The Porcine Endosalpinx at Different Reproductive Stages

Morphology, Immune Cell Infiltration and Cytokine Expression

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Cover: Immunohistochemical labelling of TGF-β1 in isthmus
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Abstract

Fertilization takes place in the lower part of the porcine oviduct and to be successful, a balance between immunological tolerance to allogeneic differences and immune reactivity to foreign pathogens is required. The thesis aims to describe immune cells and morphological changes in different segments [isthmus (Isth), ampulla (Amp) and infundibulum (Inf)] of the porcine oviduct at various stages of the oestrous cycle, after artificial insemination (AI, pre- and post-ovulatory) and during early pregnancy. The expression of pro-inflammatory and suppressive cytokines was also determined (RT-PCR and immunohistochemistry, IHC) in oviductal samples collected after AI with different components (fresh semen, spermatozoa in extender or extender alone) or only catheter inserted (control) in gilts. In cyclic sows, the epithelial morphology of Amp and Inf varied during the oestrous cycle. High levels of pseudostratification, mitotic activity and secretory granules were found at pro-oestrus/oestrus indicating influence of ovarian hormones. Lymphocytes were predominant in the epithelium whereas both lymphocytes and plasma cells were the major cell types in the connective tissue (CNT). Higher numbers of these cells were found in the Inf than in the Isth both in the cyclic sows and in sows after AI. Neutrophils were found mainly in the infundibular CNT, in cyclic sows at pro-oestrus and in pre-ovulatory inseminated sows in one group (40h after AI). In post-ovulatory inseminated sows, the numbers of neutrophils were lower but present for a longer period. In gilts, IHC-labelling for TGF-β1, IL-10 and IL-6 was apparent, especially in the epithelial cells. Higher mRNA expressions of these cytokines were found in the Isth compared with the Inf and the TGF-β1 mRNA expression was higher than that of IL-6 and IL-10 in both segments. Shortly after AI, no difference between treatments could be detected for any of these cytokines but 35-40 h after AI, the TGF-β1 mRNA expressions were higher in gilts inseminated with different semen components than in the control animals (only catheter inserted). Thus, endogenous production of TGF-β1, IL-10 and IL-6 was indicated and suggests that these cytokines have a physiological function in the porcine oviduct. The relatively higher expression of TGF-β1 mRNA indicates that this cytokine exerts an immunomodulatory role, mainly in the isthmic part of the oviduct, and this seems to be enhanced by AI. The differences between the upper and lower part of the porcine oviduct regarding presence of immune cells and expression of cytokines are likely to reflect a local immune modulation.

Keywords: Sow, Gilt, Oviduct, Immune cell, Cytokine, Seminal plasma, Insemination

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Dedication

To my parents, my wife Nok, and my sons Tee and Poo

Contents

List of Publications

The thesis is based on the following papers, which will be referred to in the text by Roman numerals:

I Jiwakanon, J., Persson, E., Kaeoket, K., and Dalin, A.-M. (2005). The sow endosalpinx at different stages of the oestrous cycle and at anoestrus: studies on morphological changes and infiltration by cells of the immune system. *Reprod Dom Anim* 40, 28-39.

II Jiwakanon, J., Persson, E., and Dalin, A.-M. (2006). The influence of pre- and post-ovulatory insemination and early pregnancy on the infiltration by cells of the immune system in the sow oviduct. *Reprod Dom Anim*, 41, 455-466.

III Jiwakanon, J., Berg, M., Persson, E., Fossum, C., and Dalin, A.-M. Cytokine expression in the gilt oviduct. Part A: effects of seminal plasma and spermatozoa shortly after insemination (submitted).

IV Jiwakanon, J., Persson, E., Berg, M., Fossum, C., and Dalin, A.-M. Cytokine expression in the gilt oviduct. Part B: effects of seminal plasma, spermatozoa and extender at 35-40 h after insemination (submitted).

Abbreviations

AIa	Artificial insemination after ovulation
AIb	Artificial insemination before ovulation
AIJ	Ampullary-isthmic junction
Amp	Ampulla
BTS	Beltsville thawing solution
CD	Cluster of differentiation
CNT	Connective tissue
CReg	Complement regulator
C_T	Threshold cycle
DAB	3, 3'-diaminobenzidine
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FasL	Fas-Fas ligand
GM-CSF	Granulocyte-macrophage colony stimulating factor
HPRT	Hypoxanthine phosphoribosyl-transferase
IELs	Intra-epithelial lymphocytes
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
Inf	Infundibulum
Isth	Isthmus
Lfn	Lymph node
LM	Light microscopy
mAbs	Monoclonal antibodies

MHC	Major histocompatibility complex
mRNA	messenger RNA
NK	Natural killer
NHS	Normal horse serum
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PMNs	Polymorphonuclear leukocytes
qRT-PCR	Quantitative RT-PCR
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase
SLC	Single layer centrifugation
SP	Seminal plasma
Sub-CNT	Sub-epithelial CNT
SWC	Swine workshop cluster
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-beta
T_H	T helper
TNF	Tumor necrosis factor
UTJ	Uterotubal junction

Introduction

The oviducts are narrow tubular organs with very complex functions in which final maturation and transport of the female and male gametes, as well as fertilization, take place (Hunter, 1998; Rodriguez-Martinez *et al.*, 2001). The mechanisms behind these diverse functions are still not fully understood (Brüssow *et al.*, 2008) and the local immune reactivity in particular needs to be explored.

The oviduct, like the other organs of the female reproductive tract, is under hormonal influence from the ovary, i.e. oestrogen from the follicles and progesterone from the corpora lutea. In pigs, the oestrogen levels increase during pro-oestrus to reach a peak at the start of standing oestrus, the stage when the animals are mated or inseminated. The progesterone levels increase after ovulation at metoestrus and reach a plateau during dioestrus. If the animal is not pregnant, the corpora lutea regress at late dioestrus and, as a result, a new cycle begins. If the animal is pregnant, luteal regression is prevented.

At natural mating or insemination, semen is deposited into the cervix/uterine body and then transported through the uterine horns up to the utero-tubal junctions and the oviducts. Semen is composed of allogeneic spermatozoa and seminal plasma (SP). SP contains various substances, e.g. oestrogens that affect uterine myometrial contraction (Claus, 1990), and proteins that interact with the spermatozoa and the oviductal environment (Topfer-Petersen *et al.*, 2008). It has been shown that approximately 25 % of the inseminated spermatozoa, and up to 70 % of the volume, are expelled retrogradely within a couple of hours (Steverink *et al.*, 1998). Large numbers of spermatozoa are also lost by intra-uterine neutrophil phagocytosis (Lovell & Getty, 1968; Matthijs *et al.*, 2003). Therefore, only a small fraction of the inseminated spermatozoa reach the lower part of the oviducts (isthmus)

where they remain viable (Mburu *et al.*, 1997) and reach final maturation before fertilization.

The mucosal surface of the female genital tract is protected by both innate and adaptive immune defense mechanisms (Wira *et al.*, 2005). The former include physical barriers, such as tight junctions of epithelial cells, antimicrobial secretions and non-specific phagocytic cells such as polymorphonuclear leukocytes (PMNs), macrophages and NK cells. The adaptive immunity depends upon lymphocytes (especially located in or under the epithelia). For the mucosal immune response to antigens, recruitment of the appropriate immune cells from regional lymph nodes is mainly determined by the cytokines/chemokines present at the site of antigen exposure (Mowat, 2003; Kaiko *et al.*, 2008). Cytokines refer to a group of soluble immunoregulatory proteins that affect a variety of cell functions. Many cytokines can be released locally, both from cells of the immune system and from non-immune tissue (e.g. epithelia and smooth muscle cell in the uterus) (Frankenstein *et al.*, 2006) including oviductal epithelial cells (Fahey *et al.*, 2005).

