Regulation of neutrophils phagocytosis for sperm by alpha 1-acid glycoprotein (AGP) in the bovine oviduct

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ウシ卵管における alpha-1-acid glycoprotein (AGP) を介した好中球による精子貪食の調節機構

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Abbreviations

AGP	:	Alpha 1 acid glycoprotein
BOEC	:	Bovine oviduct epithelial cell
BSA	:	Bovine Serum Albumin
CL	:	Corpus Luteum
CV	:	Coefficients of Variation
E2	:	Estradiol
ELISA	:	Enzyme-linked immunoabsorbent assay
EOS	:	Eosinophils
FCS	:	Fetal calf serum
fMLP	:	Ormylmethionyl-leucyl-phenylalanine
FSH:	:	Follicle Stimulating Hormone
GnRH:	:	Gonadotrophin releasing hormone
H2O2	:	Hydrogen Peroxide
IL	:	Interleukin
LH	:	luteinizing hormone
MPO	:	Myeloperoxidase
NETs	:	Neutrophil Extracellular Traps
OCL-	:	Hypochlorite
OD:	:	Optical Density
P4	:	Progesterone
PBMC	:	Peripheral blood mononuclear cell
PBS	:	Phosphate-buffered saline
PGF2α	:	Prostaglandin F-2 alpha

PMN	:	Polymorphonuclear neutrophil
real-time PCR:	:	Real-time Polymerase Chain Reaction
RT-PCR	:	Polymerase Chain Reaction
SOD	:	Superoxide Dismutase
SP	:	seminal plasma
Sp-TALP	:	Pyruvate Medium
UV	:	Ultraviolet

Chapter 1

General introduction

Reproduction is a sequence of events beginning with development of the reproductive system in the embryo. After the animal is born, it must grow and achieve puberty by acquiring the ability to produce fertile gametes. This ability must be accompanied by reproductive behavior and copulation. After copulation, the sperm and egg meet, fertilization occurs and development of the preattachment embryo follows. The conceptus attaches to the uterus by a specialized organ called the placenta. It allows the conceptus to grow and develop to term. The fully developed fetus is born and the female giving birth to it must lactate to provide nourishment for the neonate. During or after lactation, the dam must reestablish cyclicity before she can become pregnant again [1].

The immune system must detect a wide variety of agents, such as pathogens, from viruses to parasitic worms, and distinguish them from the own organism's healthy tissue. It can be classified into the innate immune system and the adaptive immune system. The immunology made a great advance towards the end of the 19th century [2], but the immune system in the local organs in cattle such as oviduct is still not fully understood and the local immune reactivity in particular needs to be explored.

1. Physiology of reproduction in dairy cattle

The normal non-pregnant cow enters the estrus every 18-24 days unless a cyclicity is interrupted by a pregnancy or pathology. Estrus lasts about 15 hours and ovulation occurs approximately 12 hours after the end of estrus. The follicular pahse consists of four major events. They are: 1) elevated gonadotropin release from the anterior lobe of the pituitary; 2) follicular growth and preparation for ovulation; 3) sexual receptivity and 4) ovulation. Estrogen is the dominant hormone that is produced by developing follicles and causes profound changes in the reproductive tract preparing it for copulation [1]. Reproductive behavior is induced by estrogen in non-primate mammals. Estrogen also controls the onset of the preovulatory luteinizing hormone （LH） surge that causes ovulation. Ovulation is a cascade of physiological and biochemical

changes that culminate in repture of dominant follicles and release of the oocyte from the ovary [1].

The estrous cycle of cows averages 21 days, with a typical range of 17-24 days in length. During approximately 283 days of gestation, the cow has no clear estrous cycle. For heifers, at about 11 months of age, they start to show the first estrus [1]. There are two endocrine phases (follicular phase and luteal phase) during the estrous cycle.

The estrous cycle starts by ovulation that releases the ovum. The ovulated site is rapidly differentiating to luteal cells (luteinization), forming the corpus luteum (CL). The CL begins to secrete progesterone (P4), a steroid hormone that maintains pregnancy, and P4 is dominant during the middle of the estrous cycle. The embryo prevents the uterus from secreting prostaglandin 2 alpha (PGF2α), thus the CL continues to release P4, and the next estrus and ovulation are blocked. On the other hand, if there is no embryo in the uterus, the uterus starts to produce PGF2α on days 17-19 after the estrus and the CL is demised within 2-3 days toward the end of the estrous cycle (luteolysis).

During the estrous cycle, the 2-3 follicular waves occur one after the other. The growth of follicles are regulated by follicle stimulating hormone (FSH) and luteinizing hormone (LH) that are secreted by a pituitary gland through the blood circulation. FSH stimulates a cohort of small follicles to grow. However, if E2 and LH are not reaching a certain concentration, the follicle fails to mature and ovulate, but it regresses, that followed by recruitment of a new group of follicles. LH is responsible for keeping the follicles growing (basal LH pulses) and also stimulating ovulation (the LH-surge). If no embryo reaches to the uterus, the CL regresses and thus P4 in the circulation drops to the basal level. Gonadortopin releasing hormone (GnRH) from the hypothalamus controls the FSH and LH release from the pituitary. Estradiool-17 β (E2) produced by the dominant follicle stimulates the final rise of LH (the LH-surge) that triggers ovulation [3, 4, 5]. The estrous cycle is a process of interaction among hormones above that regulates strictly the growth and death of follicles,

ovulation, and the CL development and regression. (Fig. 1.1).

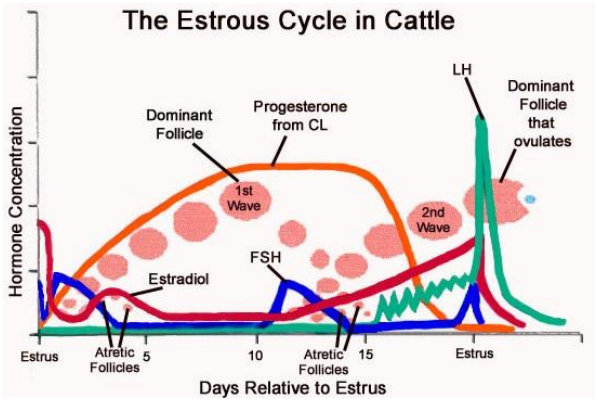

Fig. 1.1. The two phases that constitute the estrous cycle (follicular and luteal phases). Luteolysis followed by LH surge which is responsible for ovulation, marks the principal events at estrus. The luteal phase is known by high levels of P4 and ends with regression of the CL. Developing follicles produce high levels of E2 in the follicular phase. (Figure taken from http://research.vet.upenn.edu.Dairy/Reproduction/EstrousCycle/EstrousCycleFollicularWaves/tabid/3965/Default.aspx).

2. Bovine oviduct: anatomy and histology

The normal reproductive tract of the cow consists of the vulva, vestibule, vagina, cervix, uterine body, uterine horns, oviduct and ovaries. The oviduct is a coiled tube about 25 cm long when fully stretched out. Three parts was made up: infundibulum, ampulla and isthmus (Fig. 1.2) [6].

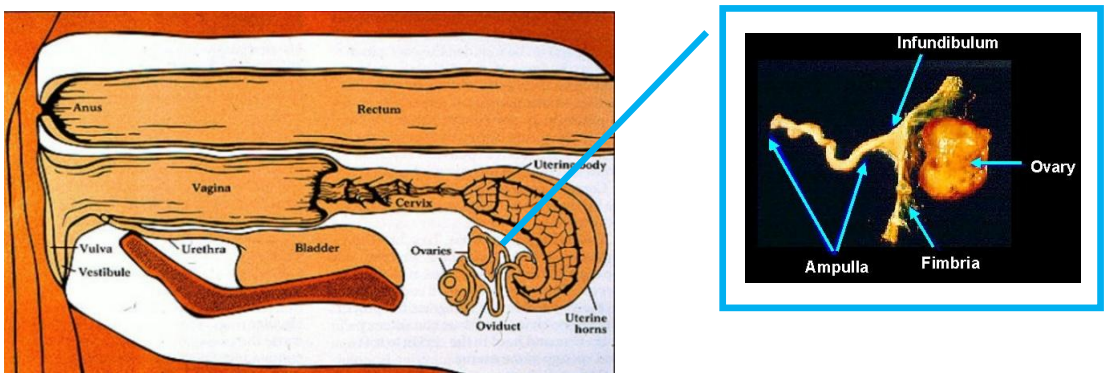

Fig. 1.2 (Figure take from http://animalsciences.missouri.edu/reprod/AnatomyFemale/bovine/sld018.htm)

The infundibulum with its finger-like projections called fimbria closed to ovary, the ampulla connects at the ovarian end with the infundibulum and at its distal end with the isthmus. The oviducts are made up of ciliated cells, secretory cells and smooth muscle cells [7]. The ciliated cells, present in greater abundance in the fimbria and infundibulum and decreasing in concentration towards the uterus, move the ovum from the ovary towards the uterus. The activity of the ciliated cells, coupled with contractions of the smooth muscle cells, keeps an ovulated ovum in constant rotation which is essential for bringing egg and sperm together and preventing oviductal implantation [8]. The secretary cells are responsible for producing mucous like substances which coat and protect the ovum. The activity of the cells in the oviduct is controlled primarily by estrogen produced by the ovary and oxytocin released by the posterior pituitary. 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*) (Fig. 1.3) [9].

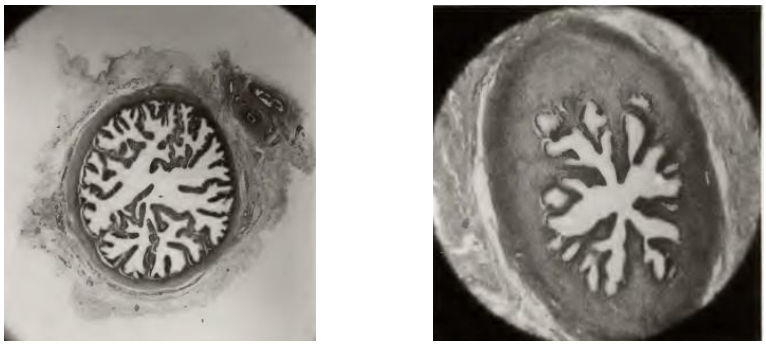

Fig. 1.3

Cross-section of the mid-region of the mature oviduct, showing the three tunies, an increase in the number of folds and a decreases in the thickness of the muscular coat. (harris' hematoxylin-eosin stain) 70× [10].

Cross-section of the isthmus of the mature oviduct, showing the formation of artefacts which resembled glanda. (Harris' hematoxylin-eosin stain) 70×[10].

3. Fertilization and early embryo development

The oviduct plays a pivotal function in early reproductive events [6, 11]. Over the past few decades, there has been a steady increase in our elementary knowledge of oviduct biology [12]. The oviduct is a small tube responsible for the transient hosting of gametes and embryos. The oocyte and spermatozoa enter the oviduct from opposite ends, meet each other via a counter-current transport system and fuse to form an embryo which represents the primal form of completeness from the genetic point of view. To meet all these demands, the oviduct features a subtle anatomy represented by the infundibulum, ampulla and isthmus, which are equipped with longitudinal and circular aligned muscle layers, endothelial ciliated and non-ciliated cells [1]. The ovum begins to divide mitotically, a process known as cleavage, immediately after fertilization is complete. Division continues so that a solid cluster of cells or blastomeres known as a morula (mulberry shape) is formed by five or six days. From about day 6 after fertilization, the ovum begins to hollow out to become a blastocyst. This consists of a single spherical layer of cells, the trophoblast, with a hollow

centre, but also with a group of cells, the inner cell mass at one edge. The sequence of these events is illustrated in. The inner cell mass is destined to form the embryo, whilst the trophoblast provides it with nutrients. At about day 8 the zona pellucida begins to fragment and the blastocyst ‘hatches’. This is then followed by a period of blastocyst elongation. Development of the so-called germ layers begins from about the fourteenth day and characterizes the beginning of the embryo phase (Fig. 1.4) [1].

