiTect™ SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany). The primers were designed using Primer3 based on bovine sequences. The primers used for real-time PCR are listed in Table 4-1 The amplification program consisted of 15 min activation at 95°C, followed by 40 cycles of PCR (15 sec denaturation at 95°C, 30 sec annealing at 52–58°C and 20 sec extension at 72°C). The values of mRNA expression were assayed by normalization to β-actin as an internal control.

Table 4-1 Bovine primers were used in real-time PCR.

Gene		Sequence of nucleotide (5'-3') *	Tm (C)
TLR-4	F	CTTGCGTACAGGTTGTTCCTAA	56
	R	CTGGGAAGCTGGAGAAGTTATG	
TLR-2	F	GCTCCTGTGACTTCCTGTCC	54
	R	CCGAAAGCACAAAGATGGTT	
AGP	F	CCAACCTGATGACAGTGGC	58
	R	GCCGACTTATTGTACTCGGG	
AGPR	F	ATGACAAAGGAGTATCAA	52
	R	AGCTTGGAGTTCTGGGAT	
*IL-1*β	F	ATGAAGAGCTGCATCCAACA	56
	R	ATGGAAGACATGTGCGTAGG	
TNF-α	F	TGACGGGCTTTACCTCATCT	56
	R	TGATGGCAGACAGGATGTTG	
β-actin	F	CCAAGGCCAACCGTGAGAAAAT	58
	R	CCACATTCCGTGAGGATCTTCA	

* F, forward; R, reverse

2.4. Statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed with Stat View 5.0 (SAS Institute Inc.). One-way ANOVA followed by Fisher's multiple comparison tests were performed, and all results were considered to be statistically significant at $P < 0.05$.

3. Results

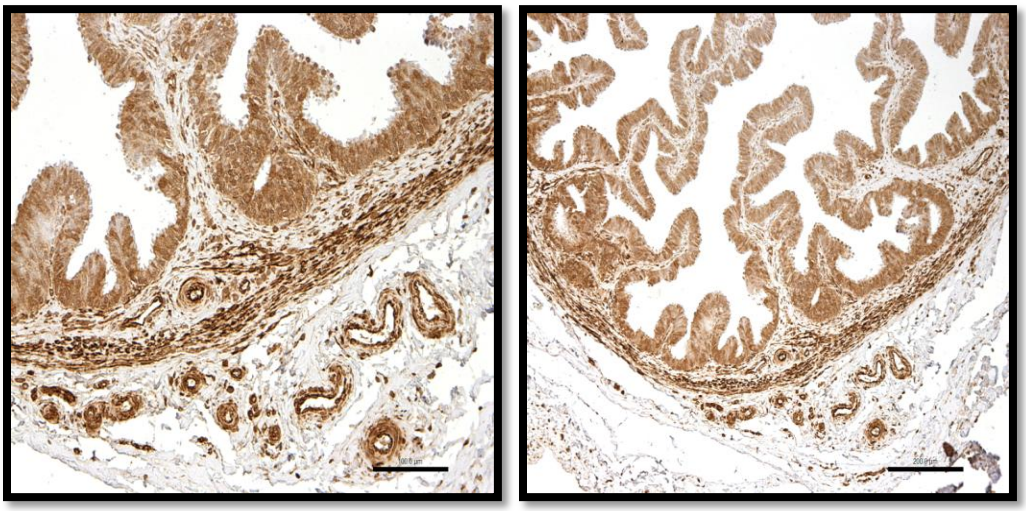
3.1. Protein expression of AGP in the oviduct

The immunohistochemistry showed that a-1-AGP is expressed in the bovine oviductal ampulla and isthmus, with weaker expression in the isthmus. a-1-AGP was expressed within epithelial cells, muscle layer and endothelial cells.

3.2. Regulation of AGP system by P4 in the BOEC

We stimulated BOEC with sex hormones at concentrations that mimic *in situ* preovulatory levels (E2, 1ng/ml; P4, 1 ng/ml; and LH, 10 ng/ml).This experiment was based on the stimulatory effects of P4 on the upregulated AGP system in the oviduct tissue. We found that P4 induced both expression and production of AGP in the BOEC (Fig. 4-1D and E, $P<0.05$).

A)



B)

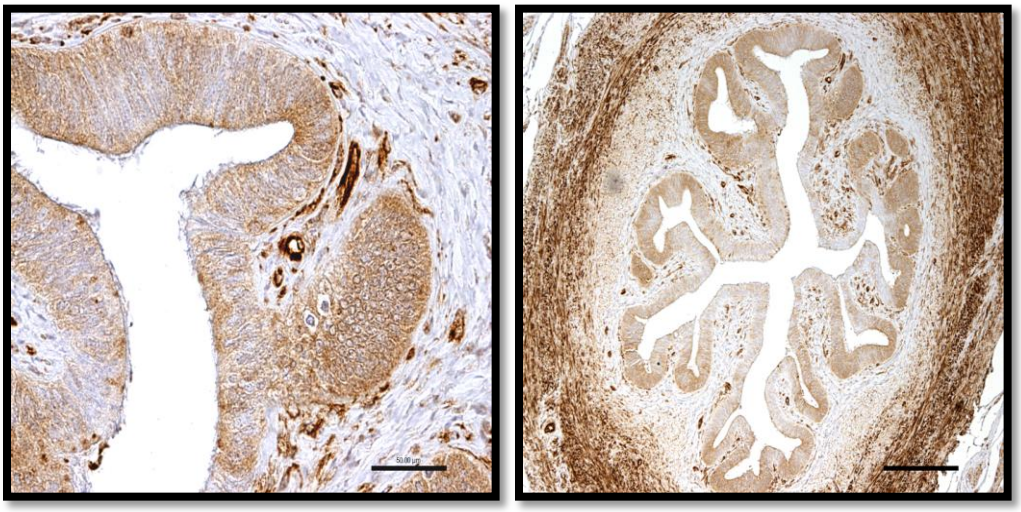


Fig. 4-1 Immunohistochemical detection of a-1-AGP in paraffin wax sections of the ampulla (A) and isthmus (B) of the bovine oviduct. Scale bars represent 50 µm.