The present study on the porcine oviduct at different reproductive stages aims to illustrate morphological changes and immune cell distribution, as well as cytokine expression after insemination with various semen components, in order to better understand the regulatory mechanisms.

The porcine oviduct

The porcine oviduct is a paired organ divided into three main segments, isthmus, ampulla and infundibulum (Fig. 1).

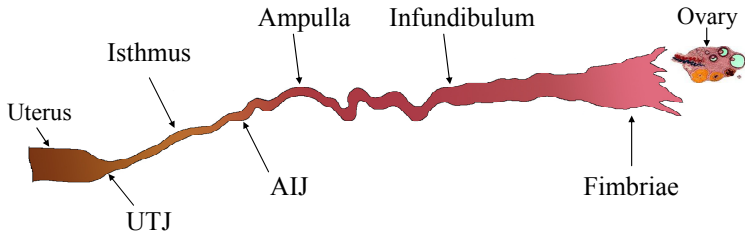

Figure 1. Different segments of the porcine oviduct

The isthmus is narrow and connected caudally (ad uterus) to the uterotubal junction (UTJ) and cranially to the wider ampulla by the ampullary-isthmic junction (AIJ). The infundibulum is funnel-shaped and

has the opening end towards the ovary. Histologically, the oviductal wall is composed of a mucous membrane, the endosalpinx; a double-layered myosalpinx, with inner circular and outer longitudinal smooth muscle layers; and a mesosalpinx, an external serous coat (Fig. 2). The muscle layer is most prominent in the isthmus but becomes thinner in the ampulla and especially in the infundibulum.

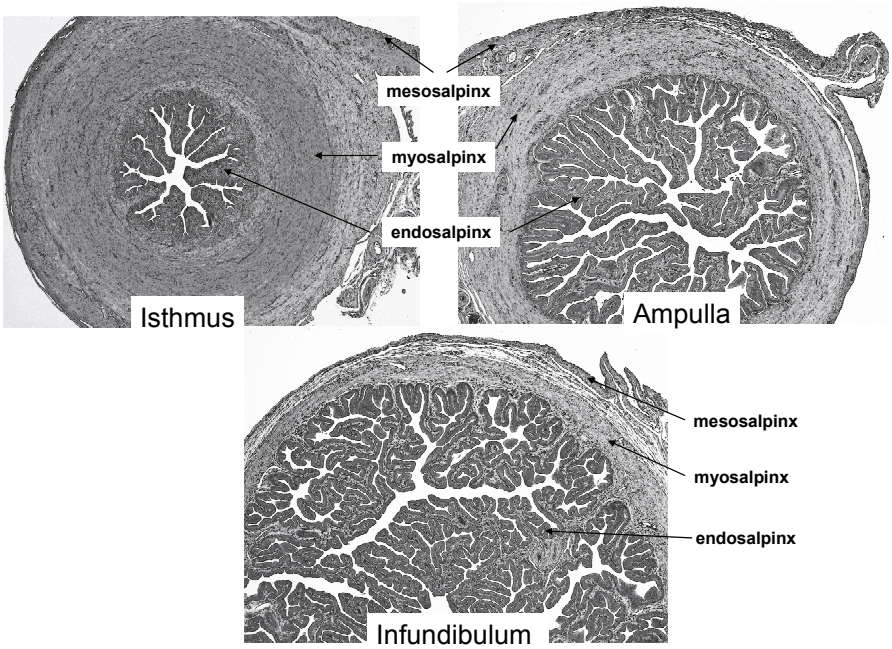

Figure 2. Cross section of different segments of the porcine oviduct at the same magnification.

Morphological aspects of the endosalpinx

The endosalpinx consists of an epithelial layer and sub-epithelial connective tissue, forming longitudinal folds along the tube (Fig. 2). The sub-epithelial connective tissue is composed of collagen and elastic fibres that are interspersed with fibroblasts and, to a lesser extent, immune cells. The epithelium includes two major cell types: secretory and ciliated cells. Both cell types reach maximum height at oestrus and metoestrus (Buhi *et al.*, 1997) but epithelial cells in different parts of the oviduct seem to respond differently to hormonal stimuli. For example, a decrease in the percentage of ciliated cells was found in infundibulum and ampulla but not in isthmus during progesterone dominance (luteal phase) compared with oestrogen dominance (follicular phase) (Buhi *et al.*, 1997; Yaniz *et al.*, 2006).

Functional aspects of the endosalpinx

The endosalpinx, especially the epithelial lining together with its secretion, forms the environment in which gamete transport, final sperm maturation (capacitation) and fertilization as well as early embryo development and transport occur (see reviews Hunter *et al.*, 1998; Rodriguez-Martinez *et al.*, 2001). The UTJ and the adjacent segment of the isthmus function as a tubal sperm reservoir (Viring *et al.*, 1980; Hunter, 1984) where spermatozoa in contact with the oviductal epithelial cells maintain intact plasma membranes (Mburu *et al.*, 1997) and viability (Hunter *et al.*, 1998; Fazeli *et al.*, 1999; Petrunkina *et al.*, 2001) during the period preceding fertilization. After ovulation, the oocytes are transported through the infundibulum and the ampulla to the AIJ by ciliary beating, in combination with an interaction (adhesion) between cumulus cells of the oocytes and extracellular matrix on the cilia (Norwood & Anderson, 1980; Talbot *et al.*, 1999). Fertilization takes place in the AIJ (Hunter, 1974) and, thereafter, the fertilized oocytes/early embryos develop and are transported along the isthmus to reach the uterine horn after about 40 h, then being in the 4-cell stage (Oxenreider & Day, 1965; Hunter, 1974).

The oviductal fluid is formed by selective transudation from the blood and a specific secretion from the epithelial cells (Leese *et al.*, 2001). The secretory activity increases during oestrus and decreases during dioestrus (Iritani *et al.*, 1974). In response to oestrogen, the oviductal epithelial cells synthesize and release proteins (Xia *et al.*, 1996). The types of glycoproteins secreted differ between the ampulla and the isthmus (Buhi *et al.*, 1990). Components in the oviductal fluid, such as bicarbonate, are important for induction of sperm capacitation (Harrison *et al.*, 1996; Tienthai *et al.*, 2004), whereas oviductal-specific glycoproteins are suggested to modulate sperm–zona pellucida interaction and to control polyspermy during fertilization (Kouba *et al.*, 2000; McCauley *et al.*, 2003; Coy *et al.*, 2008).

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 (Beer & Billingham, 1974) 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.

Studies on the immune cells in the endosalpinx have been performed in several species, e.g. humans (van Bogaert *et al.*, 1978; Kutteh *et al.*, 1988; 1990; Boehme & Donat, 1992; Suenaga *et al.*, 1998); rabbits (Otsuki *et al.*, 1989; Gu *et al.*, 2005) and small rodents (Parr & Parr, 1985; Dalton *et al.*, 1994). In the bovine oviduct (the isthmic and ampullary parts), changes in the distribution of lymphocytes (DuBois *et al.*, 1980), mast cells (DuBois *et al.*, 1980; Ozen *et al.*, 2002) and eosinophils (Matsuda *et al.*, 1983) during different stages of the oestrous cycle have been reported. In the porcine ampulla, Hussein *et al.* (1983a) observed higher numbers of immunoglobulin-containing cells at oestrus than at dioestrus. However, few studies have been made on leukocyte distribution in the porcine endosalpinx and related to different stages of the oestrous cycle. In contrast, immune cell infiltration has been widely studied in the porcine endometrium, both in gilts (King, 1988; Bischof *et al.*, 1994; Dimova *et al.*, 2007) and sows (Kaeoket *et al.*, 2001a, b; 2001c; Engelhardt *et al.*, 2002), and found to differ depending on oestrous cycle stages, i.e. to be correlated to hormonal changes (Kaeoket *et al.*, 2001c).