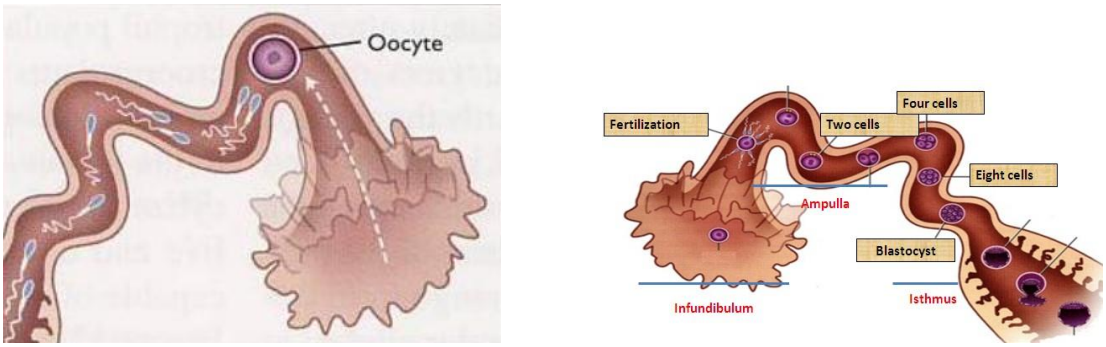

Fig. 1.4 [1]

4. The local immune system in the bovine oviduct

Oviduct should maintain an aseptic milieu and plays a pivotal role in the first step of mammalian reproduction. The microorganism such as bacteria may localize in the oviduct lumen that opens into the uterine horn and the peritoneal cavity [13]. Therefore, the oviduct represents a unique immunological site that supporting oocyte maturation, sperm capacitation, fertilization, and at the same time maintaining a permissive environment for the survival of allogeneic sperm and semi-allogeneic embryos.

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 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. The epithelial layer at the mucosal surfaces functions not only as a physical barrier, but also as an innate and adaptive immune regulator [14].

Studies on the immune cells in the oviduct have been performed in several species, e.g. humans [15] and rabbits [16]. In the bovine oviduct (the isthmic and ampullary parts), changes in the distribution of lymphocytes [17], mast cells [18] and eosinophils (EOS) [19] during different stages of the estrous cycle have been reported. As the body's first line of defense against microorganisms, neutrophils execute a variety of potent effector mechanisms for mediating innate immunity. There are little studies on the neutrophils in the bovine oviduct.

5. Sperm phagoctytosis by PMNs

Neutrophils phagocytosis for sperm is similar to that for bacteria [20]. The main focus of sperm phagocytosis by PMNs is based on the mechanism involved in the uterine environment after insemination. It was shown that the presence of sperm in the uterus induces rapid chemotaxis of PMNs, which are detected in the uterus already 0.5 h after artificial insemination in horse and pigs [21, 22]. Neutrophils either directly phagocytize sperm through cell-cell attachment or entrap them with neutrophil extracellular traps (NETs), structures consisting of neutrophil nuclear DNA and associated proteins, which ensnare sperm and hinder their motility [23]. On the other hand, it was shown that equine seminal plasma (SP) prevented NETs formation [24]. Moreover, it was found by Doty *et al*. that CRISP3 protein in equine SP was the factor that suppressed PMNs and sperm binding and regulated sperm degradation [25].

6. Biological function of Alpha 1 acid glycoprotein (AGP)

An acute-phase protein has been defined as one whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at least 50 percent during inflammatory reaction [26, 27]. AGP is one of the major acute phase proteins. It has been extensively

studied for many years, mainly due to its drugbinding behavior. In spite of this, the function of this protein is still unknown. AGP is a constitutively expressed protein that is found mainly in blood, in varying concentration, depending on the species. As with all other acute phase plasma proteins, AGP is mainly secreted by hepatocytes due to the systemic response of inflammation that follows various stressful stimuli, such as physical trauma, wounding, bacterial infections or other unspecific inflammatory stimuli [28]. The hepatic overexpression of AGP is regulated by pro-inflammatory cytokines, including IL-1, IL-6, chemokines (IL-8) and glucocorticoids [29, 30, 31, 32,33]. AGP is produced mainly by hepatic cells, but extrahepatic expression has been reported in several other tissues, including human and bovine mammary epithelial cells [34, 35], stimulated alveolar macrophages [36], human endothelial cells [37], prostate [38], and pig's nasal mucosa [39]. White blood cells, such as cultured human monocytes [40, 41] and resting and activated polymorphonuclear leukocytes [42], can also express AGP. AGP belongs to the lipocalins family, a group of proteins that bind and transport small hydrophobic molecules [43]. AGP has been further classified in a subset of lipocalins, the so-called immunocalins, a subfamily of proteins that may also immunomodulate the inflammatory reaction [26]. Therefore, AGP features at least two biological activities, apparently very different from one another. AGP may immunomodulate the inflammatory response, and, at the same time, act as a plasma transport protein.

7. Immunomodulatory functions of AGP

It is widely accepted that AGP has immunomodulatory functions [45] and a number of activities related to the immune function of different blood cells types has been described. The most important activity of AGP on macrophages is probably the induction of expression of molecules that antagonize the activity of IL-1β and TNFα, such as IL-1 receptor antagonist and soluble TNF receptor [44]. At physiological concentrations, AGP can inhibit the chemotactic response of neutrophils challenged with ormylmethionyl-leucyl-phenylalanine (fMLP), but not chemokinesis

[46]. Notwithstanding its relative abundance in blood, AGP itself does not activate lymphocytes, but it can modulate their response when evoked by other activating molecules [47]. The interaction of AGP with lymphoid cell membranes was shown to involve electrostatic forces, and is modulated by the sialic acid component of the ligosaccharide chains [48]. Experimental aggregation of platelets can be inhibited by AGP in a dose-dependent manner, although at higher concentrations (ten fold) than the amounts typically present in physiological conditions (Fig. 1.5)[49].

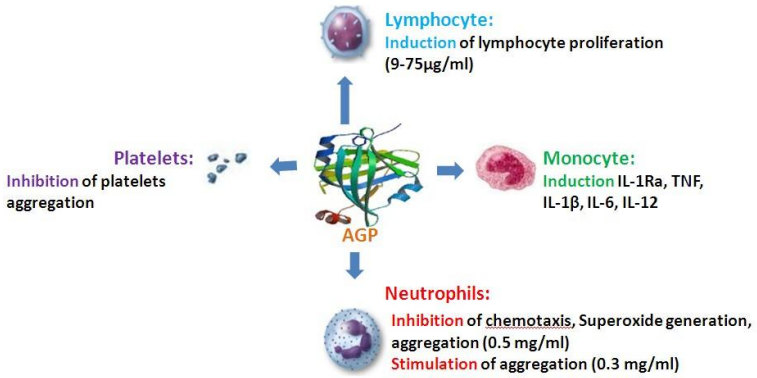

Fig. 1.5 Overview of effects of AGP on lymphocytes, platelets, mononuclear cells and neutrophils. [50]

AGP could act as a nonspecific anti-microbic agent [51], due to the fact that AGP can inhibit the infection of red blood cells by Plasmodium falciparum, possibly due to the large amount of sialic acid molecules exposed on the surface of the protein, making AGP a nonspecific competitor for cell surfaces [52]. Moreover, AGP reduces the attachment and phagocytosis of Mycoplasma pneumoniae by human alveolar macrophages [53].

8. Glycosylation of bovine AGP

Bovine AGP has been studied for its possible utilization as acute phase marker in disease, but the primary structure has only been recently discovered [35]. Very few information about AGP glycosylation in healthy animals is available [54, 55]. At least two different glycan patterns of

bovine AGP exist, one for fetal AGP and one for adult AGP [56], but any other information about glycan pattern modification during diseases are unknown. The rat model has been extensively studied to determine the effect of inflammation and some anti-inflammatory drugs on glycosylation [57, 58].

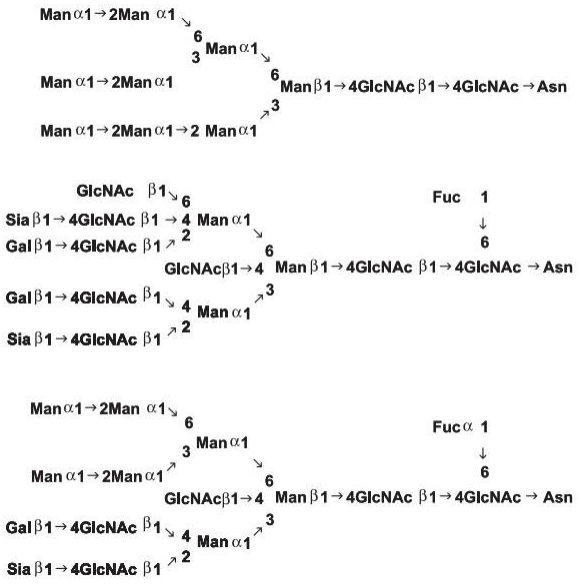

Fig.1.6. The stuctures of oligomannose (first), complex (second) and hybrid (third) glycans. All branches of an oligomannose type glycan end in mannoses. Different type glycans on every branch have two or more branches with at least one N-acetylglucosamine and galactose and possibly one sialic acid. Hybrid type of glycans is a mixture of two types and has one branch of complex structure and one, or more, oligomannose branches [59].

9. Aim of this study

The oviduct of most mammalian species is the site where spermatozoa first encounter the oocyte and the process of fertilization is initiated. The oviduct is a thin convoluted tube that opens into the uterine horn on one end and into the peritoneal cavity on the other end. Therefore, the oviduct milieu represents a unique immunological site that supports a delicate balance between protecting the oviduct from infection by potentially pathogenic ascending microorganisms and

maintaining a permissive environment for the survival of allogeneic sperm and semi-allogeneic embryos. However, little is known about how the oviduct immune system interacts with the allogeneic sperm and semi-allogeneic embryos. Therefore, the first goal of this study is to investigate the PMNs in the bovine oviduct fluid during the estrous cycle.

Once sperm escape from phagocytosis by uterine PMNs and reach the oviduct, sperm reservoirs are formed where sperm undergo capacitation [60]. Thus, the oviduct provides microenvironment for sperm capacitation [61, 62]. After capacitation, sperm are sequentially released from the reservoirs [60, 63] and are rapidly transported to the fertilization site where the oviduct microenvironment supports the viability of sperm population for >24 h until ovulation. Sperm viability until ovulation is manifested by prolonged motility and fertility [64, 65]. It was shown that in human [66], cat [67], and mouse [68] the superfluous sperm remained in the oviduct after ovulation were eliminated through phagocytosis by epithelial and immune cells. However, in cows, the presence of such immune cells, their interaction with stored sperm, and the possible regulators of local immune microenvironment in the oviduct have not been yet investigated. We have recently provided evidence that bovine oviduct epithelial cells efficiently control the balance between Th1 and Th2 cytokines [69]. A question why the fertilization could not be blocked in the physiological condition if the PMNs are existing in the bovine oviduct. Therefore, the second goal is to verify if AGP exists in the bovine oviduct fluid and regulates the sperm phagocytosis by neutrophils.