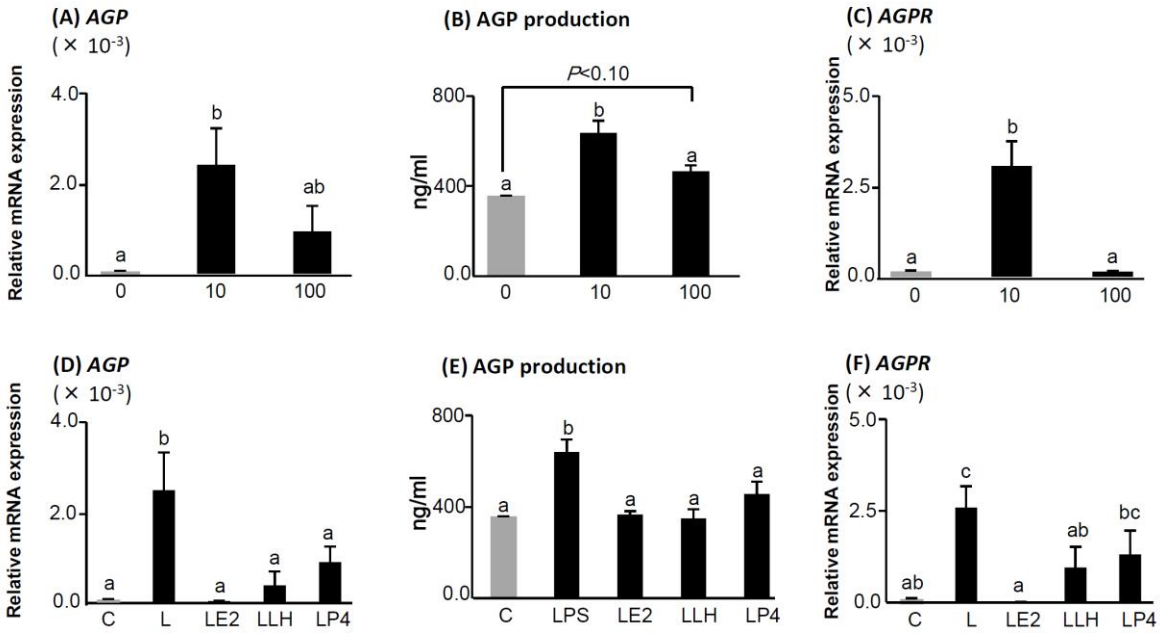


Fig. 4-2 Production and expression of *AGP* and AGP receptor *(AGPR)* in the bovine oviduct epithelial cell culture stimulated with different doses of O55:B5 *E. coli* lipopolysaccharide (10 and 100 ng/ml) (A-C), and lipopolysaccharide (10 ng/ml) together with sex hormones at concentrations observed during preovulatory *in situ* and harvested after 24 hours (D-F). C: control, L: 10 ng/ml LPS, LH: 10 ng/ml, P4:1 ng/ml, and E2:1 ng/ml. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Fisher's multiple comparison test.

3.3. Differential regulation of AGP system by LPS and sex hormones in the BOEC

The stimulation of BOEC with low dose of LPS (10 ng/ml) stimulated *AGP* system. However, 100 ng/ml LPS did not induce *AGP* and *AGPR* expression (Fig. 4-2A-C, P<0.05). The sex hormones, at concentrations observed during the preovulatory period *in situ* (E2, 1ng/ml; P4, 1 ng/ml; and LH, 10 ng/ml), completely suppressed LPS (10 ng/ml)-induced AGP system (Fig. 4-2D-F, P<0.05).

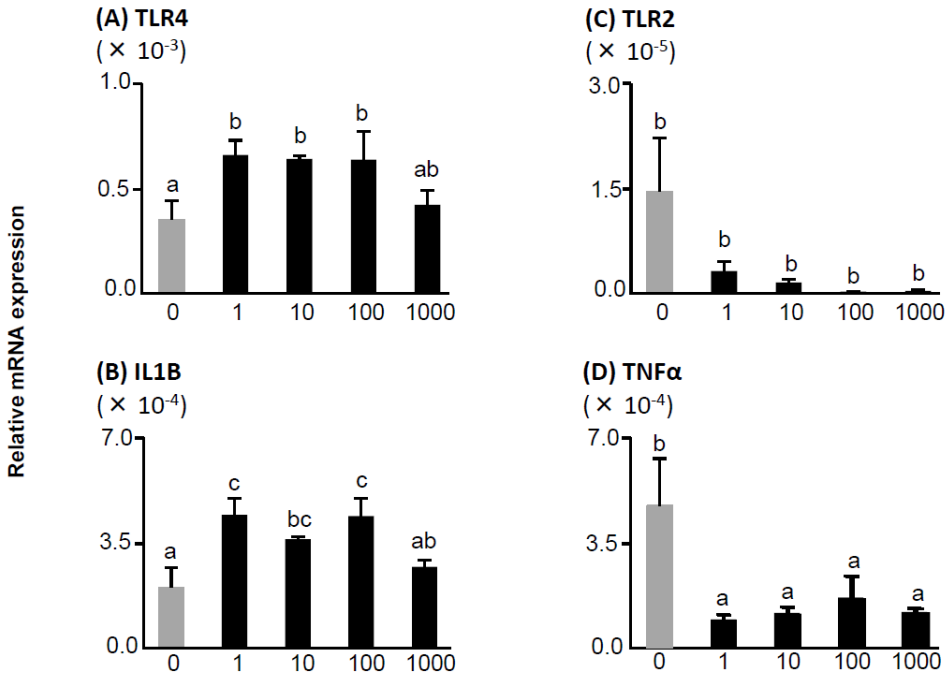


Fig. 4-3 Relative mRNA expression of *TLR4, IL1B, TNFα, and TLR2* in the bovine oviduct epithelial cell culture stimulated with different doses of AGP (0, 10, 100, and 1000 ng/ml) and harvested after 24 hours. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Fisher's multiple comparison test.

3.4. Immunological function of AGP in the BOEC

A dose-response study was performed, in which epithelial cells were incubated in 0.1% FCS medium alone or in the increasing doses of AGP (1, 10, 100, and 1000 ng/ml) for 24 hours to better understand the effects of AGP on the immune responses in the BOEC. A very small dose of AGP, 1 ng/ml, was enough to stimulate the expression of *TLR4* and *COX-2*. This phenomenon was also seen in BOEC stimulated with 10 and 100 ng/ml AGP, but not with 1000

ng/ml AGP (Fig. 4-3A and B, P<0.05). Different doses of AGP (1-1000 ng/ml) completely suppressed the expression of *TLR2*, *COX-2*, and *TNFα* (Fig. 4-3C and D, P<0.05).