In the sow endometrium, high numbers of neutrophils were found after insemination (Kaeoket *et al.*, 2003c). In contrast, Rodriguez-Martinez *et al.* (1990) found that, after insemination, neutrophils were not invading the epithelium of UTJ and isthmus, i.e. the sperm reservoir, although spermatozoa were present in the lumen. Thus, spermatozoa in the sperm reservoir escape elimination by neutrophilic phagocytosis. However, the mechanisms that create this 'privileged site' for the allogeneic spermatozoa are still unclear.

The distribution of immune cells in the sow endometrium was found to be different according to whether insemination took place before or after ovulation (Kaeoket *et al.*, 2003a). Thus, the time of insemination in relation to ovulation may also influence the immune response to semen in the oviduct due to the hormonal switch from oestrogen- to progesterone-dominance after ovulation. In accordance with this theory, the presence of an immunomodulatory cytokine, TGF-β1, and the TGF-β type II receptor was observed by immunohistochemistry in both the isthmic and ampullary segments of the porcine oviduct. However, the intensity of the labelling for TGF-β1 and TGF-β type II receptor tended to vary between dioestrus and pro-oestrus (Buhi *et al.*, 1997).

The role of seminal plasma for the immune response

Boar seminal plasma (SP) is a complex mixture of secretions from the testes, epididymides and accessory glands, especially the seminal vesicles (Ekhlasi-Hundrieser *et al.*, 2002; Manaskova & Jonakova, 2008). Data from humans and rodents show that SP can modulate a variety of immunological functions (see review by Thaler, 1989; Kelly & Critchley, 1997; Robertson & Sharkey, 2001). According to *in vitro* studies, boar SP appears to contain fractions that influence the immune cell response in both suppressive (Stanek *et al.*, 1985; Veselsky *et al.*, 1991; Dostal *et al.*, 1997) and stimulatory (Leshin *et al.*, 1998; Yang *et al.*, 1998) ways. SP proteins have been shown to both stimulate (Rodriguez-Martinez *et al.*, 2005) and suppress (Rozeboom *et al.*, 1999) migration of PMNs into the uterine lumen of sows, and a dose-dependent suppressive effect of SP was observed *in vitro*, on chemotaxis of blood-derived PMNs (Rozeboom *et al.*, 2001). It has been shown in gilts that boar SP induced expression of the cytokines granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-6 in endometrial tissue (O'Leary *et al.*, 2004).

According to Einarsson *et al.* (1980), inseminated components are rapidly distributed into the entire oviduct after insemination. This indicates that substances in semen can affect the endosalpinx in all parts of the oviduct and thereby modulate the oviductal immune response, even though only a small part of the inseminated volume enters the oviduct

It has been shown in humans that SP contains various cytokines and chemokines (Gutsche *et al.*, 2003; Basu *et al.*, 2004; Penna *et al.*, 2007; von Wolff *et al.*, 2007; Ochsenkuhn *et al.*, 2008). High concentration of TGF-β1, a cytokine generally regarded as an anti-inflammatory agent, was found in boar seminal plasma (O'Leary *et al.*, 2002) and was suggested to be an important mediator of maternal immune tolerance in the uterus (Robertson *et al.*, 2002). Therefore, seminal TGF-β1 may be of importance for the tolerance for paternal alloantigens in the oviduct as well.

Cytokine of interest to study in the oviduct

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. Because TGF-β1 has been demonstrated in the porcine oviduct (Buhi *et al.*, 1997), this cytokine, together with IL-10, was chosen as indicators of immune suppression whereas IL-1 and IL-6 were selected to reflect a pro-inflammatory response.

IL-1 and IL-6 are major pro-inflammatory cytokines which promote inflammation and stimulate an acute phase response. At the cellular level, IL-1 is critical for neutrophil recruitment by eliciting expression of chemokines and adhesion molecules (Brandolini *et al.*, 1996; Fukumura *et al.*, 1996; Jobin & Gauthier, 1997; Parsey *et al.*, 1998). IL-6 contributes to the early inflammatory response but also affects the T-cell response towards a T_H type 2, and is, in combination with TGF-β, involved in the development of T_H17 cells, as recently reviewed (Dienz & Rincon, 2009). In the porcine endometrium, the expression of mRNA for IL-6 increased during pregnancy (Chabot *et al.*, 2004) and also after intra-uterine infusion with seminal plasma (O'Leary *et al.*, 2004).

IL-10 is considered to be a potent suppressor of the effector functions of macrophages, T cells and NK cells (Moore *et al.*, 2001). It acts to terminate the inflammatory response and limits inflammation-induced tissue changes by de-activating macrophages and inhibiting their synthesis of pro-inflammatory cytokines and chemokines (de Waal Malefyt *et al.*, 1991; Fiorentino *et al.*, 1991). A role of IL-10 during pregnancy was observed by Chaouat *et al.* (1996), who found that recombinant IL-10 prevented fetal absorption in mice but not after neutralization with anti-IL-10. Robertson *et al* (2006) demonstrated, also in mice, that IL-10 modulates resistance to inflammatory stimuli by down-regulating pro-inflammatory cytokines in the uterus and placenta.

The transforming growth factor β (TGF-β) is a multifunctional cytokine that influences numerous cellular processes. TGF-β is traditionally regarded as an anti-inflammatory agent (Shull *et al.*, 1992; Kulkarni *et al.*, 1993; Gorelik & Flavell, 2000) and was recently also found to be an important regulator of T-cell differentiation (Bommireddy & Doetschman, 2007; Rubtsov & Rudensky, 2007; Sundrud & Rao, 2007). At the site of implantation, TGF-β is believed to play a significant role in establishing a maternal tolerance to the semi-allogenic conceptus (see review Godkin & Dore, 1998).

General aspects on leukocyte subsets

The cells of the immune system arise from stem cells in the bone marrow through two main lines of differentiation. The lymphoid lineage produces lymphocytes, natural killer (NK) cells and a subpopulation of dendritic cells (DC). Another subpopulation of DC differentiates from the myeloid lineage that also generates monocytes, neutrophils, eosinophils, basophils and mast cells. Most of these cell populations can be distinguished by histochemical

labelling based on their morphology and granular content. For further characterization into subsets, a number of phenotypic markers are used. Most common is the use of monoclonal antibodies (mAbs) that react with cell surface proteins identified as clusters of differentiation (CD) antigens, as recently reviewed for porcine cells (Piriou-Guzylack & Salmon, 2008; Ezquerra *et al.*, 2009; Summerfield & McCullough, 2009).

Lymphocytes consist of B and T cells that, typically, are small- to medium-sized cells with densely stained nuclei surrounded by a thin rim of cytoplasm (see Fig. 4a). When activated, B lymphocytes differentiate into plasma cells (Calame, 2001) that locally produce antigen-specific antibodies in the tissue. Plasma cells appear as large cells with an eccentric round or oval nucleus (see Fig. 4c). The chromatin is clumped in a characteristic 'cartwheel' or 'clock face' pattern. By light microscopy, the cytoplasm appears purple due to its large content of ribosomal RNA and proteins which are stained by both acidophilic and basophilic dyes (amphophilic).