Chapter 2

Existence of PMNs in the bovine oviduct fluid during the estrous cycle

1. Introduction

As the body's first line of defense against microorganisms, PMNs execute a variety of potent effector mechanisms for mediating innate immunity. In the oviduct tissue, PMNs were detected in response to infection in mice and human [70, 71]. In fact, it is not surprising that neutrophils can be detected in the bovine oviduct since the oviduct opens into uterine on one end and into the peritoneal cavity on the other end. PMNs migration can be induced by IL-8, a neutrophil-specific chemoattractant. IL-8 is a small protein (8.4 kDa) and produced by macrophages, endothelial cells and neutrophils. In the ovary the IL-8 is detected in theca, granulosa, granulosa-lutein cells in human and rabbit [72, 73]. In the bovine, IL-8 was observed during the early luteal phase and it induced the neutrophil migration into the early corpus luteum.

In the present study, I investigated the PMNs in the bovine oviduct fluid during the estrous cycle and first time show the localization of IL-8 production in the bovine oviduct fluid which can indirectly verify the PMNs migtration into the oviduct fluid through the oviduct tissue.

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 PBS without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 0.3% amphotericin B (Sigma-Aldrich), and 0.3% gentamicin (Sigma-Aldrich) 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 [74, 75]. 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.

2.2 Identification of PMNs in oviduct fluid from the different stages of the estrous cycle

Thirty bovine oviducts in different stages of the estrous cycle were collected within 15 min of sacrifice in the slaughterhouse. The identification of PMNs in the oviduct fluid was performed according to the method previously described [76]. Leukocytes were separated according to the protocol of Cotter and Muruve [77] with minor modifications. Basically, the collected fluid was passed through a 40-μm pore cell strainer (BD Biosciences, Durham, USA), and centrifuged at 300 ×g for 6 min at 20 °C. To purify leukocytes, the cell pellet was then suspended in 10 ml of 35% Percoll (Sigma-Aldrich, Tokyo, Japan) and centrifuged at 360 ×g for 10 min at 20 °C. After removal of the supernatant, the leukocyte pellet was washed with 5 ml $PBS^{-/-}$ and suspended in 1 ml $PBS^{-/-}$. Giemsa-stained PMNs were detected by light microscope (2-5 lobes of nuclear and finely-granular). For a total cell count, a sample of the leukocyte suspension was diluted (1:10) with 0.1% acetic acid (Sigma-Aldrich, Tokyo, Japan), and mounted on a haemocytometer. To determine PMNs proportions in leukocyte populations, a 20 μl sample of the leukocyte suspension was diluted in Macs separation buffer (MACS Miltenyi Biotec, Tokyo, Japan) and analyzed by flow cytometric evaluation (Beckman Coulter, Inc., CA, UAS).

2.3 Primary bovine oviduct epithelial cell (BOEC) isolation

The isolation and cultivation of BOECs was based on the method described previously [78, 79]. 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). Oviducts were cut, separated from the connective tissue, and separated into ampulla and isthmus. BOECs from the ampulla and the isthmus, 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. BOECs from the ampulla and the isthmus, were mechanically dislodged and purified then, placed in 1.5-mL microcentrifuge tubes with 500 μL TRIzol reagent (Invitrogen Corporation) and stored at −80°C until RNA extraction. The purity of the epithelial cell preparations was evaluated by reacting the cells with monoclonal antibodies to cytokeratin (anti-cytokeratin-CK1) and immunostaining. Approximately 92–95% of the cells were positive for anti-cytokeratin (CK1) antibodies.

2.4 IL-8 gene expression in BOECs

Total RNA was extracted from the BOEC using TRIzol (Invitrogen Corporation) as described in the protocol of Chomczynski and Sacchi [80]. 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). 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.

The synthesized cDNA was stored at –30°C. The primers used for real-time PCR were as follows: IL-8 (NM_173925, 170 bp), forward, 5-CCTCTTGTTCAATATGACTTCCA-3’ and reverse, 5-GGCCCACTCTCAATAACTCTC-3’; β-actin (K00622, 256 bp) forward, 5-CCAAGGCCAACCGTGAGAAAAT-3’ and reverse, 5-CCACATTCCGTGAGGATCTTCA-3’.

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.

2.5 Immunohistochemistry for IL-8

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. The sections were then deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. 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 10 mM 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 for non-specific protein binding sites. The antibody used for immunohistochemistry was rabbit anti-IL-8 (1:300, PB0273B-100, Kingfisher Biotech, USA). 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-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 with DAB (Sigma, Steinheim Germany) as the substrate for 5 min at room temperature. Sections were counterstained with hemalum, dehydrated, and mounted with DPX (Fluka, Buchs, Switzerland). To analyze unspecific

binding, the primary antibody was replaced with rabbit IgG (Sigma) at the same concentration of the primary antibody.

2.6 Statistical analysis

Data are presented as the mean ± S.E.M. of 3–9 experiments. Statistical analyses were performed with StatView 5.0 (SAS Institute, Inc., Cary, NC, USA). Statistical significance between groups was made using a one-way ANOVA followed by multiple comparison tests (Fisher's test for three groups, and Bonferroni's test for more than three groups). Results were considered to be statistically significant at $P < 0.05$.

3. Results

3.1 Identification of PMNs in oviduct fluid

We have previously shown that, during the preovulatory stage, PMNs are present in the bovine oviduct fluid [76]. Therefore, in this study, I investigated the possible changes in the number of PMNs throughout the different stages of the estrous cycle. PMNs were present in oviduct fluid at 3 different stages of the estrous cycle, and their numbers did not vary across the cycle (average $5–7\times10^3$ cell/oviduct flush). PMNs were found to constitute approximately 12–16% of the total leukocytes in the oviduct flush (Fig. 2.1 a, b).

3.2 The expression of the IL-8 in bovine oviduct

IL-8 is a strong chemoattractant for PMNs [81]; therefore, we investigated IL-8 gene expression in BOECs. The IL-8 gene was expressed in BOECs that were freshly separated from bovine oviduct without any culture, and IL-8 gene expression was approximately 10 times higher in ampullary epithelial cells than in isthmic epithelial cells (Fig 2.2 a, b).

The intensity of IL-8 staining in ampullary epithelial cells was also much stronger than that

detected in isthmus cells (Fig. 2.3 c – h).

4. **Discussion**

Neutrophils play a central role in the first defense reaction of the body due to their high tendency to infiltrate different tissues where chemotactic agents are released. Previously, we showed that neutrophils infiltrate the oviduct fluid during the preovulatory stage [76]. The present data indicates that, under physiological conditions, neutrophils infiltrate the oviduct fluid not only during the preovulatory stage, but also throughout the different stages of the estrous cycle, and that their numbers remain relatively constant over the estrous cycle. The average number of PMNs were $5–7\times10^3$ cell/oviduct flush, constituting approximately 12–16% of the total leukocyte population in the oviduct flush. Our results also show that BOECs constantly produce IL-8, a strong chemotactic agent for neutrophils, throughout the whole estrous cycle. IL-8 has also been found to be produced in human oviduct fluid [82]. The stable production of IL-8 could be one of the factors contributing to the continuous recruitment of PMNs into the oviduct fluid and the maintenance of PMNs at a relatively constant level through the different stages of the estrous cycle. These results indicate that PMNs are normal constituents of the local immunological microenvironment in the bovine oviduct that protect the oviducts from potentially pathogenic microorganisms and maintain a sterile environment.

5. Figures

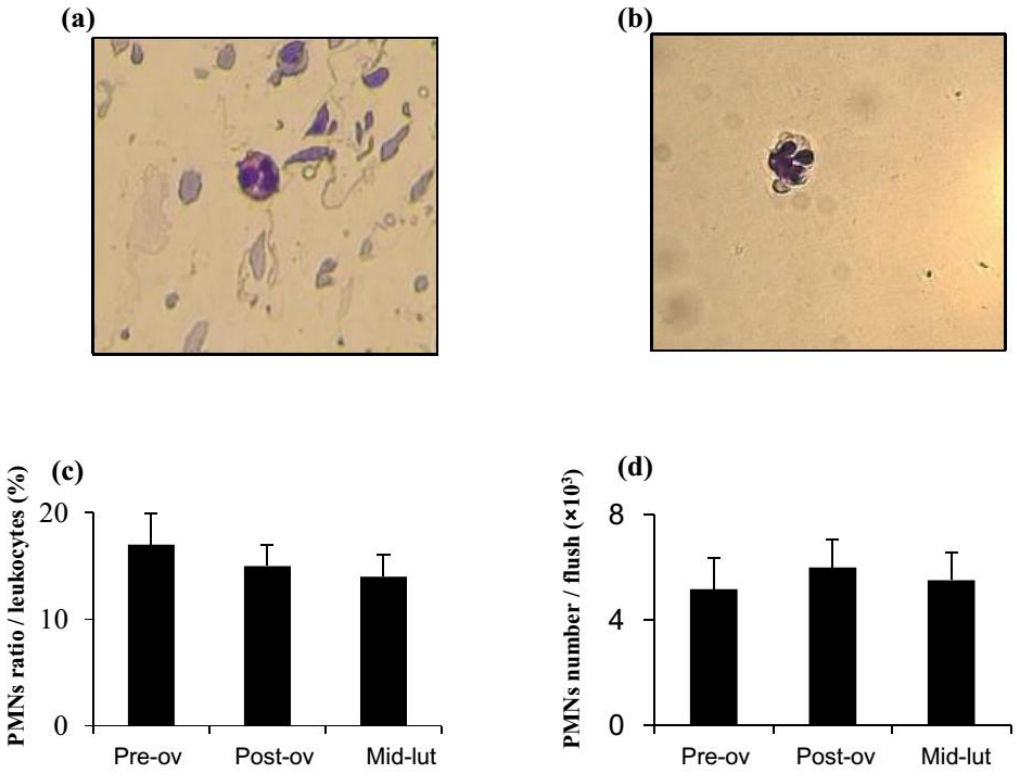

Fig. 2.1 (a, b) Light micrographs (×400) of neutrophils in oviduct fluid. (c) The ratio of PMNs in leukocyte populations isolated from oviduct flushes, and (d) the numbers of PMNs per oviduct flush during the different stages of estrous cycle, pre-ovulatory (pre-ov, *n* = 10), post-ovulatory (post-ov, *n* = 10), mid-luteal stage (mid-lut, *n* = 10).