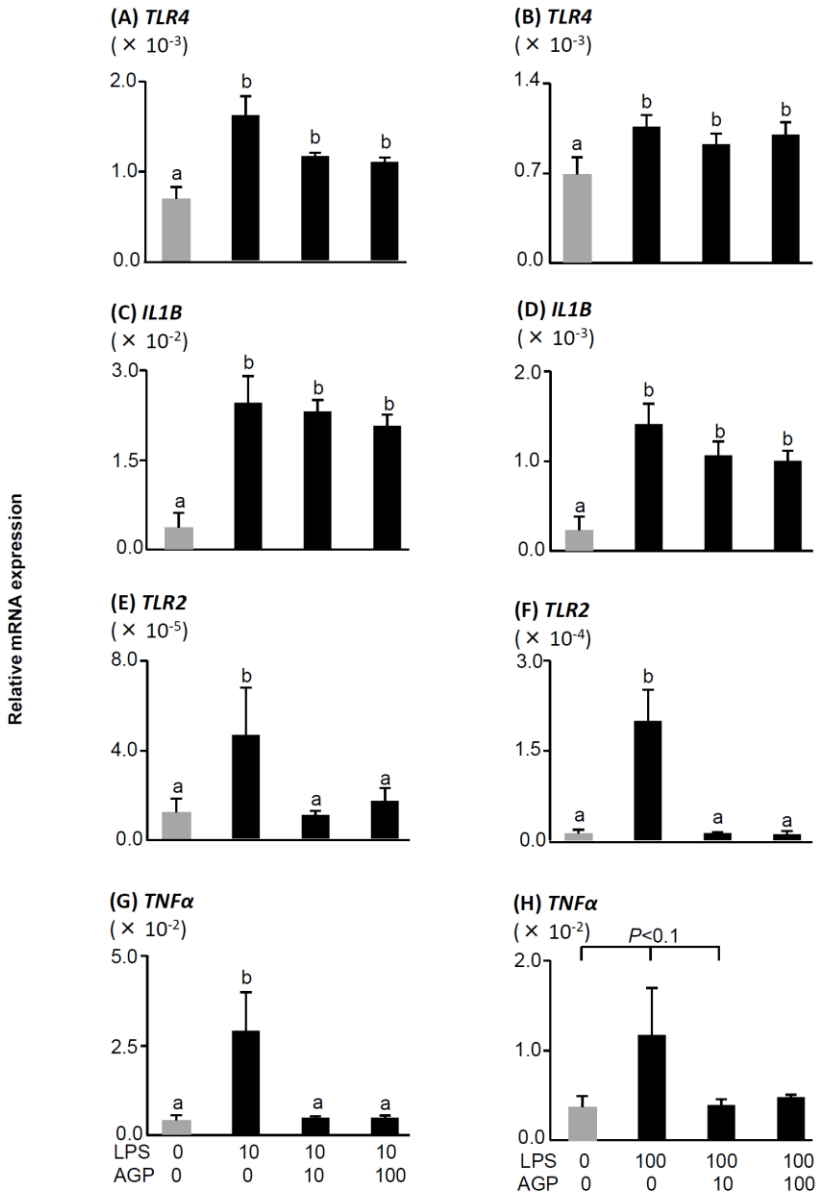


Fig. 4-4 Relative mRNA expression of *TLR4, IL1B, TNFα, and TLR2* in the bovine oviduct epithelial cell culture stimulated with different doses of O55:B5 *E. coli* lipopolysaccharide (10 and 100 ng/ml) together with different doses of AGP (10-100 ng/ml) and harvested after 24 hours. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Fisher's multiple comparison test.

3.5. Immunological interaction between AGP and LPS in the BOEC

AGP (10 or 100 ng/ml) failed to downregulate the LPS-induced *TLR4* (Fig. 4-4A-D, $P<0.05$). However, AGP (10 or 100 ng/ml) completely suppressed the LPS-induced *TNFα* and *TLR2* expression (Fig. 4-4E-H, $P<0.05$).

4. Discussion

The results of this study provide the first evidence showing that the bovine oviduct expresses genes and proteins for AGP. The immunohistochemistry showd that a-1-AGP is expressed in the bovine oviductal ampulla and isthmus, with weaker expression in the isthmus. a-1-AGP was expressed within epithelial cells, muscle layer and endothelial cells of the bovine oviduct.

The present data showed that 10 ng/ml LPS, but not 100 ng/ml LPS, induced AGP system. The LPS-TLR4 complex stimulates signal transduction pathways that initiate the production of inflammatory cytokines, chemokines and, after cell activation, APPS such as AGP that are important components of the inflammatory immune response to the invading microbe (116). LPS is known to be one of the initial inducing agents associated with the induction of APPs *via* TLR4 in the liver after injury (100). Kimura et al. (117) reported that the protective effects of zinc against LPS-induced lethality were caused by AGP. The present study indicated that the early induction of AGP by low dose of LPS (10 ng/ml) is a part of mucosal defense in the bovine oviduct while it seems that high dose of LPS (100 ng/ml) is able to overcome this immune defense in BOEC.

The results show that sex hormones LH, E2, and P4 suppressed the expression and production of *AGP* in LPS-stimulated model while in physiological model, P4 alone up-regulated AGP system. Moreover, Lebreton et al. (118) found that sex steroids, estrogens, raise the concentration of serum AGP in rats. Indeed, the complexity of the LPS-mediated acute phase response involves activation and differential glycosylation of AGP in the stimulated vs. resting environment (119).Thus, the specific functions of AGP may be affected by the biological environment in which the acute phase response is activated (119).

Interestingly, the different doses of AGP (1-100 ng/ml) up-regulated the expression of *TLR4* and *ILB*, with down-regulation of *TLR2* which could be being strong mediator of anti-inflammatory effects (57). Moreover, the administration of AGP (10-100 ng/ml) did not affect on LPS-induced *TLR4* and *IL1B* expression while suppressed the LPS-induced *TLR2* expression. To our knowledge, there is no literature showing the effect of AGP on *TLR4* and *TLR2* expression. However, AGP can potentiate LPS-induced IL1B production by human monocytes and macrophages (110). This could result in a positive feedback loop; the local concentration of AGP can induce the expression of *TLR4* and pro-inflammatory cytokine *IL1B*, which, in turn, is important for the propagation of the inflammatory response (120), and could stimulate leukocyte recruitment and activation (81). The present data show a protective and pro-inflammatory role for AGP via up-regulation of *TLR4* and *IL1B* in the bovine oviduct, and suggest that local production of AGP *in vitro* at the low dose of LPS may activate cytokine secretion.

Of note, different doses of AGP (1-1000 ng/ml) suppressed the expression of *TNFα*. Moreover, administration of AGP (10-100 ng/ml) also inhibited the stimulatory effect of LPS on *TNFα* expression. TNFα is considered to be the major mediator of the shock-inducing activities of endotoxins (121). Libert et al. (122) found that AGP was able to very significantly protect

animals from lethal shock induced by TNFα or LPS. Moreover, human AGP can reduce inflammation directly through reducing TNFα production by stimulated mononuclear leukocytes (123). Our results show that AGP plays an anti-inflammatory role through suppression of *TNFα* expression in the BOEC to prevent tissue damage.