NK cells are morphologically large granular lymphocytes, which lack antigen-specific receptors and are, therefore, regarded as a part of the innate immune system. In the pig, NK cells are activated, for example, during placental development and are considered to be important in the early interactions between the conceptus and the maternal immune system (Croy *et al.*, 1998).

Neutrophilic granulocytes, constituting the major population of PMNs, contain highly lobulated nuclei stippled with some purplish granules in the cytoplasm (see Fig. 4d). They are triggered to leave blood vessels as a result of the endothelial cells expressing adhesive proteins that bind the neutrophils to the walls of small blood vessels. The neutrophils are then squeezed out and are attracted to the site of infection. The neutrophils are the earliest phagocytic cells to be recruited in response to inflammation, they engulf and destroy foreign substances, and then, after a short time, die.

Eosinophilic granulocytes are the second major population of PMNs. They are larger than neutrophils and are easily recognized by their large granules which are stained bright red with eosin. The granules contain major basic protein and eosinophil peroxidase, known to have profound effects on tissue and vascular remodeling, for example, when associated with endometrial physiology (Jeziorska *et al.*, 1995; Kaeoket *et al.*, 2001c, 2003c).

Mast cells are long-living cells usually found in connective tissue where they are able to proliferate. The mast cell can be recognized by its content of metachromatic granules when fixed and stained with metachromatic dyes such as toluidine blue (Fig. 4b). Histamine, the major active substance found in the mast cells, is a vasoactive amine, which promotes increased

vascularization, permeability and causes contraction of smooth muscles. A role of the mast cell as a potential regulatory link between innate and adaptive immunity has been suggested (Frossi *et al.*, 2004; Heib *et al.*, 2008).

Circulating monocytes migrate into tissue where they mature to become macrophages. Most of the macrophages are attracted to the site of inflammation after the first line defense of neutrophils. Morphological features of macrophages, i.e. an irregular surface with pleats, protrusions and indentations together with engulfed materials in their cytoplasm, correspond to their phagocytic activities. Macrophages are efficient producers of pro-inflammatory/regulatory cytokines when activated/deactivated and can also act as antigen-presenting cells.

Aims of the study

The general aims of this thesis on the porcine oviduct were to illustrate histological changes of the endosalpinx, focusing on immune cells, as well as to study effects of inseminated components on the local cytokine response.

The specific aims were to investigate:

I isthmus, ampulla and infundibulum of the sow oviduct regarding:

- morphological changes at different stages of the oestrous cycle and anoestrus as well as during early pregnancy;
- the distribution of leukocytes at different stages of the oestrous cycle and anoestrus, as well as after pre-ovulatory (including early pregnancy) and post-ovulatory insemination.

II isthmus and infludibulum of the gilt oviduct regarding:

- the expression of pro-inflammatory (IL-1β and IL-6) and suppressive (IL-10 and TGF-β1) cytokines in these segments;
- the effects of seminal plasma and spermatozoa on the cytokine responses shortly (5-6 h) and at 35-40 h after insemination, including analyses of cytokines (IL-6, IL-10 and TGF-β1) in boar seminal plasma.

Materials and Methods

The materials and methods used in the present thesis are described in detail in Papers I-IV. A more generalized description with specific comments is presented below. All procedures involving the use of animals were approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden.

Animals and the different experimental parts

All animals used in present study (Papers I-IV) attained natural oestrus and ovulation, i.e. no hormonal induction was used. The studies on morphological changes and immune cell distribution in the oviduct (Papers I and II) together included 53 sows (Fig. 3) with known normal reproductive performance and with a mean parity number 3.4 ±0.7 (Paper I) and 3.5 ±0.6 (Paper II). The sows were slaughtered at five different stages of the oestrous cycle and anoestrus (Paper I), at five different stages after pre-ovulatory insemination (AIb), or at four stages after post-ovulatory (AIa) insemination (Paper II). Studies on immune cell distribution in the endometrium had been performed earlier in these sows (Kaeoket *et al.*, 2001a,b,c; 2003a,b,c).

To evaluate effects of semen components and/or extender on immune cell distribution and cytokine production in the oviduct, gilts were slaughtered at 5-6 h (Paper III, 16 animals) or 35-40 h (Paper IV, 16 animals) after treatment (Fig. 3). Gilts were chosen instead of sows in order to exclude any potential effects of earlier exposure to semen/seminal plasma. To ensure maturity of the gilts, the studies were performed at the second oestrus at the earliest (Schnurrbusch & Erices, 1979).

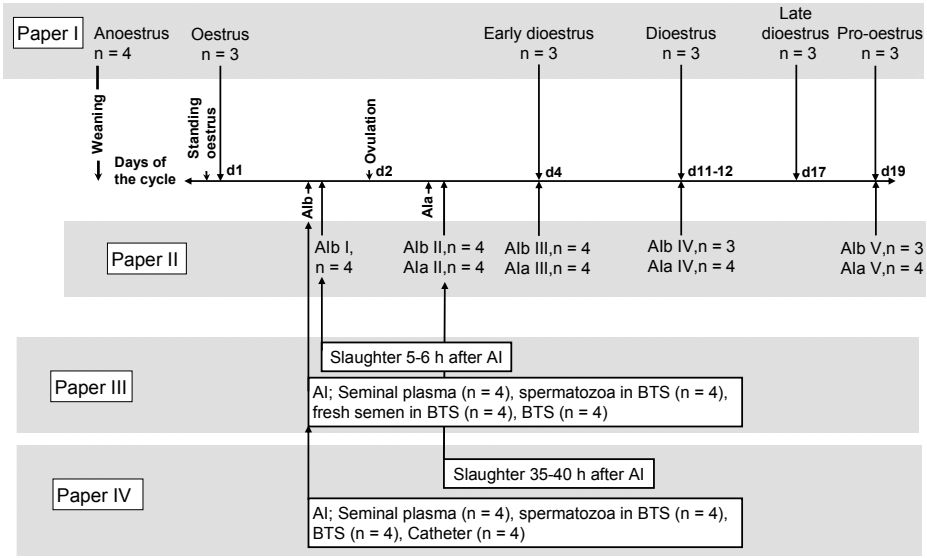

Figure 3. Distribution of experimental groups of sows (Paper I and II) and gilts (Paper III and IV) according to the time of sample collection (slaughter); AI = artificial insemination, b = 20-15 h before ovulation, a = 15-20 h after insemination, BTS = Beltsville Thawing Solution

Oestrus detection was done in the presence of a boar (control of standing reflex). In sows, ovarian follicular development and/or ovulation was checked with transrectal ultrasonography (Kaeoket *et al.*, 2001c, 2003c, a). In gilts, transcutaneous sonography was performed instead, due to their small body size and narrow rectum. Ultrasonography was done every 4 h after the start of standing oestrus in the gilts (Paper IV), and from 16 h after the start of standing oestrus (2nd oestrus after weaning) in the sows. To confirm the ovarian activity during the different stages of the oestrous cycle/early pregnancy, blood samples were collected one hour prior to slaughter for analyses of plasma oestradiol-17β (E_2) and progesterone. To confirm the presence of spermatozoa and pregnancy (fertilized oocytes/early embryos), the oviducts were flushed (Papers II and IV) and/or the uterine horns were examined (Paper II) after slaughter.