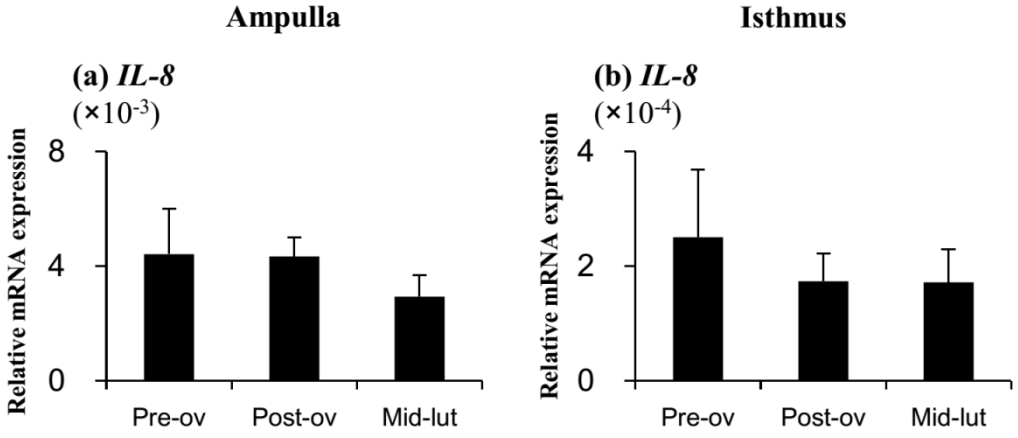

Fig. 2.2 IL-8 gene expression in BOECs freshly separated from bovine oviduct without any culture ((a) ampulla, *n* = 5, and (b) isthmus, *n* = 5,). Note that the relative concentration of IL-8 mRNA expression in ampulla is expressed as × 10^{-3}, while in isthmus it is × 10^{-4}.

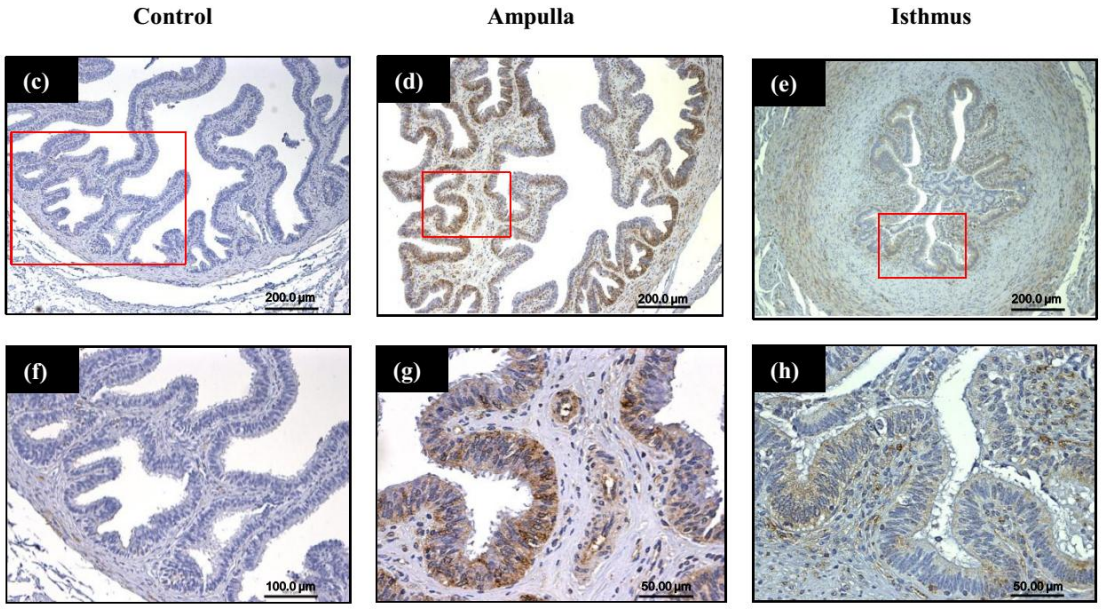

Fig.2.3 Location of IL-8 in bovine oviduct tissue (control section without first antibody (c, f), ampulla (d, g), and isthmus (e, h)). Note that the relative concentration of IL-8 mRNA expression in ampulla is expressed as × 10^{-3}, while in isthmus it is × 10^{-4}. Immunohistochemistry of IL-8 in bovine oviduct tissue (control section without first antibody (c, f), ampulla (d, g), and isthmus (e, h)). The scale bars indicate 200 µm.

Chapter 3

Alpha 1-acid-glycoprotein regulates sperm survival in the bovine oviduct

1. Introduction

The oviduct is a key component of the female reproductive tract, where essential states such as oocyte maturation, sperm capacitation, fertilization, and initial embryonic development take place [83, 84]. The oviduct is classically described as a sterile milieu, even though pathogens and endotoxins could invade the mucosal surfaces of the oviduct via the uterus, peritoneal cavity, and follicular fluid. Therefore, the oviduct environment should be equipped with an efficient and strictly controlled immune system [85]. We have recently shown that polymorphonuclear neutrophils (PMNs), the first line of defense against microorganisms, are present in the bovine oviduct fluid during preovulatory stages. Moreover, the findings of our recent study strongly suggest that the bovine oviduct provides a prostaglandin E_2 (PGE_2)-rich microenvironment to protect sperm from phagocytosis by PMNs that they possibly face *in vivo*, thereby supporting sperm survival in the oviduct [76].

Alpha 1-acid glycoprotein (AGP), a major acute-phase protein produced mainly in the liver, is a single polypeptide chain of 20.4 kDa, with a carbohydrate moiety that accounts for 40% of its total mass [86, 87]. Hepatic production and serum concentrations of AGP are increased in response to systemic injury and inflammation [88]. The precise biological functions of AGP are not completely understood, but numerous activities of potential physiological significance have been described, particularly its effects on immunomodulation and its ability to bind basic drugs [89]. These activities of AGP have been shown to be mostly dependent on carbohydrate composition, and changes in glycosylation can affect the biological properties of AGP [90]. AGP mRNA is expressed in extrahepatic organs, such as the lung, kidney, spleen, lymph node, uterus, and ovary [91]. Therefore, we hypothesized that AGP is secreted locally in the bovine oviduct, and is involved in regulation of the phagocytic activity of neutrophils for sperm.

To test our hypothesis, we investigated, 1) the local production of AGP in the bovine oviduct, 2)

the effects of AGP on the phagocytic activity of PMNs for sperm and superoxide production; and 3) the impact of AGP desialylation on PMN phagocytosis for sperm.

2. Materials and methods

2.1. Oviduct preparation

Paired oviducts along with their ipsilateral ovaries were collected from a local slaughterhouse, and they were closed from both ends to prevent leakage or contamination of the oviduct contents. The stages of the estrous cycle were determined macroscopically by assessing ovarian morphology through the observation of color, size, and weight of the corpus luteum, as described previously [92]. Furthermore, oviducts as well as the attached uteri were macroscopically examined to ensure that they were healthy and free from inflammation. Following these examinations, oviducts were immersed in PBS without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$) (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 0.3% amphotericin B (Sigma-Aldrich), and 0.3% gentamicin (Sigma-Aldrich).

2.2. Primary bovine oviduct epithelial cell (BOEC) isolation and cultivation

The isolation and cultivation of BOECs was based on the method described previously [78, 79]. Briefly, oviducts were cut and separated from the surrounding connective tissue. BOECs were mechanically dislodged, purified, and cultured in D-MEM/F12 culture medium supplemented with 2.2% $NaHCO_3$, 0.1% gentamicin, 1% amphotericin, and 10% fetal calf serum (FCS; BioWhittaker, Walkersville, MD, USA), in 6-well culture dishes (Nalge Nunc International, Roskilde, Denmark) at 38.5 °C in 5% CO_2 and 95% air. After monolayer formation, cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH, USA), replated in 12-well culture dishes at a density of 3×10^4 cells/ml, and cultured until formation of a subconfluent monolayer. The growing BOEC monolayer was then cultured in medium supplemented with 0.1% FCS and incubated for 24 h with AGP (bovine AGP; Life Diagnostics, Inc., West Chester, PA, USA) (0, 1, 10 or 100 ng/ml). Finally, the

culture medium was collected and stored at −80 °C until PGE_2 determination. Finally, 500 µl TRIzol reagent (Invitrogen Corporation) was added to the wells, and the cells were collected, placed in 1.5 ml microcentrifuge tubes and then, stored at −80°C until RNA extraction. The purity of epithelial cell preparations was evaluated with monoclonal antibodies to cytokeratin (anti-cytokeratin-CK1) and immunostaining. Approximately 98% of the cells were positive for anti-cytokeratin (CK1) antibodies.

2.3. AGP gene expression

AGP gene expression in PMNs, BOECs and the liver was detected by reverse transcription polymerase chain reaction (RT-PCR), as described previously [93]. The primers used for PCR were as follows: 5'- CCAACCTGATGACAGTGGC-3', forward and 5'-GCCGACTTATTGTACTCGGG-3', reverse, for AGP (NM_001040502, 109 bp) and 5-CCAAGGCCAACCGTGAGAAAAT-3', forward, and 5-CCACATTCCGTGAGGATCTTCA-3', reverse, for β-actin (K00622, 256 bp).

2.4. AGP concentration determination

Fourteen oviducts at different stages of the estrous cycle (preovulatory, n = 4; postovulatory, n = 5; and mid-luteal stage, n = 5) were very gently flushed with 200 µl $PBS^{-/-}$ and the flushing media were collected into 1.5 ml microcentrifuge tubes. Extreme precautions were taken to avoid any outside contaminations. The flushing media were centrifugated at 1000 g for 10 min at 4°C to remove cellular debris. The AGP concentrations were measured directly in the flushing media using an ELISA kit (Uscn Life Science Inc., Wuhan, China) according to the manufacturer's protocol. The AGP concentrations were quantified based on a standard curve with optical density (OD) measurements at 450 nm on an ELISA reader (Multiskan MS plate reader, Thermo Labsystems, Vantaa, Finland). The intra- and inter-assays coefficients of variation (CVs) were 10% and 12%,

respectively. The range of the standard curves for these assays was 15.5-1000 ng/ml.

2.5. PGE_2 concentration determination

Previously, we have shown that the bovine oviducts provide a PGE_2-rich microenvironment to protect sperm from phagocytosis by PMNs [76]. Therefore, we investigated the effect of AGP on PGE_2 production from cultured BOECs. The BOECs were incubated with AGP (0, 1, 10 or 100 ng/ml) for 24 h and then, the culture medium was collected and used for measuring of PGE_2 concentrations by using a second antibody enzyme immunoassay as previously described [92]. Thirty-six BOEC supernatants were used for PGE_2 concentration determination. The intra and inter-assays CVs were 7.3 and 11.4%, respectively. The ED_{50} was 260 pg/ml, and the range of the standard curves for these assays was 20–20000 pg/ml.

2.6. Preparations of PMNs

Heparinized blood from a multiparous Holstein cow during the luteal stage was collected, and PMNs were isolated as previously described [81]. The PMNs were suspended at a density of 15×10^6 cells/ml in culture medium supplemented with 0, 1, 10 or 100 ng/ml of AGP (bovine AGP; Life Diagnostics, Inc., West Chester, PA, USA) and incubated at 37 °C in 5% CO_2 and 95% air for 4 h with gentle shaking. After PMNs incubation, PMNs were washed 2 times with $PBS^{-/-}$ and used for a phagocytosis assay.