Interestingly, AGP induced *IL1B* expression and suppressed *TNFα* expression. It is likely that in certain condition these cytokines are regulated differentially. For example, the exposure of macrophages to low doses of LPS suppresses the production of TNFα, but not of IL1B (119). Ligresti et al. (115) also found that AGP can also inhibit TNFα-, but not vascular endothelial growth factor (VEGF)-mediated angiogenesis. One possible explanation for these anti-TNFα effects is that AGP blocks TNFα by inducing expression of soluble tumor necrosis factor receptor 1(sTNFR1), which is an antagonist to *TNFα* (115, 124). Thus, these findings suggest that expression of *TNFα* and *IL1B* are regulated differently by AGP in BOEC. More studies are however needed to better define the mechanisms by which AGP influences TNFα. A better understanding of the AGP/TNFα system may provide novel insights into the mechanisms that regulate local inflammation-dependent pathologies.

Taken together, we observed 2 opposite responses to AGP in BOEC: first, up-regulation of pro-inflammatory expression of *TLR4* and *IL1B*, and second, downregulation of *TNFα* and *TLR2* expression. AGP is considered to function as a natural pro-inflammatory and anti-inflammatory agent (111). Pukhal'skii et al. (125) reported that AGP included both anti- and pro-inflammatory properties and induced production of both IL10 and TNFα in human lymphocyte. The present data imply that AGP also possess both anti-inflammatory and pro-inflammatory functions in BOEC. Thus, the pro-inflammatory responses, manifested by up-regulation of *TLR4* and *IL1B*, rise to eliminate pathogens and dead tissue, and in doing so, often cause injury to the

host while the timely arrival of anti-inflammatory responses such as AGP response seeks to limit the damage while not interfering with the pathogen elimination.

Chapter V

Interferon-tau (IFN-τ) Stimulates the Expression of Interferon Stimulated Gene 15 (ISG15) mRNA and Favors a T helper (Th) 2 Response by Bovine Oviduct Epithelial Cell (BOEC) Culture

1. Introduction

The semi-allogeneic embryo expresses paternal antigens but is not rejected by the maternal immunity. However, the exact mechanism explaining that how the new embryo escapes from the mother immunity, particularly in the oviduct, is not clear.

In domestic ruminant species, IFN-τ provides the primary fetal signal for maternal recognition of pregnancy. IFN-τ is shown to induce expression of *ISG15* in the uterus, corpus luteum (CL) and blood cells in ewes and cows (126-127). IFN-τ also plays an important role in cytokine production, such as IL4 (128), to prepare the uterine environment suitable for the growth and differentiation of conceptus. Obviously, during pregnancy the immune system shifts toward a Th2 response rather a Th1 response. It has been shown that Th1 cytokines such as TNF-α are toxic and induce apoptosis of human trophoblast cells (129). In contrast, Th2 cytokines such as IL-10 and TGF-β play a critical role in preventing fetal loss (130). Moreover, The Th2 cytokine profile can block the activation of Th1 cells, while Th1 cytokines inhibit Th2-cell proliferation. Therefore, this appears that a delicate balance between Th1 and Th2 is crucial for reproductive events in the oviduct. Regarding that each cytokine profile regulating the other, any disruption of the Th1/Th2 balance may be implicated in infertility.

Recently, it is reported that IFN-τ mRNA is first expressed in 16-cell stage IVF embryos on day 4, and its protein is detected in the trophectoderm of non-hatched blastocytes on day 7 (131-132). Moreover, we have recently reported that mRNA expression of *ISG15* is higher on day 5 in pregnant cows (126). It is therefore hypothesized that possibly very small and undetectable amount of IFN-τ secreted by 4 to 5-day embryo in the oviduct may start changes in maternal immunity and in Th1/Th2 balance before normal maternal recognition period.

2. Materials and Methods

2.1. Bovine Oviduct Epithelial Cell (BOEC) Isolation and Culture

Epithelial cells were isolated as previously described (133). In brief, fifteen oviducts immersed in PBS solution without Ca^{2+}/Mg^{2+} ($PBS^{-/-}$) (Sigma, St. Louis, MO, USA) were transported in an ice box from slaughterhouse to the laboratory. BOECs were mechanically dislodged while being flushed with PBS. The pooled sheets of BOECs from 2-3 cows were washed by a medium consisting of D-MEM/F12, 0.1% gentamicin, 1% amphotericin and 2.2% $NaHCO_3$. Thereafter, the cells were harvested by centrifugation at 300 g for 10 min, suspended in 10 ml PBS, layered over 10 ml Percol and centrifuged at 900 g for 20 min. Finally, the cell pellets were washed once with the abovementioned medium, harvested by centrifugation at 300 g for 10 min, and cultured in culture medium (D-MEM/F12, 0.1% gentamicin, 1% amphotericin and 2.2% $NaHCO_3$ supplemented with 10% fetal calf serum [FCS; BioWhittaker, Walkersville, MD, USA]) in 6-well culture dishes (Nalge Nunc International, DK-4000 Roskilde, Denmark). The following day, the BOEC culture was washed twice with PBS and incubated with culture medium supplemented with 5% FCS. The medium was renewed every 48 hours. After monolayer formation, cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH, USA) until single cells appeared, and these cells were again plated in 12-well culture dishes at a density of 1.5×10^4/ml and incubated in culture medium supplemented with 5% FCS. The purity of epithelial cell preparations was evaluated by reacting the cultured cells with monoclonal antibodies to cytokeratin (anti-cytokeratin-CK1) and immunostaining. The cells in the culture medium showed characteristic epithelial morphology. Approximately 98% of the cells were positive for anti-cytokeratin (CK1) antibodies.

2.2. Hormonal Treatments

After the second passage but before the growing BOEC monolayer covered 70%–80% of the bottom of the culture plate, the BOEC monolayer was cultured with medium supplemented with 0.1% FCS and incubated for 24 hours with 4 doses (1, 10, 100 and 1000 ng/ml) of IFN-τ, LH (10 ng/ml, USDA-bLH-B6, USDA Animal Hormone Program, Bethesda, MD, USA), P4 (1 ng/ml, Sigma) or E2 (1 ng/ml, Sigma). As a control, culture medium without any stimulant was added to the BOEC. The concentrations of hormones in this study were maintained similar to their physiological level in the bovine oviduct during the preovulatory period *in situ* (34).