Semen preparation and artificial insemination

The sows and gilts were inseminated with a standard volume of 100 ml but with a relatively high sperm number (10×10^9 in sows and 5×10^9 in gilts) per dose as compared to conventional artificial insemination (Crabo & Dial, 1992), since the insemination was done only once. Beltsville Thawing

Solution (BTS), one of the most widely used extenders, developed by Pursel *et al.* (1973), was chosen for the fresh semen and sperm preparations. The pure spermatozoa were prepared using a colloid single layer technique provided by Dr Jane Morrell, Department of Clinical Sciences, Swedish University of Agricultural Sciences. This method has been proven to be efficient in separating motile, normal spermatozoa from the rest of the sperm sample (Morrell *et al.*, 2008). For seminal plasma preparation, i.e. centrifugation, as described in Paper III, pooled semen from four boars was used to diminish individual variation. The first 10 ml of the sperm-rich fraction of each ejaculate was removed (total volume ranged between 200-500 ml) and used in another study (Saravia *et al.*, 2008). Thereafter, the rest of each ejaculate was used for the SP preparation employed in the present study.

A standard disposable catheter (Goldenpig™, IMV, L'Aigle, France) was used for insemination. In practice, the best pregnancy result is obtained when insemination is done within 24 h before ovulation (Soede *et al.*, 1995; Nissen *et al.*, 1997). Therefore, in Paper II (sows), insemination was performed at either 20-15 h before expected ovulation (pre-ovulatory insemination, AIb; their ovulation time was estimated from the results of ultrasound examination after the 1st post-weaning oestrus) or, in Papers III and IV (gilts), at 15-20 h after the start of the standing reflex since the ovulation time could not be predicted. In paper II, one group of sows was inseminated after ovulation (post-ovulatory insemination). The time of ovulation was then determined by ultrasound examination. The interval from ovulation to insemination (15-20 h) was chosen deliberately to be outside the time for normal fertilization.

Cytokine determination in seminal plasma and oviductal fluid (Paper IV)

To determine the presence of some selected cytokines in boar seminal plasma, the content of interleukin (IL)-6, IL-10 and transforming growth factor (TGF)-β1 in pooled SP and SP from five individual boars was analyzed. In addition, the presence of these cytokines in oviductal flushing fluid was also examined (Paper IV). All analyzes were performed with commercially available ELISA kits (P6000, IL-6; P1000, IL-10; and MB10B, TGF-β1; Quantikine Porcine Immunoassays, R&D System Europe Limited, Abingdon, UK) according to the manufacturer's instruction.

Tissue collection and fixation

Three different segments from the oviduct (isthmus, ampulla and infundibulum) were included in Papers I and II. As the major differences presented in Papers I and II were between isthmus and infundibulum, these two segments were selected for the studies in Papers III and IV.

Samples were immersion-fixed in 3% glutaraldehyde for light microscopy (LM, Papers I and II) and transmission electron microscopy (TEM, Paper I) or in 2% paraformaldehyde for immunohistochemical staining (Papers III and IV). Corresponding samples, frozen by plunging into liquid nitrogen, were stored at -70 to -80 °C until analysis by immunohistochemical staining (Papers I-IV) and for quantitative analysis of mRNA (Papers III and IV). Specimens of lymph nodes (located in the mesometrium) were also collected and fixed as above. The different tissues collected and methods used in Papers I-IV are summarized in Table 1.

Table 1. *Methods used for analysis of various immune parameters, Papers I-IV*

Paper	Groups	Sample	Fixation	Method	Detection
I	All groups	Isth, Amp, Inf, Lfn	Glutaraldehyde	LM	Leukocytes
	Pro-oestrus and anoestrus	Isth, Amp, Inf, Lfn	Frozen	IHC	Cell markers
	Dioestrus	Inf	Glutaraldehyde	TEM	Epithelial layer
II	AIb and AIa	Isth, Amp, Inf, Lfn	Glutaraldehyde	LM	Leukocytes
	AIb I-IV	Isth, Amp, Inf, Lfn	Frozen	IHC	Cell markers
	AIb V	Inf	Frozen	IHC	Cell markers
III & IV	All groups	Isth, Inf, Lfn	Paraformaldehyde	IHC	Cytokines
			Frozen	qRT-PCR	Cytokine mRNA
			Frozen	IHC	Cell markers
IV	All groups	Oviductal flushing fluid	Frozen	ELISA	Cytokines
	Boars	Pooled SP & SP from individual boars			

LM = light microscopy, TEM = transmissible electron microscopy, IHC = immunohistochemistry, Isth = isthmus, Amp = ampulla, Inf = infundibulum, Lfn=lymph node and SP = seminal plasma.

Preparation of oviductal samples for morphological examination (Papers I and II)

Semi-thin sections (3-4 μm on glass slides), from plastic-embedded tissue samples (immersion-fixed in 3% glutaraldehyde and embedded in water-soluble methylmetacrylate, Historesin®, LKB, Bromma, Sweden), were stained with buffered toluidine blue and used for evaluation of general morphology and presence of leukocytes in the oviductal tissue (Papers I and II). This method gives a better resolution by LM compared to the use of paraffin sections stained with Hematoxylin and Eosin. In addition, some cells, e.g. mast cells, can be recognized by their content of metachromatic granules.

To further clarify the morphology of protruding epithelial cells and the unidentified intra-epithelial leukocytes (Paper I), transmission electron microscopy was used.

Immunohistochemistry (IHC)

Immunohistochemistry on cryo-fixed samples was used to characterize leukocyte subpopulations according to their expression of cell markers [CD2, CD3, CD79, CD14, SWC3 (only in Paper I) and MHC class II] in three segments of the oviduct (isthmus, ampulla and infundibulum, Papers I and II). In Papers III and IV, CD8- and CD25-positive cells were detected in two segments of the oviduct (isthmus and infundibulum). In order to phenotype lymphoid cells, the presence/absence of more than one type of membrane molecule (CD marker) is often needed. In addition, the level of expression may vary between subpopulations of cells or due to their activation status, and this fluctuation is commonly used for further characterization of the cells as 'dim' or 'bright', preferably by flow cytometry (see review by Gerner *et al.*, 2009). When using IHC on frozen sections, cells are lost at each washing and unless a cocktail of antibodies can be applied simultaneously, such unspecific cellular losses might confound the results. Therefore, only single labelling was performed in the present study.

Sections from a regional (mesometrial) lymph node were included as a positive control for the CD markers used. However, due to technical reasons, samples from only seven sows in Paper I (four anoestrus and three pro-oestrus) and samples from only groups of AIb I-IV and infundibular part of group AIb V in Paper II, could be run for IHC analysis.

For detection and localization of porcine cytokines (Papers III and IV), paraffin sections from isthmus, infundibulum and lymph node were subjected to IHC. Available antibodies were tested on cryo-fixed sections

but there were no antibodies that reacted specifically to any of the cytokines IL-1β, IL-6, IL-10 or TGF-β1. Therefore, sections from chemical-fixed (2% paraformadehyde), paraffin-embedded tissue samples were tested. An antigen-retrieval method was needed to unmask epitopes which normally are masked during the paraffin embedding process (Cattoretti *et al.*, 1993). Heat-induced antigen retrieval by the microwave technique was used in the present studies. After optimizing the method for the different cytokines, the method worked for IL-6, IL-10 and TGF-β1 but not for IL-1β. Alternatively, the levels of IL-1β were below the detection limit.