2.7. Preparation of sperm

In parallel with the PMN preparation, sperm preparation was also carried out. Frozen straws were obtained that contained semen from three highly fertile Holstein bulls of the Genetics Hokkaido Association (Hokkaido, Japan). All semen straws were obtained from a single ejaculate from each bull separately. *In vitro* capacitation was induced by 4 h incubation of bull sperm in modified Tyrode's albumin, lactate, and pyruvate medium (Sp-TALP) supplemented with 10 μg/ml

heparin, according to the method previously described [94, 95]. Capacitation was verified by the induction of acrosome reactions using 100 μg/ml lysophosphatidylcholine for 15 min. Acrosome reactions were detected by performing a dual staining procedure with Trypan blue supravital stain and Giemsa stain as described by Kovacs and Foote [96]. After the treatment for capacitation, sperm were washed and suspended in Tyrode's medium containing lactate, pyruvate, and HEPES (TL-HEPES) [97, 98], and they were then used in the phagocytosis assays.

2.8. Coating of plates with desialylated AGP (as-AGP)

Plates were coated as described previously [99], with minor modifications. Three 96-well ELISA plates (Nunc, Denmark) were coated with AGP (0, or 10 μg/well) dissolved in 150 μl of PBS for 2 h at 37 °C. They were then washed three times with PBS and blocked with 0.1% bovine serum albumin (BSA) in PBS for 2 h at 37 °C. Plates were coated with as-AGP by incubating the AGP-coated plates with 200 mU/ml neuraminidase (Streptococcus 6646K, EC 3.2.1.18, Seikagaku corporation, Tokyo, Japan) in 0.1 M PBS (pH 5.2) for 2 h at 37 °C. Next, the plates were washed three times with PBS and blocked with 0.1% BSA in PBS for 2 h at 37 °C. The plates were washed again three times with PBS before use. PMNs were cultured on the coated plates for 4 h and then, used in the detection of the phagocytic activity and superoxide generation of PMNs in response to sperm.

2.9. Phagocytosis assay

The phagocytic activity of PMNs for capacitated sperm was assayed according to the method previously described [100], with minor modifications. Briefly, PMNs incubated for 4 h were suspended in TL-HEPES. A 50 μl aliquot of PMN suspension was mixed with an equal volume of the treated sperm suspension in a 96-well untreated polystyrene microtest plate (Thermo Scientific, Roskilde Denmark) and incubated at 38 °C in 5% CO_2 and 95% air for 60 min with gentle swirling

on a test-plate shaker. The final concentrations of PMNs and the treated sperm were 15×10^6 and 30×10^6 cells/ml, respectively. After incubation, an equal volume of heparin (40 mg/ml in TL-HEPES) was added to facilitate the dissociation of agglutinated PMNs. Subsamples of 75 µl were fixed by adding 25 µl of 2% (v/v) glutaraldehyde. The fixed samples were mounted into glass slides and examined at × 400 magnification using a phase-contrast microscope connected to a digital camera and a computer system (Suite, Leica Microsystems, Wetzlar, Germany). At least 400 PMNs were counted in different areas of the specimens. The percentage of PMNs with phagocytized sperm was recorded as the phagocytosis rate. Quantification of the number of PMNs with phagocytized sperm was performed independently by two observers.

2.10. Superoxide generation determination

PMNs were incubated with AGP (0 or100 ng/ml) for 4 h, suspended in TL-HEPES either with or without the treated sperm and directly used for measuring superoxide generation. Briefly, 10 µl luminol reagent (Sigma) was pipetted into a 96-well FluoroNunc plate (Nunc, Roskilde, Denmark). Next, 100 µl of PMNs incubated for 4 h (0.5×10^6 cells/ml) and treated sperm (1×10^6 cells/ml) were added. Superoxide generation was detected at 425 nm using an AB-2350 Phelios (ATTO, Tokyo, Japan).

2.11. Scanning electron microscopy (SEM)

Neutrophils either directly phagocytize sperm through cell-cell attachment or entrap them within neutrophil extracellular traps (NETs), structures consisting of neutrophil nuclear DNA and associated proteins, that act to ensnare the sperm and hinder their motility [101, 102]. We therefore used SEM to investigate the effect of AGP on NET formation by PMNs for sperm entanglement. Basically, PMNs were incubated in culture medium without any stimulation, or with AGP (100 ng/ml) for 4 h, and then a phagocytosis assay was performed by incubation of PMNs (15×10^6

cells/ml) together with capacitated sperm (30×10^6 cells/ml) for 60 min with gentle swirling on a test-plate shaker. For SEM, each sample, after phagocytosis, was placed onto cover glass coated with 0.1% neoprene in toluene, dried at room temperature, and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After fixation, the samples were washed in PB, postfixed in 1% osmium tetroxide in PB and dehydrated in a graded series of ethanol solutions. The specimens were then freeze-dried with *t*-butyl alcohol using a freeze dryer (ES-2030, Hitachi, Tokyo, Japan). Each dried sample was mounted on a specimen stub with cover glass and sputter-coated with platinum (Pt) (Ion sputter coater E-1045 ion sputter coater, Hitachi High-Technologies Corporation, Tokyo, Japan). The specimens were observed using a scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at an accelerating voltage of 5 kV.

2.12. Statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed with StatView 5.0 (SAS Institute, Inc., Cary, NC, USA). Statistical significance between groups was determined using a one-way ANOVA followed by multiple comparison tests (Fisher's test for three groups, and Bonferroni's test for more than three groups). Results were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. AGP mRNA expression and the AGP concentration in oviduct fluid

Our results demonstrate local gene expression of AGP by cultured BOECs *in vitro* that was well comparable to that for the liver and PMNs (Fig. 3.1a). Thus, the local concentrations of AGP in the oviduct fluid were determined. The AGP concentrations in bovine oviduct flushing media were not significantly changed during different stages of the estrous cycle and were ranged from 30–60 ng/ml (Fig. 3.1b).

3.2. Dose-dependent effect of AGP on the phagocytic activity and superoxide generation of PMNs for sperm

The AGP system has immunomodulatory functions [90], and may be involved in regulation of the local immune response in the bovine oviduct. It is therefore of interest to determine the effect of AGP on PMN-mediated phagocytosis of sperm. A 4-hour preexposure of PMNs to AGP (1, 10 or 100 ng/ml) resulted in a dose-dependent decrease in the PMN phagocytosis activity for capacitated sperm (Fig. 3.2c). Moreover, the stimulation of PMNs with 100 ng/ml AGP prior to the superoxide assay reduced superoxide production by PMNs incubated with capacitated sperm compared with unstimulated PMNs (Fig. 3.2d).

3.3. Observation of NET formation by SEM

During the phagocytosis assay, the addition of sperm to PMNs induced NET formation (Fig. 3.3a, b, d, e). However, the exposure of PMNs to AGP (100 ng/ml) prior to the phagocytosis assay reduced NET formation by PMNs (Fig. 3.3c, f).

3.4. Effect of desialylated-AGP on phagocytic activity and superoxide production by PMNs for sperm

The immunomodulatory functions and binding activities of AGP have been shown to be mostly dependent on carbohydrate composition, and changes in the glycosylation of AGP can affect its biological properties [90]. A four-hour incubation of PMNs on AGP-coated plates prior to the phagocytosis assay resulted in a decrease in the phagocytic activity of PMNs for capacitated sperm (Fig. 3.4a). However, incubation of PMNs in the as-AGP-coated plates resulted in complete abolishment of the suppressive effect of AGP on the phagocytosis of sperm by PMNs (Fig. 3.4a). Moreover, the incubation of PMNs in the as-AGP-coated plates resulted in removal of the suppressive effect of AGP on the superoxide production by PMNs in response to sperm (Fig. 3.4b).

3.5. The effect of AGP on PGE2 production from cultured BOECs

Incubation of BOECs with AGP (0, 1, 10, or 100 ng/ml) for 24 hours resulted in stimulation of PGE_2 production in BOECs *in vitro* in a dose-dependent manner (Fig. 3.5a, $P < 0.05$).

3.6. The effect of AGP and PGE2 in combination on the phagocytic activity of PMNs for sperm

A four-hour incubation of PMNs with the local concentration of AGP detected in oviduct flushing media (50 ng/ml), along with that of PGE_2 (10^{-8} M, 3.52 ng/ml, [76]), resulted in an additive effect in suppression of the phagocytic activity of PMNs for treated sperm (Fig. 3.5b).

4. Discussion

Bovine AGP is the main acute phase protein; its concentration in the peripheral blood circulation increases from approximately 0.3 mg/ml to 0.9 mg/ml during disease [55, 103]. AGP is mainly produced by the liver, from which it diffuses into the general circulation [35]. AGP mRNA was detected in extrahepatic organs such as the lung, kidney, spleen, lymph node, uterus, ovary, placenta, and decidua [91, 104]. Additionally, AGP protein has been previously detected in sow oviduct fluid [105]. In this study, we have provided the first evidence for the local gene expression of AGP by bovine oviduct epithelial cells *in vitro*. The oviduct flushing media contained AGP in the range of 20–60 ng/ml, which is much lower than that seen in bovine plasma. Generally, it has been shown that AGP has immunomodulatory effects [45, 47, 89, 106]. This prompted us to investigate the effect of AGP on the phagocytosis of sperm by PMNs. Our results show that detectable concentrations of AGP were found in the oviduct flushing media, and AGP dose-dependently suppressed PMN phagocytosis of sperm *in vitro*. Additionally, SEM analysis demonstrated that AGP drastically reduced NET formation, preventing sperm from being fixed and trapped by PMNs, and thus indirectly resulted in the suppression of PMN phagocytosis for sperm.

Previously, it has been shown that AGP induced a dose-dependent inhibition of superoxide

generation in PMNs stimulated by phorbol-12-myristate-13-acetate [107]. Additionally, the ability of PMNs to release superoxide has been used as an indicator for evaluating their phagocytic activity on sperm [108]. Our results show that only in the presence of sperm did AGP (100 ng/ml) significantly suppress superoxide release by PMNs. These findings suggest that the AGP secreted in bovine oviducts contributes to the protection and maintenance of sperm survival through the suppression of phagocytic activity and superoxide release by PMNs. In humans, it has been shown that leukocytes are the predominant source of superoxide production in sperm preparations and that the contribution of spermatozoa was either undetectable or was a small fraction of that contributed by leukocytes [109]. In general, PMNs form NETs for pathogen uptake depending on superoxide release through reactive oxygen species (ROS)-generating pathways [110]. NADPH decomposition results in the release of superoxide, which is converted to hydrogen peroxide (H2O2) either by superoxide dismutase (SOD) or spontaneously [110]. The generated H2O2 is then used in the formation of halogenated ROS, such as hypochlorite (OCL-) by myeloperoxidase (MPO), which induces NETs formation [110]. Therefore, we hypothesize that AGP, via the suppression of superoxide generation, could affect the ROS-generating pathways that lead to suppression of NETs formation, altering the phagocytic behavior of PMNs for sperm.

The mechanism by which AGP suppresses the phagocytosis of sperm by PMNs is still unknown. The binding and immunomodulatory activities of AGP have been shown to be mostly dependent on its carbohydrate composition [90]. Importantly, bovine AGP is one of the most heavily glycosylated proteins [86], and AGP glycosylation is modified during disease [111]. It is conceivable that the terminal sialic acid residues exposed on the surface of AGP block phagocytosis by binding phagocyte sialic acid-binding immunoglobulin-type lectins (Siglec) [112]. Therefore, we hypothesized that the sialic acid contributes to the suppressive effect of AGP on PMN phagocytosis for sperm. In fact, our results show that desialylating AGP completely abolished the

AGP-suppressive effect on both PMN phagocytosis of sperm and superoxide release. These results are in agreement with previous studies showing that desialylating AGP abolished an AGP hepato-protective effect [113] and anti-apoptotic activity [114], whereas hyposialylated AGP completely inhibited the phagocytosis of *E. coli* by feline neutrophils [115].