2.3. Extraction of RNA, Production of cDNA and Real-Time PCR

TRIzol (Invitrogen Corporation) was used to extract mRNA as described in our recent study (133). The RNA concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at absorbances of 260 and 280 nm. DNase treatment was carried out using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA). Quantifications of mRNA expression were performed using synthesized cDNA via real-time PCR with a LightCycler (Roche Diagnostics, Mannheim, Germany) using a QuantiTect™ SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany). The primers used for real-time PCR were as follows: 5'-GGTATGATGCGAGCTGAAGCACTT-3', forward, and 5'-ACCTCCCTGCTGTCAAGGT-3', reverse, for ISG15 (Accession No. NM_174366); 5'-TTCTGCCCTGCGAAAACAA-3', forward, and 5'-TCTCTTGGAGCTCACTGAAGACTCT-3', reverse, for IL10 (Accession No. NM_174088.1); 5'-GCCACACGTGCTTGAACAAA-3', forward, and 5'-TGCTTGCCAAGCTGTTGAGA-3', reverse, for IL4 (Accession No. NM_173921.2); 5'-

CTGCTGAGGCTCAAGTTAAAAGT-3', forward, and 5'-CAGCCGGTTGCTGAGGTAG-3', reverse, for TGFB (Accession No. NM_001166068.1); 5'-CTTTCTTCAAATGCAGCATTGG-3', forward, and 5'-GGGTCTGGGTGATACAACGAA-3', reverse, for IL12 (Accession No. NM_174355.2); 5'-TGACGGGCTTTACCTCATCT-3', forward, and 5'-TGATGGCAGACAGGATGTTG-3', reverse, for TNFα (Accession No. NM_173966.3); 5'-GCTCCTGTGACTTCCTGTCC-3', forward, and 5'-CCGAAAGCACAAAGATGGTT-3', reverse, for TLR2 (Accession No. NM_174197.2); 5'-CTTGCGTACAGGTTGTTCCTAA-3', forward, and 5'-CTGGGAAGCTGGAGAAGTTATG-3', reverse, for TLR4 (Accession No. NM_174198.6); 5'-TCCTGAAACCCACTCCCAACA-3', forward, and 5'-TGGGCAGTCATCAGGCACAG-3', reverse, for COX-2 (Accession No. AF031698); 5'-AGGACGCTCAGAGACATGGA-3', forward, and 5'-TTCGGTCCGAGGAAAGAGTA-3', reverse, for mPGES1 (Accession No. NM_174443); 5'-GCATGTCAGTGTTGGTGCTT-3', forward, and 5'-CAG TCAGCGGTTTCAAGTCA-3', reverse, for IFNAR1 (Accession No. NM_174552.2); and 5'-ATGGGCTCTTTCTTCCTGGT-3', forward, and 5'-ACTGTTCCCTGCATGCTCTT-3', reverse, for IFNAR2 (Accession No. NM_174553.2). The values of mRNA expression were assayed by normalization to β-actin as the internal standard. The expression of *β-actin* was stable in all experiments, and no significant difference was detected in the levels of *β-actin* expression between treatments.

2.4. Enzyme Immune-Assay of PGE2

The PGE2 measurements in the medium were performed without further preparation of medium, using a second antibody enzyme immunoassay (EIA) according to our previous study

(133). The assay was performed using 96-well immuno plates (Corning, NY, USA) coated with anti-rabbit IgG (Seikagaku Corporation, Tokyo, Japan).

2.5. Statistical Analysis

Data are presented as the mean ± SEM of 5 experiments. Statistical analyses were performed with StatView 5.0 (SAS Institute Inc.). One-way ANOVA followed by multiple comparison tests, Bonferroni, was performed, and differences were statistically considered significant at $P < 0.05$, or trended to be significant at $P < 0.15$.

3. Results

The preovulatory level of progesterone (1 ng/ml) induced ($P < 0.05$) *INFAR1* and *IFNAR2* expression (Fig. 5.1A-B). IFN-τ dose-dependently induced mRNA expression of *ISG15*, *TGFB*, *IL10*, *TLR2* (Fig. 5.1C-E and I), and PGE2 production (Fig. 5.2C) while at doses 1-100 pg/ml suppressed ($P < 0.05$) *TNFα* expression (Fig. 5.1G). IFN-τ at 1000 pg/ml stimulated ($P < 0.05$) expression of *IL4*, *TLR4* (Fig. 5.1F and J), *COX-2*, *mPGES1* (Fig. 5.2A-B). IFN-τ did not affect IL-12 expression (Fig. 5.1H).

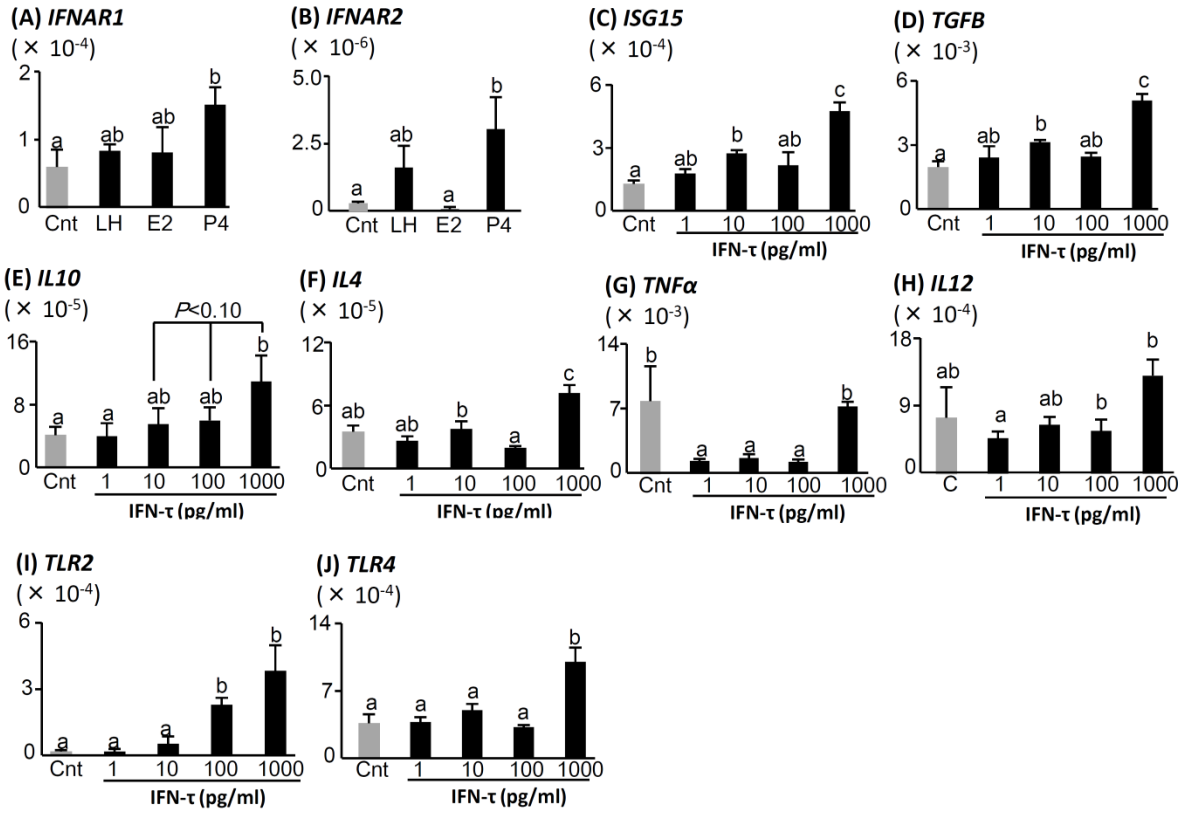


Fig. 5.1. (A-B) Relative mRNA expression of *IFNAR1* and *IFNAR2* in the bovine oviduct epithelial cell culture stimulated with estradiol (E2, 1 ng/ml), progesterone (P4, 1 ng/ml) and luteinizing hormone (LH, 10 ng/ml) at concentrations observed during the preovulatory period *in situ;* (C-J) Relative mRNA expression of *ISG15, TGFB, IL10, IL4, TNFα, IL12, TLR2*, and *TLR4* in the bovine oviduct epithelial cell culture stimulated with different doses of IFN-τ (1-1000 pg/ml) and harvested after 24 hours. Cnt (gray bar): control without any stimulant. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Bonferroni's multiple comparison test.