For visualization, a standard avidin-biotin immunoperoxidase technique (Vectastain® ABC kits, Vector Laboratories, Inc., Burlingame, CA, USA) was performed. The sections were incubated overnight at 4 °C with the primary antibodies (see Table 1 in Paper I and Table 1 in Paper IV) or with isotype-matched negative controls. In the final step, 3, 3'-diaminobenzidine (DAB, Dakopatts AB, Älvsjö, Sweden) was added as chromogen. All sections were counterstained with Mayer's haematoxylin followed by mounting in glycerine-gelatin.

For reliable semi-quantitative comparison on cytokine immunolabelling, factors that may affect immunolabelling were standardized as follows: IHC analyses were always performed within 1 week after sectioning; washing with PBS was always performed in the same manner (4 times, 5 min each); and the time for counterstaining with Mayer's haematoxylin was kept constant. In addition, sections from all treatments were performed in the same IHC run for the respective antibodies and one reference section was always included in each IHC run as an inter-assay control.

Evaluation of morphology and leukocyte distribution

In Papers I and II, toluidine blue-stained sections of the three oviductal segments were examined for morphology and leukocyte distribution (lymphocytes, plasma cells, neutrophils, eosinophils, macrophages and mast cells, see Fig. 4). Leukocyte subpopulations were also examined by IHC (Papers I-IV; see above). The morphological changes occurring in the endosalpinx were evaluated by a scoring system as described in Paper I. Quantitative evaluation of the different immune cells and some morphological parameters, i.e. vessels and mitotic figures, was performed by manual counting. All slides were coded before examination and the counts (on morphological parameters and cells) were performed under LM with 400× magnification using an ocular reticule divided into 100 small squares placed in one eyepiece of the light microscope (Fig. 4).

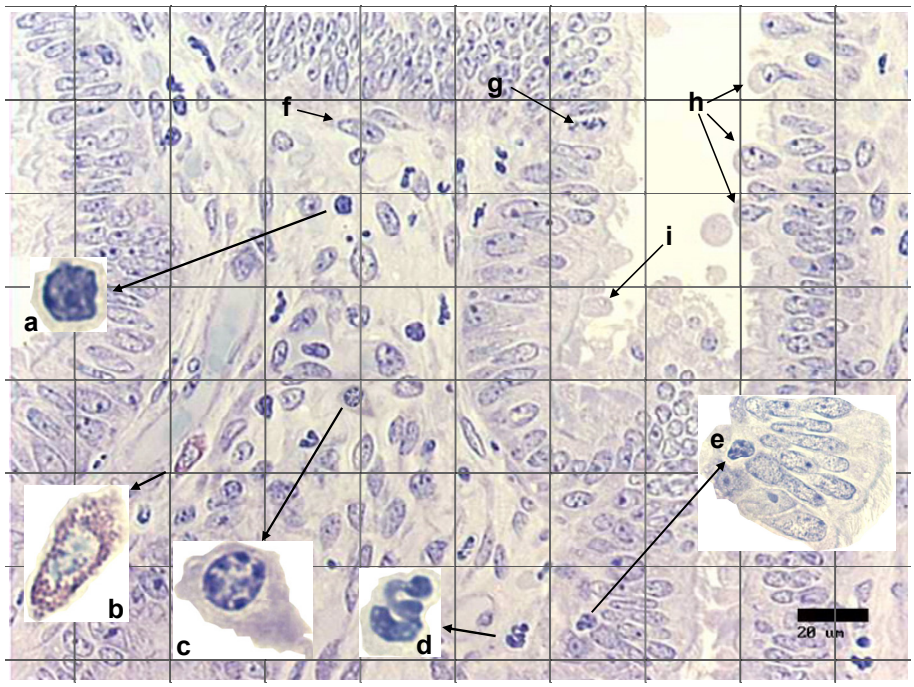

Figure 4. Morphology of the porcine oviductal mucosa (infundibulum at pro-oestrus, toluidine blue stained section); a = lymphocyte, b = mast cell, c = plasma cell, d = neutrophil, e = intra-epithelial lymphocyte, f = fibroblast, g = mitotic figure, h = nucleated protruding cells and i = cytoplasmic protrusion.

For cell counting, each section was divided into two compartments – epithelium and sub-epithelial connective tissue. Counting was carried out over as long a length of the epithelial layer and in as large an area of the sub-epithelial connective tissue as possible in each section (Papers I-IV). For quantitative comparison, the data were adjusted into 100 small squares, i.e. of positive cells per 2.5 mm for the epithelial length and per 6.25×10^{-2} mm^2 for the sub-epithelial connective tissue area. An advantage with this cell counting method is that it provides information not only about the numbers of different immune cells but also about their distribution in the different tissue layers. However, the manual counting is very time-consuming, which is a disadvantage. An automated method to evaluate IHC-labelling is by computerized image analysis, as has been used, for example, for quantification of pro-inflammatory cytokines in biopsies from the porcine mammary gland (Zhu *et al.*, 2007). However, to facilitate the comparison with the differential leukocyte counts performed, the IHC-labelling of leukocyte subpopulations was also enumerated manually (Papers I-IV).

Different degrees of oedema at various reproductive stages may have affected the cell density in the sub-epithelial connective tissue. The level of oedema was, however, indirectly estimated by the scoring of fibroblast density performed in the present study.

A particular cell type, referred to as intra-epithelial mononuclear cells, with abundant cytoplasm and spherical euchromatic nuclei was found in the toluidine blue-stained sections but could not be further identified. These cells were, therefore, not counted but studied in detail by transmission electron microscopy (Paper I).

Since a high number of cells were positively stained for MHC class II in the connective tissue of all segments, MHC class II-positive leukocytes could not be clearly distinguished from positive endothelial cells. Therefore, the number of MHC class II-positive cells was not counted but their presence was described.

Evaluation of cytokines (Papers III and IV)

With IHC, localization of proteins in different tissue compartments can be visualized, although a proper quantification may be difficult to make even when image analysis is applied (Taylor & Levenson, 2006). In papers III and IV, the presence of cytokines was evaluated semi-quantitatively using a scoring system (see Table 1 in Paper III). By this scoring system, the average results of both the intensity of labelling and proportion of positive cells in each compartment (surface epithelium and sub-epithelial connective tissue of isthmus and infundibulum) were estimated.

Quantitative RT-PCR (Papers III and IV)

Real-time reverse transcription (RT) followed by polymerase chain reaction (PCR) is the best technique available for analyzes of mRNA expression for various applications. It is an extremely sensitive and accurate method which allows quantification of very low, and very high, levels of transcripts (see review Giulietti *et al.*, 2001). One can also compare small differences and changes in mRNA levels between samples. In addition, this method is easy to use, producing reliable and rapid results. Therefore, in the present study, real-time RT-PCR was performed both to analyze an endogenous cytokine production after different treatments and to be able to quantify it (Papers III and IV).

Quantitative one-step real-time RT-PCR with a fluorescence-labelled probe was carried out to evaluate IL-1β, IL-6, IL-10, and TGF-β1, for

reasons mentioned elsewhere, as well as the housekeeping genes cyclophilin and hypoxanthine phosphoribosyl-transferase (HPRT) mRNA expression. Reverse transcription of total RNA and PCR amplification was performed using the GenAmp r*Tth* DNA polymerase (Applied Biosystems, CA, USA), *Taq*Man fluorescent probes, and gene-specific primers (DNA Technology, A/S, Aarhus C, Denmark). To allow a comparison between tissues (isthmus, infundibulum and lymph node) in both paper III and IV, one randomly selected sample was included in every run as a reference sample. The amplification specificity was checked by gel electrophoresis of randomly selected samples from each assay. The bands obtained were always found to be of the predicted molecular weight indicating specific amplification.