We previously demonstrated that luteinizing hormone stimulates BOECs to secrete PGE_2, which plays a major role in suppressing the phagocytic activity of PMNs for sperm [76]. Interestingly, our results showed that AGP dose-dependently stimulated PGE_2 secretion from BOECs. Moreover, AGP and PGE_2, within the physiological concentrations detected in oviduct flushing media, additively suppressed the phagocytosis of sperm by PMNs. Thus, these findings suggest that AGP not only directly suppresses sperm phagocytosis but also works cooperatively with PGE_2 to suppress the phagocytosis of sperm by PMNs in the bovine oviduct.

Taken together, our findings suggest that AGP has immunomodulatory functions in the bovine oviduct. It is proposed that under physiological conditions, the local AGP system in the bovine oviduct may aid sperm survival through direct suppression of the phagocytic activity of PMNs for sperm, through reduction of superoxide production by phagocytizing PMNs, and by limiting NET formation.

5. Figures

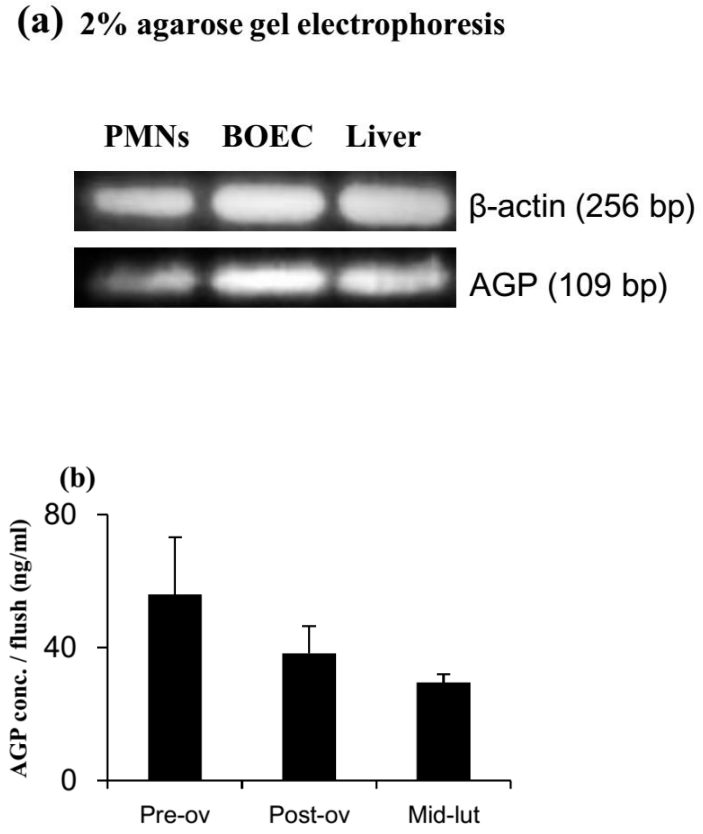

Fig. 3.1 (a) AGP gene expression in PMNs, BOECs, and liver. (b) The AGP concentrations per oviduct fluid during the estrous cycle. (pre-ovulatory, *n* = 4, post-ovulatory, *n* = 5, and mid-luteal stage, *n* = 5).

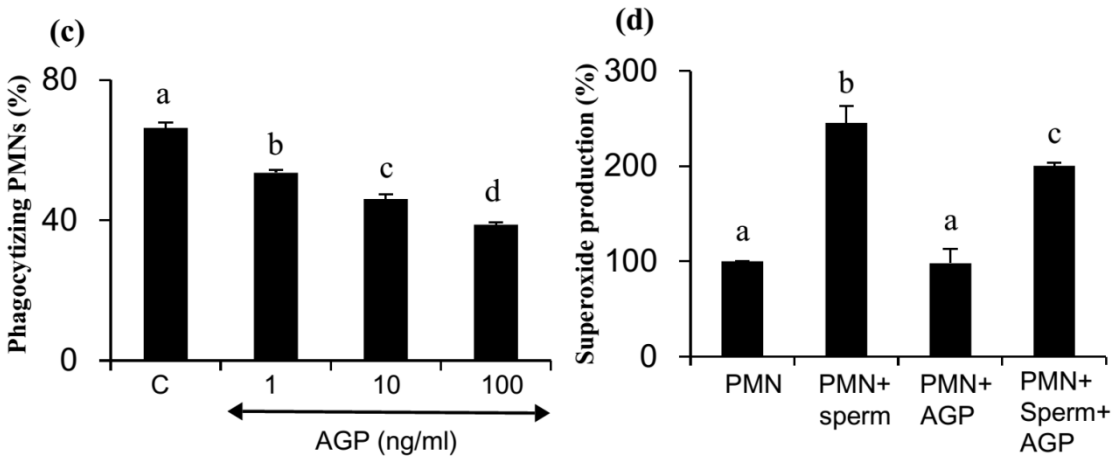

Fig. 3.2 (c) Percentage of PMNs phagocytosis for sperm treated to induce capacitation *in vitro*. PMNs were incubated for 4 h in culture medium supplemented with 0, 1, 10, or 100 ng/mL AGP, followed by the 1-h phagocytosis assay. Numerical values are presented as means ± S.E.M. of three experiments. The different letters indicate significant differences between treatments at $P < 0.05$. (d) The effect of AGP on percentage of superoxide production by PMNs undergoing *in vitro* phagocytosis of sperm treated to induce capacitation. Numerical values are presented as means ± S.E.M. of four experiments. The different letters indicate significant differences between the marked treatments at $P < 0.05$.

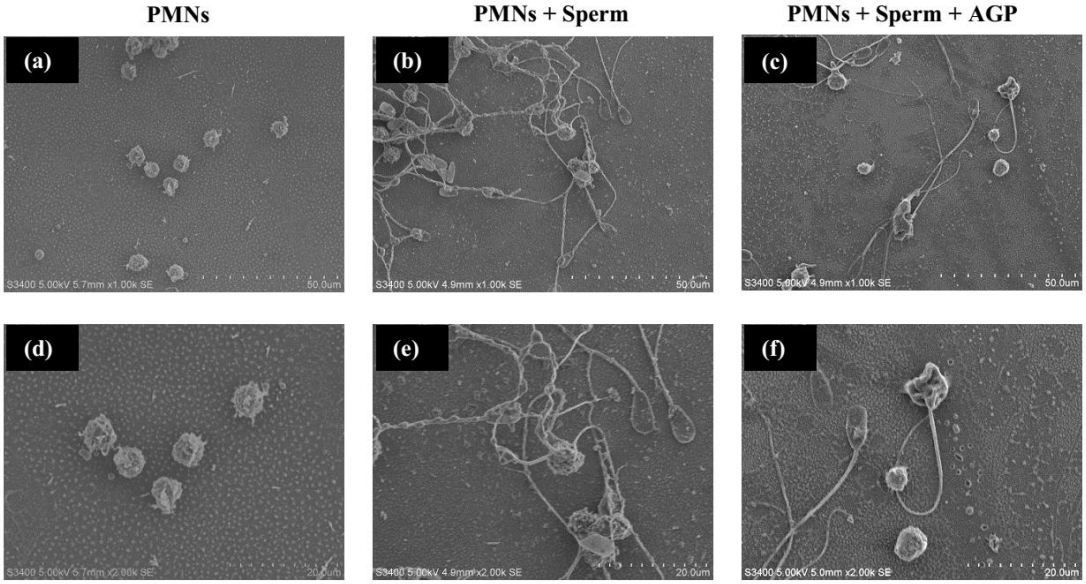

Fig. 3.3 Scanning electron microscopy of sperm phagocytosis by PMNs. The upper panels by ×1,000 (a,b,c), and equivalent lower panels by ×2,000, respectively. PMNs were incubated without any stimulant (a, d), or with sperm addition to induce neutrophil extracellular traps (NETs) (b, e). NET formation was suppressed in PMNs incubated with AGP (100 ng/mL) prior to phagocytosis assay (e, f).

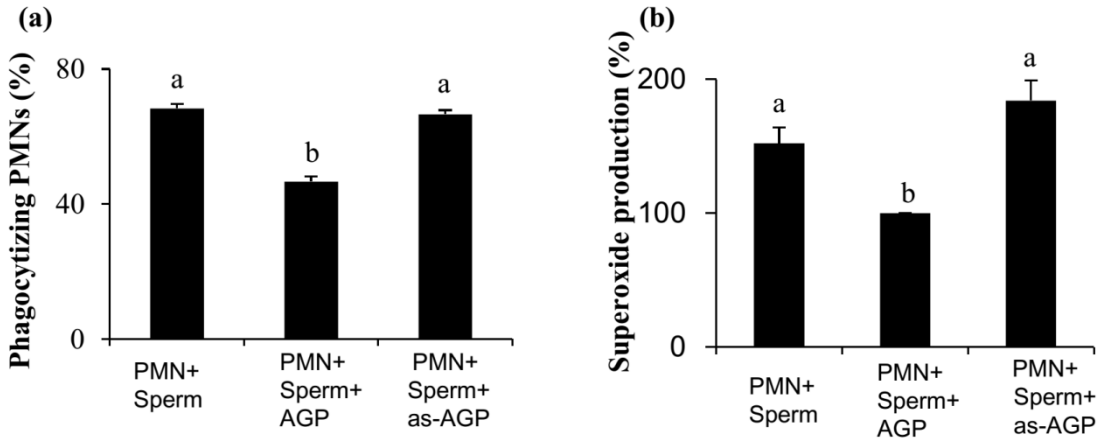

Fig 3.4 (a) The percentage PMNs undergoing the phagocytosis of sperm treated to induce capacitation, *in vitro*. The different letters indicate significant differences between the marked treatments at $P < 0.001$. (b) The percentage of superoxide production by PMNs undergoing *in vitro* phagocytosis of sperm treated to induce capacitation. The different letters indicate significant differences between the marked treatments at $P < 0.05$. Numerical values are presented as means ± S.E.M. of four experiments.

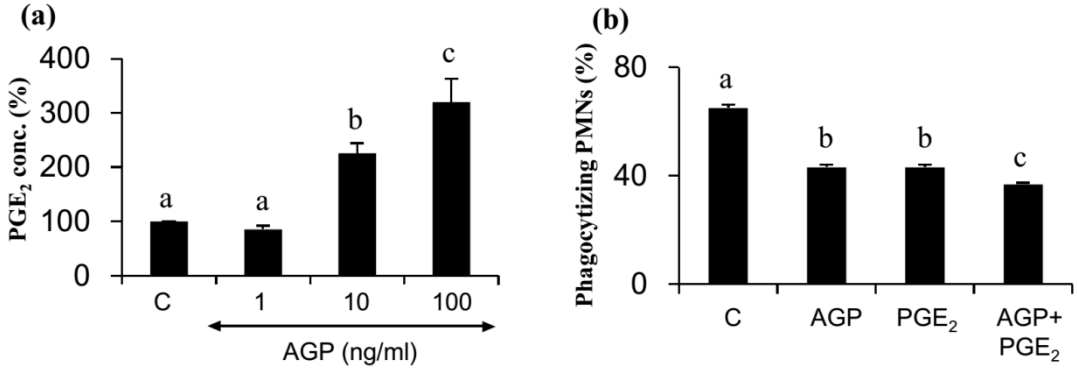

Fig. 3.5 (a) The percentage of PGE_2 production in BOEC culture medium supplemented with AGP (0, 1, 10 or 100 ng/mL) for 24 h (*n* = 9 /group, 100% = 48.1 ± 4.1 ng/mL, means ± S.E.M.). (b) The effect of AGP (50 ng/mL) and PGE_2 (10^{-8} M, 3.52 ng/mL) in combination, on the phagocytic activity of PMNs for sperm treated to induce capacitation. Numerical values are presented as means ± S.E.M. of four experiments. The different letters indicate significant differences between the marked treatments at *P* < 0.05.