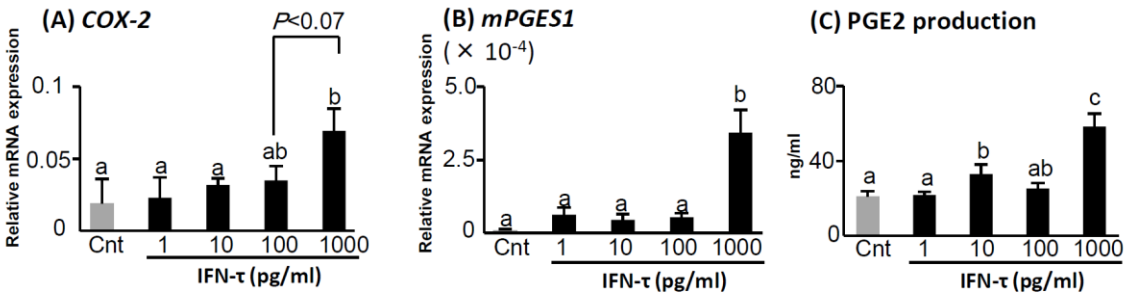


Fig. 5.2. Effect of different doses of IFN-τ (1-1000 pg/ml) on (A) mRNA expression of *COX-2*, (B) mRNA expression of *mPGES1*, and (C) PGE2 production in the bovine oviduct epithelial cell culture. Cnt (gray bar): control without any stimulant. Numerical values are presented as the mean ± SEM of 5 experiments. Different letters indicate significant differences between the treatments at $P < 0.05$ as determined by ANOVA followed by Bonferroni's multiple comparison test.

4. Discussion

Progesterone induced both *IFNAR1* and *IFNAR2* expression in BOECs, implying that progesterone may increase the sensitivity of oviductal epithelial cells to IFN-τ. This phenomenon also suggests that early pregnancy-related increase in concentration of P4 and IFN-τ might work together to start changes in gene expression related to IFN-τ.

The stimulation of BOEC with IFN-τ dose-dependently induced *ISG18* expression. Interestingly, 10 pg/ml of IFN-τ was enough to stimulate a significant increase in *ISG15* expression. In cows, synthesis of endometrial ISG15 is concomitant with the secretion of IFN-τ by conceptus (127). Of note, embryonic loss rates during bovine early pregnancy could be consistent with the insufficient expression of *ISG15* in CL (127). We have recently reported that 100 pg/ml IFN-τ upregultes the expression of *ISG15* in PMN on day 4-5 of the estrous cycle (126). The present data suggest that BOECs via upregulation of *ISG15* respond to very small amount of IFN which may start early changes in maternal immunity.

IFN-τ stimulated mRNA expression of Th2 cytokines such as *TGFB, IL4* and *IL10,* as well as expression of *COX-2* and *PGES* expression and PGE2 production in the BOEC. This phenomenon was accompanied by a decrease in *TNFα* expression without any effect on *IL12* expression. It is reported that immunomodulators such as TGF-β may play a key role in conceptus protection (134). Moreover, PGE2 selectively suppresses effector functions of macrophages and neutrophils and the Th1-mediated immunity, but it promotes Th2 and regulatory T cell responses (135). The present data suggest that IFN-τ favors a Th2 response and might involve in immune tolerance at very early stage of pregnancy.

It is suggested that dendritic cells (DCs) regulate immune responses directed against the conceptus (136). In normal pregnancy, tolerogenic stimuli from trophoblasts such as PGE2 influence the resident DCs to produce anti-inflammatory cytokines (e.g., IL-10), which in turn promote the induction of tolerance at the fetal-maternal cross-talk (136). Therefore, our results suggest that IFN-τ may regulate the immune system via elevated PGE2 production, which may prime resident DCs toward suppression of the immune system, thereby contributing to embryo tolerance.

Recently, we showed that bovine oviduct intensively expresses gene and protein for TLR2 and TLR4 in apical pole of epithelial cells (133). Therefore, to gain a better understanding of IFN-τ function, BOECs were stimulated with IFN-τ, showing a dose-dependent upregulation of TLR2 expression. Also, IFN-τ at 1000 pg/ml stimulated TLR4 expression. The exact reason of this phenomenon is not clear. However, it is speculative that IFN-τ via upregulation of *ISG15* might influence the expression of these receptors, since *ISG15* expression preceded the expression of *TLR2* and *TLR4*. These data suggest that TLR2 and TLR4 may involve in

physiology of oviduct as well as in early embryonic development. However, further investigation in this field is necessary.

Taken together, very small amount of IFN-τ upregulates *ISG15* expression and favors a Th2 response. This suggests that secretion of IFN-τ possibly from 4 to 5-day embryo can start changes in maternal immune system before normal maternal recognition of pregnancy. It is still unclear that whether a 4 to 5-day embryo which expresses IFN-τ mRNA can also secrete very small amount of IFN-τ or not. Further studies and higher technologies are needed to confirm this possibility.

Chapter VI

General Discussion and Conclusions

Epithelial surfaces, including the female reproductive tract, skin, respiratory tract, gastro-intestinal tract and urinary tract, make up the main interfaces between the host and external environment. The primary function of these epithelial surfaces is to provide a physical and immunological barrier to prevent invasion by microorganisms, with which these surfaces are in constant contact.