For real-time RT-PCR, there are two ways to quantify the amounts of mRNA, a relative quantification based on the relative expression of a target gene versus a reference gene, or an absolute quantification, based either on an internal or an external calibration curve (Morrison *et al.*, 1998; Tichopad *et al.*, 2003). To investigate physiological changes in the gene expression, the relative expression ratio is adequate in most cases (Pfaffl, 2001). For relative quantification (Serazin-Leroy *et al.*, 1998), the common standardization is to use a gene involved in the basic function and constitutively expressed in the cells, the so-called housekeeping genes (their expression is believed to be constant even under experimental treatments) (Thellin *et al.*, 1999; Suzuki *et al.*, 2000). However, numerous studies have shown that various housekeeping genes can, in fact, vary under experimental conditions (Schmittgen & Zakrajsek, 2000; Janovick-Guretzky *et al.*, 2007; Tanic *et al.*, 2007). Vandesompele *et al.* (2002) demonstrated that the conventional use of a single housekeeping gene for normalization may lead to relatively large errors in a significant proportion of samples tested. Therefore, in the present study, the cytokine mRNAs were determined in relation to geometric mean of two housekeeping genes: HPRT and cyclophilin (Papers III and IV).

For RNA isolation, a mixture of cells representing the mean of all cell types from the endosalpinx (epithelium and sub-epithelial connective tissue), as well as from the myosalpinx, was used. The ration between epithelial cells, smooth muscle cells and cells in the connective tissue is not the same for the isthmus and the infundibulum which could confound the result of qRT-PCR analyses. For instance, the isthmus has a thicker myosalpinx than the ampulla and infundibulum. In addition, immunolabelling also demonstrated the presence of the three cytokines analyzed in the cells of myosalpinx. Therefore additional methods such as *in situ* hybridization are needed for a closer tissue-related illustration of cytokine expression.

Statistical analyses

The statistical analyses were carried out using the SAS statistical package (SAS Institute, Inc., Carry, NC, USA). Normal distribution of residuals from the statistical models was tested using the UNIVARIATE procedure option NORMAL. Differences in mean numbers of immune cells, morphological variables or cytokine ratio were tested using analysis of variance (Proc MIXED). The statistical model included the fixed effects of group and segments, and the interaction between groups and segments, as well as the random effect of gilt/sow nested within group. The Bonferroni *t*-test was used to compare least-square means between groups when overall significance for the effect was found. The variables (Paper I and II) in which the distribution was not normal were analyzed using NPAR1WAY procedure (Wilcoxon's rank-sum test) with the effect of group and segment included monofactorially. A *P*-value ≤ 0.05 was considered statistically significant.

General Results and Discussion

The studies included in the present thesis were conducted to elucidate histological variations of the porcine endosalpinx, with focus on immune cells and the effects of inseminated components on the local cytokine response. The first part (Papers I and II) was performed to gain more knowledge about the distribution of immune cells (as identified by light microscopy and immunohistochemistry) in three segments (isthmus, ampulla and infundibulum) of the porcine oviduct. The studies were conducted during different stages of the oestrous cycle (including anoestrus) as well as after pre- and post-ovulatory insemination and during early pregnancy. Furthermore, changes in the tissue morphology of endosalpinx were studied. The second part (Papers III and IV) aimed to understand more about the immune regulation in the oviduct. The expression of pro- and anti-inflammatory cytokines was studied by IHC and quantitative real time RT-PCR in two segments (isthmus and infundibulum) after insemination of gilts with various components commonly used at artificial insemination (AI).

Studies on sows (Papers I and II)

Morphological findings in the sow oviduct (Papers I and II)

The morphology of the epithelial cells in the endosalpinx changed depending on the stage of the oestrous cycle (Paper I, Fig. 1). The changes (pseudostratification, mitotic activity, secretory granules and cytoplasmatic protrusions) were especially observed in the ampulla and the infundibulum, i.e. in the upper part of the oviduct. Hormonal levels, but not insemination and pregnancy as such (Paper II), seemed to induce the morphological changes. From oestrus and up to Day 11, the hormones (oestradiol-17β and

progesterone) were at similar levels in non-pregnant (Paper I) and pregnant (Paper II) sows.

Pseudostratification of the epithelial cell layer in the ampulla and infundibulum was at a high level at pro-oestrus and oestrus i.e. the stages when the plasma levels of oestradiol-17β were high (Paper I). This is in agreement with a study by Nayak & Zimmerman (1971a) who found that oestradiol treatment increased the height of the epithelium in the ampulla of ovariectomized gilts. Ciliated cells comprise the major part of all oviductal epithelial cells (Buhi *et al.*, 1997), and their pseudostratification may reflect proliferation and a preparation of the epithelium for oocyte transport (Odor & Blandau, 1973) and sperm transport (Hunter *et al.*, 1991).

Most secretory granules were found at oestrus, especially in the ampulla (Paper I, Fig. 1), in accordance with the findings of Nayak & Zimmerman (1971b) who observed secretory granules to be more abundant during oestrus in the ampullae and fimbriae of gilts. In all segments of the oviduct, a marked presence of epithelial cells undergoing mitosis was observed at pro-oestrus (Paper I). This mitosis, in combination with pseudostratification and secretory granules at oestrus, indicate a higher functional activity which, at this stage, is needed for the fertilization process in the oviduct. For instance, the granules of oviductal secretory cells contain glycoproteins which are released into the lumen (Buhi *et al.*, 1993) and it has been suggested that porcine oviductal specific glycoproteins modulate sperm-zona pellucida interaction and control polyspermy during fertilization (Kouba *et al.*, 2000; McCauley *et al.*, 2003; Coy *et al.*, 2008).

Nayak & Zimmerman (1971a) observed that progesterone treatment of ovariectomized gilts resulted in prominent cytoplasmic protrusions in the ampullar epithelium. Abe & Oikawa (1992) also found protrusions, i.e. bulbous processes of secretory, epithelial cells (ampulla and infudibulum) when progesterone was high (i.e. in the luteal phase). In contrast, Palmer *et al.* (1965) found epithelial protrusions concomitant with a low progesterone level (lactational anoestrus). In the present study, protrusions of ampullar and infundibular epithelial cells were found in non-pregnant sows (Paper I) at various concentrations of progesterone, from high levels (at dioestrus), to low levels (at pro-oestrus, oestrus and anoestrus) and also in pregnant sows (Paper II). According to the scoring, the least prominent amount of protrusions in cyclic sows was found at oestrus (when proliferation is dominating) and early dioestrus. Electron microscopy showed that the protrusions contained cytoplasm but also some nuclei (Paper I, Fig. 3). The appearance of cytoplasmic protrusions has been explained in different ways. Mburu et al. (1996), studying the isthmus of sows, suggested it to be an

artifact as a result of luminal flushing prior to fixation. Abe & Hoshi (2007) suggested that the drastic reduction in the height of ciliated cells during progesterone caused the extrusion of most secretory cells beyond the ciliated cells. In other species, shedding of extruded cells into the oviductal lumen has been suggested to be a process of cell death (Odor *et al.*, 1980; Sawyer *et al.*, 1984; Murray, 1995). The apical protrusions shown in the present study (Paper I and II), are suggested to be a part of the process during which dying epithelial cells are eliminated in the oviduct, since only a very low number of macrophages (that normally phagocytose dying cells) were found in the oviductal epithelium.