Chapter 4

General discussion and conclusion

1. PMNs in the bovine oviduct fluid

PMNs are the first cells to invade inflamed tissues. They are present in high numbers in blood and are rapidly recruited because of the kinetics of expression of leuko-epithelial adhesion molecules on leucocytes and epithelial cells. PMNs migrate following a chemo-attractant gradient initiated in the injured tissue [116].

Anatomically, the oviduct is a thin convoluted tube that opens into the uterine horn on one end and into the peritoneal cavity on the other end. PMNs were found in the peritoneal fluid by Wright's-Giemsa stained smear [117]. Even a small increase in PMNs can be detected, these PMNs in the peritoneal fluid play a central role in the first defense reaction of bacterial infection [117]. In the bovine uterus, the PMNs increase significantly in the subepithelial connective tissue, preparing the endometrium for a rapid inflammatory reaction. In mares and gilts, the onset of PMN chemotaxies is rapid and the duration of PMN infiltration relatively short. This ensures effective removal of sperm and bacteria and subsequent return of the endometrium to a normal state, prepared to receive the embryo. Therefore, PMNs may migrate into the oviduct from peritoneal fluid or/and uterus when the chemotactic factors existing in the oviduct fluid.

In chapter 2, relatively constant numbers of PMNs were found to exit in the oviduct fluid during the pre-ovulatory stage under physiological conditions [76]. Thus, I evaluated the PMNs ratio and number during the different stages of the estrous cycle, suggesting that the oviductal PMNs contribute to the local innate immunity that protects the oviduct from potential pathogenic microorganisms. These results together with fact that the oviduct creates the optimal microenvironment for survival of allogeneic sperm prompted us to investigate the effect of the oviduct secretions on the phagocytic activity of PMNs for sperm. The presence of sperm in the uterus induces rapid chemotaxis of PMNs, which are detected in the uterus already 0.5 h after artificial insemination in horse and pigs [21]. The first sperm have been found in the oviducts within

minutes after mating, but these rapidly transported spermatozoa may not participate in fertilization [62]. Thousands of sperm is not a small number in the oviduct compare with the uterus. The thousands of sperm may induce PMNs recruiting. I also detected the IL-8, for PMNs a chemoattractant is produced by BOEC and existed in the bovine oviduct tissue. In vitro study showed that the PMNs migration was stimulated by the supernatant derived from the early CL while neutralizing this activity with an anti-IL-8 antibody reduces the PMN migration [81]. IL-8 has also been found to be produced in human oviduct fluid [82]. Therefore, the continuous recruitment of PMNs in the bovine oviduct fluid may due to the production of IL-8 in BOEC (Fig. 4.1).

2. Future aspects of immune cells in the bovine oviduct

Thousands of sperm and $5–7\times10^3$ cell/oviduct flush of PMNs are not a small number in the oviduct compare with the uterus however, fertilization seems always successful to be occurred without affected by sperm phagocytosis by PMNs. PBMCs were also detected existing in the bovine oviduct fluid (data not shown). PBMCs were the major type of immune cells to react to anti-inflammatory control. For example, transforming growth factor β (TGFβ) produced by activated T cells inhibits T-helper-cell proliferation and differentiation. This contribute to control immune responses and maintains immune homeostasis [118](Fig. 4.2 a c). Therefore, I hypothesized that after ovulation or sperm reaching to the oviduct, the immune cells in the bovine oviduct may provide an immune tolerance for supporting fertilization and early embryo development. In the future, the factors to regulate immune tolerance or related cytokines production in the oviductal immune cells should be investigated (Fig. 4.2 b).

3. Suppression of AGP on phagocytic activity of PMNs for sperm

AGP is primarily synthesized by the liver, but can be produced also in the extra-hepatic organs [91]. Thus, the acute phase response may take place in the extra-hepatic cell types. In chapter 3, I

investigated the gene expression of AGP in BOEC and showed its local concentration in the oviduct flushing. AGP suppressed the superoxide generation in PMNs which was induced by the phagocytosis of sperm. A sperm-PMN binding is mediated by direct cell membrane attachment or by NET entanglement. My results showed that AGP suppressed direct cell membrane attachment and NET entanglement generation during the sperm phagocytosis by PMN through the sialic acid fragment of AGP. Therefore, AGP is a regulator of sperm survival in the bovine oviduct.

Luteinizing hormone stimulates BOECs to secrete PGE_2, which plays a major role in suppressing the phagocytic activity of PMNs for sperm [76]. Both PGE_2 and AGP exist in the bovine oviduct fluid, so that it was necessary to investigate the possible additive effect of these two factors for sperm phagocytosis by PMN. The data showed that AGP not only stimulated PGE_2 secretion from BOECs, but also suppressed additively the phagocytosis of sperm by PMNs together with PGE_2. Therefore, AGP could directly regulate sperm pahgocytosis, and stimulats BOEC to produce physiological factors such as PGE_2 in regulating the sperm survival in the bovine oviduct (Fig. 4.2b).

4. Future aspects of the sperm survival in the bovine oviduct.

In this study, it became clear that AGP and PGE_2 in the bovine oviduct protect the sperm from phagocytosis by PMNs. It is known that a large number of pro/anti-inflammatory factors are secreted in the bovine oviduct, thus, their direct and indirect functions on sperm phagocytosis by PMNs should be investigated in detail (Fig.4.2).

5. Figures

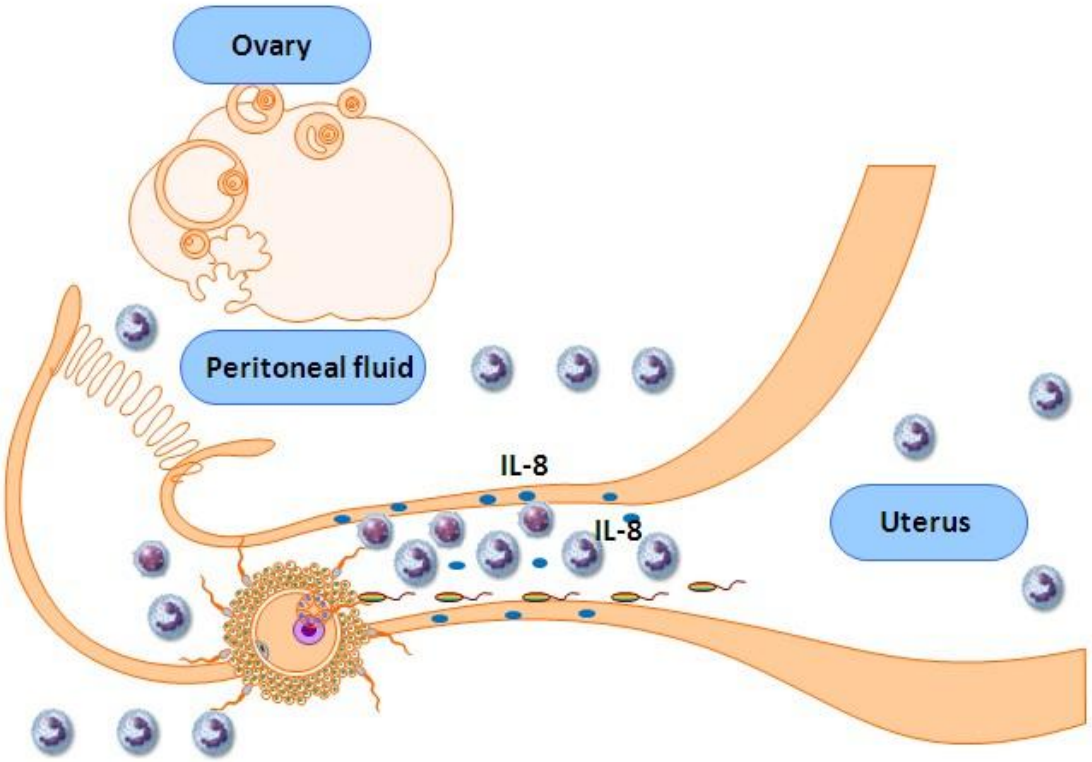

Fig. 4.1

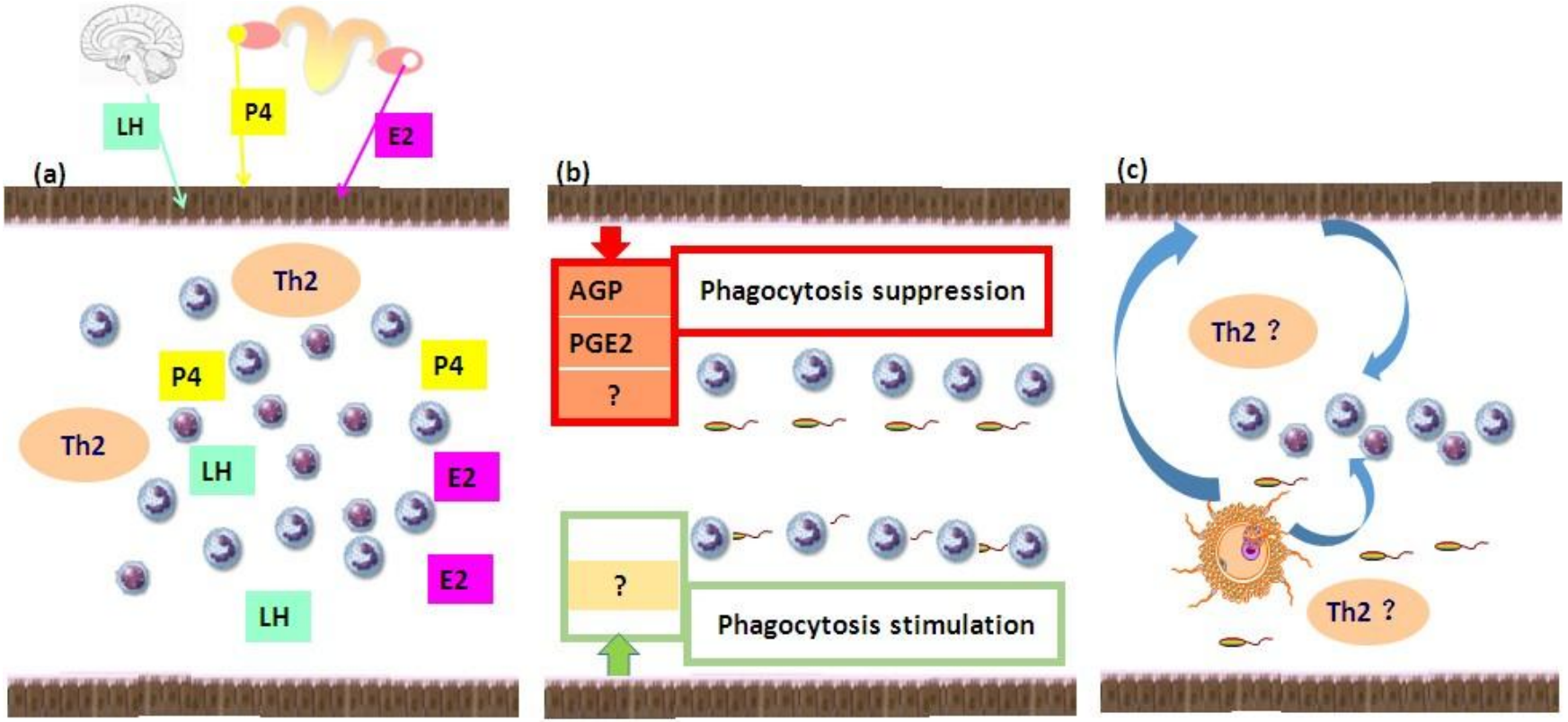
(a)
LH
P4
E2
Th2
P4
P4
Th2
LH
E2
E2
LH
(b)
AGP
PGE2
?
Phagocytosis suppression
?
Phagocytosis stimulation
(c)
Th2 ?
Th2 ?
Pre-ovulation
After Insemination
After Fertilization