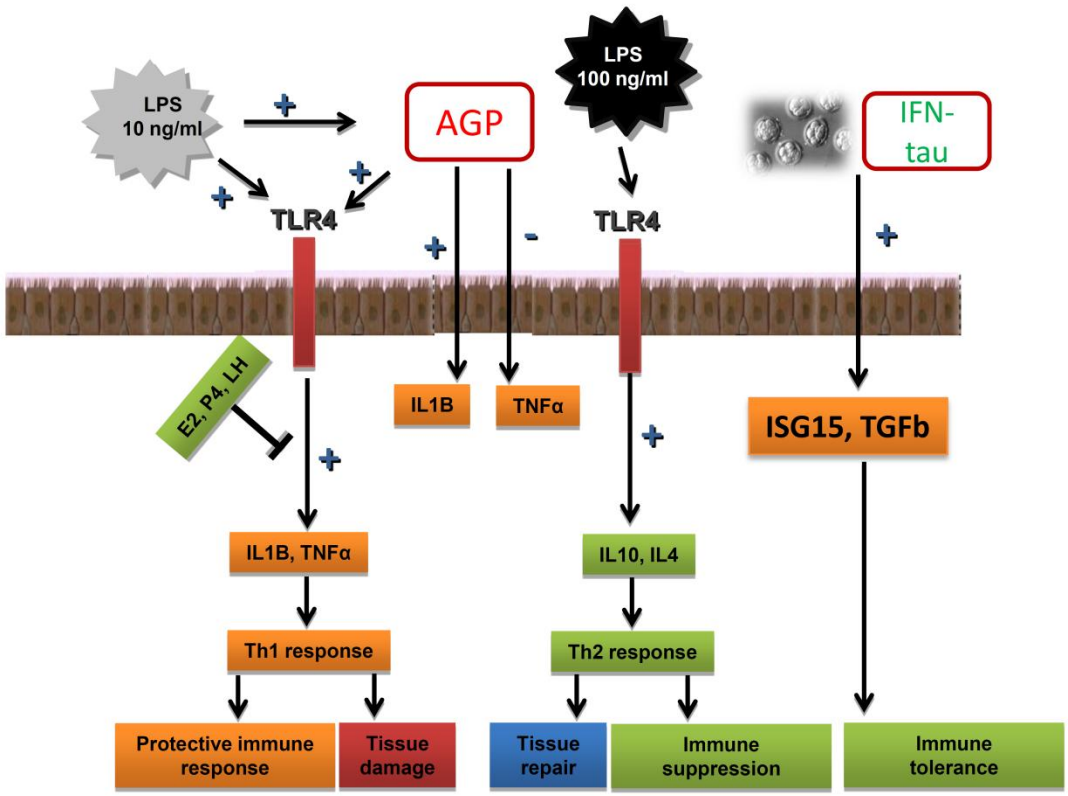
The quantity and composition of these allogeneic factors vary based on the anatomical site. In particular, the female reproductive tract, the most important place of reproduction process including sperm capacitation, fertilization and embryo and conceptus development in the mammalian body, is exposed to various stimuli such as allogeneic sperm, semi-allogeneic embryo and allogeneic pathogens from peritoneal cavity, uterus and follicular fluid.

Cross-talk between bacteria and host cells is mediated by PRRs. TLRs are a family of PRRs that recognize PAMPs. While the general term PAMP is used for these TLR ligands, the molecular patterns that are actually recognized by these TLRs in the FRT are not just from pathogens, but sperm and embryo, viruses and endogenous proteins as well. The TLRs are primarily responsible for recognizing bacterial products, including TLR2 (bacterial lipoproteins) and TLR4 (lipopolysaccharide, LPS).

TLRs are inducible or constitutively expressed, at various levels and combinations, in all cell-types. However, given the close association between epithelial cells and the stimulants, TLR signaling by epithelial cells provide the first line of communication between bacteria and host. Upon ligation of TLRs with an appropriate ligand, signaling cascades are activated, resulting in the innate immune effector responses as well as the initiation of adaptive immune responses. Stimulation of TLRs leads to the recruitment of cytoplasmic adaptor proteins. These adaptor proteins initiate signaling cascades that ultimately lead to the activation of the NF-κB signaling

pathways. Activation of these signaling pathways leads to the induction of pro-inflammatory chemokines, cytokines and antimicrobial peptides. TLR signaling has been shown to enhance epithelial barrier function by inducing expression of tight junction proteins, mucin production, antimicrobial peptides and protection against epithelial apoptosis.

It was hypothesized that oviduct epithelial cells express TLRs and respond to various stimuli such as LPS and embryo while these responses are under action of ovarian steroids and LH. We were to investigate the immune response of BOEC to LPS, oviduct-derived protein stimulation, AGP, and embryo media.



The present data showed that BOEC is very sensitive to various stimuli, suggesting that BOEC responds to low concentration of LPS (10 ng/ml) via induction of proinflammatory

cytokines. Interaction of 10 ng/ml *E. coli* LPS with TLR-4 induces expression of Th1 cytokines toward protective immune response, while activation of TLR-4, together with possible TLR-2 signaling by 100 ng/ml *E. coli* LPS, induces the expression of Th2 cytokines toward immune suppression. This results in inhibition of host defense and increased susceptibility to infection. On the other hand, Th2 cytokines and TLR-2 play important roles in preserving tight junction and tissue repair. Moreover, ovarian steroids and LH markedly influence innate immune protection by epithelial cells in the oviduct through inhibition of pro-inflammatory responses.

As mentioned in the chapter 4, I observed two opposite responses to AGP in BOEC: first, up-regulation of pro-inflammatory expression of *TLR-4* and *IL1-B*, and second, downregulation of *TNFα* and *TLR-2* expression. AGP is considered to function as a natural pro-inflammatory and anti-inflammatory agent. It is reported that AGP included both anti- and pro-inflammatory properties and induced production of both IL-10 and TNF-α in human lymphocyte. The present data imply that AGP also possess both anti-inflammatory and pro-inflammatory functions in BOEC. Thus, the pro-inflammatory responses, manifested by up-regulation of *TLR-4* and *IL-1β*, rise to eliminate pathogens and dead tissue, and in doing so, often cause injury to the host while the timely arrival of anti-inflammatory responses such as AGP response seeks to limit the damage while not interfering with the pathogen elimination.

I investigated the effect of very small amount of IFN-τ on BOEC immune response. The results suggest that IFN-τ favors a Th2 response in the BOEC.

Taken together, bovine oviduct epithelial cells show the respond to various stimuli differentially and act to keep the appropriate and constant condition that is necessary for a successful fertilization. This series of study suggests that bovine oviduct epithelial cells act to

protect against pathogens while regulating the strength of proinflammatory responses that is harmful for cells. In this way, bovine oviduct epithelial cells employ ovarian steroids and LH to preclude over-expression of pro-inflammatory cytokines and Th1 response. These hormones as well as IFN-τ also favor a Th2 response, showing a critical role in the oviduct.