Fig.4.2

Abstract

The oviduct is a key component of the female reproductive tract, where essential states such as oocyte maturation, sperm capacitation, fertilization, and initial embryonic development take place. An intriguing question of oviductal function is how the oviduct mounts local immune responses against microbial pathogens, while allowing spermatozoa and the early embryo, both of which carry foreign proteins, to escape detection by the local immune system of the oviduct.

We have previously shown that polymorphonuclear neutrophils (PMNs) are present in bovine oviductal fluid under physiological conditions during the different estrous cycle. Therefore, in this study, I investigated the possible changes in the number of PMNs throughout the different stages of the estrous cycle. The bovine oviduct was very gently flushed by 200 µL PBS without Ca^{2+} and Mg^{2+} ($PBS^{-/-}$) without any touch from the outside but with just handing from ampulla to isthmus, and the oviduct fluid was accumulated into a 1.5 mL microcentrifuge tube. Bovine oviduct epithelial cells （BOEC） and the immune cells were separated from the oviductal fluid by 10 ml of 35% Percoll. For a total cell count, a sample of the leukocyte suspension was diluted (1:10) with 0.1% acetic acid, and mounted on a haemocytometer. To determine PMNs proportions in leukocyte populations, a 20 µl sample of the leukocyte suspension was diluted in Macs separation buffer and analyzed by flow cytometric evaluation. Neutrophils infiltrate the oviduct fluid not only during the preovulatory stage, but also throughout the different stages of the estrous cycle, and that their numbers remain relatively constant over the estrous cycle. The average numbers of PMNs were $5–7\times10^3$ cell/oviduct flush, constituting approximately 12–16% of the total leukocyte population in the oviduct flush. The interleukin 8 (IL-8), a strong chemotactic agent for neutrophils, gene was expressed in BOECs that were freshly separated from bovine oviduct without any culture. BOECs continuously expressed IL-8 mRNA, and IL-8 gene expression was approximately 10 times higher in ampullary epithelial cells than in isthmic epithelial cells. Ampullary epithelial cells and isthmic

epithelial cells were intensively stained for IL-8 by immunohistochemistry (IHC). BOECs constantly produce IL-8 throughout the whole estrous cycle. The stable production of IL-8 could be one of the factors contributing to the continuous recruitment of PMNs into the oviduct fluid and the maintenance of PMNs at a relatively constant level throughout the different stages of the estrous cycle. These results indicate that PMNs are normal constituents of the local immunological microenvironment in the bovine oviduct that protect the oviducts from potentially pathogenic microorganisms and maintain a sterile environment.

Alpha 1-acid glycoprotein (AGP) is a major acute-phase protein produced mainly in the liver. Hepatic production and serum concentrations of AGP are increased in response to systemic injury, inflammation, or infection and its effects on immunomodulation have been described. AGP mRNA is expressed in extra-hepatic organs, such as the lung, kidney, spleen, lymph node, uterus, and ovary. Therefore, I hypothesized that AGP is secreted locally in the bovine oviduct, and is involved in the regulation of the phagocytic activity of neutrophils for sperm. To test my hypothesis, I investigated, 1) the local production of AGP in the bovine oviduct; 2) the effect of AGP on the phagocytic activity of PMNs for sperm and superoxide production; and 3) the impact of AGP desialylation on the PMN phagocytosis of sperm. I have provided the first evidence for the local gene expression of AGP by bovine oviduct epithelial cells *in vitro*. The oviduct flush solutions contained AGP in the range of 20–60 ng/mL, which is much lower than that seen in cow plasma. AGP, at the detectable range of concentrations seen in the oviduct flush experiments, dose-dependently suppressed PMN phagocytosis of sperm *in vitro*. Neutrophils either directly phagocytize sperm through cell-cell attachment or entrap them with neutrophil extracellular traps (NETs), structures consisting of neutrophil nuclear DNA and associated proteins, which ensnare sperm and hinder their motility. The four-hour incubation of the PMNs with AGP (1, 10, or 100 ng/mL) prior to phagocytosis assay, resulted in a dose-dependent decrease in the phagocytosis by

PMNs of sperm treated to induce capacitation. Additionally, SEM analysis demonstrated that AGP (100ng/mL) drastically reduced NET formation, preventing sperm from being fixed and trapped by PMNs, and thus indirectly results in the suppression of PMNs phagocytosis for sperm. The ability of PMNs to release superoxide has been used as an indicator for evaluating their phagocytic activity on sperm. my results show that only in the presence of sperm did AGP (100 ng/mL) significantly suppress superoxide release by PMNs. These results suggested that the AGP secreted in bovine oviducts contributes to the protection and maintenance of sperm survival through the suppression of phagocytic activity and superoxide release by PMNs. It has been shown that NET formation may depend on superoxide release through reactive oxygen species (ROS)-generating pathways. Therefore, I hypothesize that AGP, *via* the suppression of superoxide generation, could affect the ROS-generating pathways that lead to the generation of NETs, altering the phagocytic behavior of PMNs for sperm. It is conceivable that the terminal sialic acid residues exposed on the surface of AGP block phagocytosis by binding phagocyte sialic acid-binding immunoglobulin-type lectins (Siglec). Desialylating AGP abolished an AGP hepato-protective effect and anti-apoptotic activity. Therefore, I hypothesized that the sialic acid contributes to the suppressive effect of AGP on the PMNs phagocytosis for sperm. A four-hour incubation of PMNs on the AGP-coated plates prior to phagocytosis assay resulted in a decrease in the phagocytic activity of PMNs for treated sperm. However, incubation of PMNs on the as-AGP-coated plates resulted in complete abolishment of the suppressive effect of AGP on the phagocytosis of sperm by PMNs. This phenomenon was also seen in the superoxide production following incubation of the PMNs with sperm on AGP- or as-AGP-coated plates. My results show that desialylating AGP completely abolished the AGP-suppressive effect on both PMN phagocytosis of sperm and superoxide release. Previously, we demonstrated that luteinizing hormone stimulates BOECs to secrete PGE_2, which plays a major role in suppressing the phagocytic activity of PMNs for sperm. A dose-response study was performed, in which BOECs were incubated with AGP (0, 1, 10, or 100 ng/mL) for 24 hours. My results showed

that AGP stimulated PGE_2 production in BOECs *in vitro* in a dose-dependent manner. Moreover, A four-hour incubation of PMNs with the local concentration of AGP detected in oviduct flush (50 ng/mL), along with that of PGE_2 (10^{-8} M, 3.52 ng/mL), resulted in an additive effect in the suppression of the phagocytic activity of PMNs for treated sperm. Thus, these findings suggest that the AGP not only directly suppresses sperm phagocytosis but also works cooperatively with PGE_2 to suppress the phagocytosis of sperm by PMNs in the bovine oviduct.

Taken together, the results demonstrate that PMNs constantly infiltrate bovine oviducts throughout the estrous cycle, possibly under the effects of the chemotactic cytokine, IL-8, that secreted from oviduct epithelial cells. Additionally, the present findings shed a light on the immunomodulatory functions of AGP in the bovine oviduct. It is proposed that under physiological conditions, the local AGP system in bovine oviduct may aid sperm survival through the direct suppression of the phagocytic activity of PMNs for sperm, the reduction of superoxide production by phagocytizing PMNs, and by limiting NET formation.

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要 旨

卵管は卵子の成熟、精子の受精能獲得、受精、そして初期の胚発生が起こる雌性生殖器官である。卵管は、病原体に対して局所の免疫反応で対応し、一方で、異物とも考えられる雄を由来とするタンパク質を有する精子や初期胚を受け入れていると考えられるが、その詳細はまったく不明である。本研究は、ウシ卵管について、まず、通常の発情周期中の卵管液中に、自然免疫の最初の反応に関わる好中球が一定数で常在することを示した。この事実から、受精のために卵管にたどりつく少数の精子が、好中球の貪食の攻撃をかわすメカニズムが存在する事が必要であると考えた。

卵管液から白血球を分離し、光学顕微鏡観察、フローサイトメトリー分析によって、発情周期中のウシ卵管液中には、卵管1つあたり5-7千の好中球が存在する事がわかった。免疫組織化学による組織染色によって、好中球の強力な走化性因子であるインターロイキン-8（IL-8）は、卵管上皮細胞（BOEC）で強く発現している事がわかった。このことは、BOEC は IL-8 を常に強く分泌する事で、発情周期中の卵管腔内に好中球を誘引していることが伺われた。

Alpha 1-acid glycoprotein（AGP）は、主に肝臓で分泌される急性タンパク質である。体内で炎症が起きると、すぐに分泌量が増加し、血中の免疫細胞や末梢器官に作用して、免疫機能の調節に関わる事が示されている。本研究では、ウシ卵管液中に、血液中よりずっと低い濃度の AGP が存在する事を ELISA 法によって示した。検出された濃度の AGP は、濃度依存的に好中球が精子を貪食するのを抑制した。同時に、AGP はこの貪食作用の際に放出される好中球の活性酸素の量を抑制した。走査型顕微鏡による観察の結果、AGP は好中球が自らの DNA とタンパク質で形成する細胞外トラップ（NETs）の誘導を抑制して、精子を絡めとるのを抑制している事も明らかとなった。重要な事に、AGP 分子の脱シアル化は、これらの好中球の精子貪食活性の抑制をできなくなることが示された。さらに、AGP は BOEC 培養系において、PGE2 分泌を刺激し、AGP と PGE2 は好中球の精子貪食を相加的に刺激することがわかった。

以上の一連の知見は、発情時期のウシ卵管において、上皮細胞が分泌する IL-8 で好中球は卵管液中に誘引されて常在し、病原体などの感染に備えると同時に、遺伝的には雄由来の異物とも考えられる精子に対しては、卵管上皮細胞が分泌する急性タンパクである AGP を介して、好中球が貪食するのを強く抑制する事で、受精が起こりやすい局所環境を維持することに貢献している事が示唆された。