1. Summary

The mammalian oviduct provides an optimal microenvironment for the activation and transportation of gametes, sperm capacitation, fertilization, and early embryonic development, which is critical for the establishment of a successful pregnancy. The oviduct is classically described as a sterile milieu even though pathogens and endotoxins can invade the mucosal surfaces of the oviduct via the uterus, peritoneal cavity, and follicular fluid. In addition, Sperm and the new embryo, which are allogeneic and semi-allogeneic agents, respectively, directly contact the oviduct epithelial cells, that could induce the immune responses in female. Therefore, the oviduct should be equipped with an efficient and strictly controlled immune system that would maintain optimal conditions for fertilization and early embryo development while provides protection against pathogens. In this study I aimed to reveal the oviductal immune responses to the different stimuli such as gram-negative bacteria endotoxin (LPS), sex steroids, LH and embryo-derived protein (IFN-tau) pathophysiology vs. physiology.

In chapter 2, first, I investigated the regional distribution of immune cells (i.e eosinophils) in the bovine oviduct *in vivo.* Histological studies revealed more abundant eosinophils **(**EOS**)** in the infundibula of the oviducts ipsilateral to the preovulatory dominant follicle and the ovulated ovary. The number of EOS was higher in the infundibula of the oviducts

ipsilateral to the ovulated ovary than those of the oviducts contralateral to the ovulated ovary. The infundibula of the oviducts ipsilateral to the preovulatory dominant follicle had higher number of EOS than those of the oviducts ipsilateral to the mid-cycle corpus luteum. The number of EOS in the isthmus was higher in the outer layers (*tunica muscularis* and *tunica serosa*) than in the inner layers (*tunica mucosa* and *tunica submucosa*) during the estrous cycle. Thus, the EOS number varied with the region of the bovine oviduct, with greater number in the infundibula of the oviduct ipsilateral to the ovulated ovary, suggesting the impact of ovulation.

In chapter 2, I tried to reveal immunological responses of oviduct epithelial cells to the different stimuli. I studied the effect of *Escherichia coli* lipopolysaccharide (LPS) and its interaction with ovarian steroids, estradiol (E2) and progesterone (P4), and luteinizing hormone (LH) at concentrations observed during preovulatory period on immune responses in BOEC culture. Immunohistochemistry of oviduct tissue showed intensive expression of Toll-like receptor-4 (TLR-4) and TLR-2 in epithelial cells. A dose of 10 ng/ml LPS stimulated *TLR-4*, cyclooxygenase-2 (*COX-2*), nuclear factor-kappa B inhibitor A (*NFKBIA*), interleukin 1β (*IL-1β*), and tumor necrosis factor α (*TNF-α*) expression, indicating an early pro-inflammatory response. A dose of 100 ng/ml LPS did not induce expression of these genes but stimulated *TLR-2*, *IL-10*, *IL-4*, and microsomal prostaglandin E synthase-1 (m*PGES-1*) expression and PGE2 secretion, indicating an anti-inflammatory response. Ovarian steroids and LH completely block LPS (10 ng/ml)-induced *TLR-4*, *IL-1β*, and *TNF-α* expression as well as LPS (100 ng/ml)-induced *TLR-2* expression. These data suggest the existence of an early signaling system to infection in the BOEC. In addition, ovarian steroids and LH may play a critical role in inducing homeostasis and in controlling hyperactive pro-inflammatory responses detrimental to the epithelial cells, sperm and the embryo.

In chapter 3, I investigated function and regulation of an acute phase protein (APP), alpha-1-acid glycoprotein (a-1-AGP) in the bovine oviduct and BOEC. Alpha-1 acid glycoprotein (AGP) is one of the main acute phase proteins (APPs) that are mainly produced by liver. Little is known about the local production and function of AGP. This study aimed to investigate the expression of AGP system *in vivo* and possible immune function of AGP in bovine oviduct epithelial cell (BOEC) *in vitro*. The results showed that the bovine oviduct expresses both AGP and AGP receptor (*AGPR*), with the highest expression during postovulatory phase. Concentration of AGP in the oviduct fluid was lowest during the mid luteal phase. Progesterone (P4 1 ng/ml) stimulated the expression of *AGP* and AGP receptor (*AGPR*) in the BOEC. Lipopolysaccharide (LPS) (10 ng/ml, but not 100 ng/ml) stimulated the expression of *AGP* and *AGPR* in the BOEC. Sex hormones, estradiol (E2), progesterone (P4), and luteinizing hormone (LH) at concentrations observed during preovulatory period, suppressed LPS-induced *AGP* and *AGPR* expression. AGP (1-100 ng/ml) induced the expression of Toll-like receptor 4 (*TLR4*) and interleukin 1β (*IL-1β*), indicating a proinflammatory role for AGP. AGP (1-1000 ng/ml) suppressed the expression of *TLR2* and tumor necrosis factor α (*TNF-α*), an anti-inflammatory function for AGP. The co-stimulation of BOEC with LPS (10-100 ng/ml) and AGP (10-100 ng/ml) did not influence the LPS-induced *TLR4* and *IL-1β* expression but inhibited LPS-induced *TLR2* and *TNFα* expression. To our knowledge, this is the first evidence showing the expression of AGP system in the bovine oviduct, indicating that AGP could be considered as a part of regulatory responses with the aim to silence some acute pro-inflammatory genes, and to maintain the possible expression of certain genes involved in the anti-infectious process.

In chapter 4, after investigation of immune response to LPS in the BOEC, pathophysiology, to reveal indirect response of oviduct immune system to the existence of a new

embryo, BOEC was stimulated with interferon-tau which is a type I IFN, synthesized and secreted by the trophectoderm of the blastocysts. IFN-tau is regarded as the initial fetal signal required for maternal recognition of pregnancy in ruminant species. Recently, it has been shown that interferon-tau mRNA and protein are expressed in embryos on day 4 and on day 7 respectively. Therefore, I aimed to investigate the possible effect of very small amount of IFN-tau on the Th1/Th2 balance in the BOEC. IFN-tau dose dependently induced the expression of TLR2, ISG15, TGF-β and IL1β; While, IFN-tau could not induce TLR4 and IL12. The results suggest that possibly very small amount of IFN-tau in the oviduct starts the change in the immune response toward Th2 response which is related to a successful pregnancy.

Taken together, bovine oviduct epithelial cells respond to various stimuli differentially and act to keep one appropriate and constant condition that is necessary for a successful fertilization. This project suggests that bovine oviduct epithelial cells act to protect against pathogens while regulating the strength of proinflammatory responses that is harmful for cells. In this way, bovine oviduct epithelial cells employ ovarian steroids and LH to preclude over-expression of proinflammatory cytokines and Th1 response. These hormones as well as IFN-tau also favor a Th2 response, showing a critical role in maintaining an optimal Th1/Th2 balance in the oviduct. This study shows that oviductal epithelial cells via sex hormones act to stabilize the local immune status toward a Th2 response to support reproductive process like sperm capacitation, fertilization, embryo development and zygotes transportation. Therefore, the endocrine and autocrine systems in the oviduct influence reproductive health in dairy cattle as well as cow's soundness, and eventually result in higher cattle production.

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