Distribution of immune cells in the sow oviduct (Papers I and II)

The most common leukocyte type in the *epithelial layer* of all three oviductal segments, and in all sows investigated, was the lymphocyte (Paper I, Fig. 4 and Paper II, Fig. 2), which is in agreement with observations in humans (van Bogaert *et al.*, 1978; Morris *et al.*, 1986). Only occasional intra-epithelial neutrophils and very low numbers of macrophages were found. In the *sub-epithelial connective tissue* (Paper I, Fig. 5 and Paper II, Fig. 6 and 7), lymphocytes and plasma cells were the most common immune cells. Lymphocytes were found in numbers at least double that of plasma cells, which has been described for the human oviduct as well (Kutteh *et al.*, 1990). The numbers of lymphocytes and plasma cells were significantly higher in the infundibulum than in the isthmus and this difference has not been shown previously, either for the pig or for other species. The numbers of mast cells, eosinophils and macrophages in connective tissue were very low and did not vary between segments or stages/groups in the different studies (oestrous cycle stages, groups after pre-ovulatory insemination or groups after post-ovulatory inseminations). In summary, the most important findings were the clear differences between isthmus and infundibulum, and also that there were very few phagocytic cells in the oviduct, with those found being mainly in the infundibulum.

Subpopulations of cells identified by cell markers

The leukocyte subpopulations were further identified by IHC, although not in all experimental groups due to technical reasons, and only with a limited number of membrane markers. In general, the results showed that CD2-positive cells (e.g. T, B or NK cells) were the most common lymphocyte subpopulation in both epithelial and connective tissue layers (Papers I and II). T cells, (CD3-positive cells), probably made up the major part of the

CD2-positive cells in both epithelium and connective tissue. In order to fully elucidate that point, double labelling would have been necessary.

B cells, identified as CD79-positive cells, were few and were observed only in the sub-epithelial connective tissue layer. These results agree with those of Boehme & Donat (1992) who also found that the predominant cell type in human oviductal mucosa was the CD3-positive cell, and that few B cells could be detected.

In all groups and segments studied with IHC (Papers I and II), high numbers of cells in the connective tissue were positively stained for MHC class II, particularly endothelial cells, indicating an antigen-presenting environment that is capable of providing strong stimuli to local T cells. Endothelial cells also express MHC class II in other types of porcine mucosal connective tissue such as endometrial (Kaeoket *et al.*, 2001b; 2003b) and intestinal (Wilson *et al.*, 1996). This is in contrast to the epithelium of the oviduct (Papers I and II), where few cells were MHC class II-positive, a result that also corresponds to the low numbers of macrophages found in the oviductal epithelium. In the porcine endometrium, where macrophages were found in the surface epithelium (especially around oestrus), a relative high number of cells were positively stained for MHC class II (Kaeoket *et al.*, 2001b; 2003b). However, there seem to be species differences since the human oviductal epithelium has been shown to express MHC class II (Bulmer & Earl, 1987; Edelstam *et al.*, 1992; Imarai *et al.*, 1998).

Effect of oestrous cycle stage (Paper I)

Taking all oviductal segments together (isthmus, ampulla and infundibulum), no differences between oestrous cycle stages were found for the numbers of intra-epithelial lymphocytes, IELs, or the main cell types (lymphocytes and plasma cells) in the connective tissue. The numbers of CD2- and CD3-positive cells did not differ between pro-oestrus and anoestrus in either the epithelium or connective tissue. The results show that there is no apparent influence of hormonal changes on the presence of lymphocytes and plasma cells in the oviduct of cyclic sows. This is in contrast to a previous study on the endometrium from the same cyclic sows (Kaeoket *et al.*, 2001c), where the infiltration in the endometrial connective tissue of lymphocytes and plasma cells (low numbers), as well as T lymphocyte subpopulations, varied significantly during different stages of the oestrous cycle.

Neutrophils were found in the oviductal connective tissue mainly at pro-oestrus (Paper I, Fig. 5), when the number was significantly higher in the infundibulum than in the ampulla, and the isthmus where they were seen only occasionally. This result was supported by the IHC labelling for

SWC3, e.g. present on porcine granulocytes, monocytes and plasmacytoid dendritic cells (Ezquerra *et al.*, 2009), at the same stage and in the infundibular connective tissue. The presence of neutrophils further emphasizes the differences between the isthmus and the infundibulum, and indicates that oestradiol influences innate immune reactions in the upper part of the oviduct, which is in agreement with the results from the endometrium of the same sows (Kaeoket *et al.*, 2001c). The very low numbers of neutrophils in the isthmus thus possibly illustrates a local modulation of the immune reactivity that may be necessary to allow certain reproductive events to occur.

Effects of pre-ovulatory insemination/early pregnancy (Paper II)

After pre-ovulatory insemination, neutrophils were found almost exclusively in the infundibular connective tissue, and a significantly higher number was observed approximately 40 h after insemination (group AIb II) compared to the other groups (only occasional neutrophils). This result indicates that insemination activates the first line of defense mechanism in the upper part of the oviduct but not in the isthmus. Since the infundibulum has its opening end towards the abdominal cavity, oviductal response to pathogens after, for example, mating/insemination, may be important for the protection of the abdomen and also, *vice versa,* i.e. for the protection of the oviduct from abdominal contents. In addition, considering the time in relation to insemination, the response in the oviduct occurred later than in the endometrium (of the same sows) where the highest number of neutrophils (both epithelium and sub-epithelial connective tissue) was found 5-6 h after insemination (Kaeoket *et al.*, 2003c). Moreover, the phagocyte response in the endometrium was more active than in the infundibulum as shown by the presence of neutrophils in the endometrial epithelium and uterine lumen. In the infundibular epithelium only occasional neutrophils were observed. The utero-tubal junction represents a barrier that considerably limits the amount of semen entering the oviduct (see review Rath *et al.*, 2008). Therefore, one reason for the different results seen for neutrophils may be the higher stimulation by fluid volume and/or semen components on the endometrium compared with the infundibular endosalpinx.

In the infundibular *epithelium*, a lymphocyte infiltration due to pregnancy was observed. The mean numbers of IELs in infundibulum after pre-ovulatory insemination/at early pregnancy (Paper II, Fig. 2) were higher than the mean number of IELs in the infundibulum of the cyclic sows (Paper I, Fig. 4). In addition, the infundibular number of IELs was

significantly higher in pregnant sows at Day 19 (group AIb V) than in most of the earlier groups after insemination. The numbers of epithelial lymphocytes positive for CD2 or CD3 were significantly higher in the infundibulum than in the ampulla and isthmus (Paper II, Fig. 4). One effect of insemination/early pregnancy therefore seems to be a recruitment of lymphocytes to the infundibular epithelium.

In the *sub-epithelial connective tissue*, the morphological examination did not reveal any effect of early pregnancy on the number of lymphocytes, but IHC labelling showed that the numbers of CD2- and CD3-positive cells in all three segments were fewer soon after insemination (group AIb I) than later (groups AIb II-IV, Paper II, Fig. 4). However, this difference was only significant for CD2-positive cells. The higher numbers of CD2- than CD3-positive cells in all segments and in both tissue compartments indicate the presence of NK cells. A study on lymphocyte subpopulations in porcine blood and lymphoid tissue has shown that a large proportion of non-T and non-B lymphocytes with $CD2^{+}3^{-}4^{-}8^{lo}$ phenotype have NK cell activity (Yang & Parkhouse, 1996). In man and mouse, NK cells have been classified as $CD16^{+}CD56^{dim}$ and $CD16^{-}CD56^{bright}$ cells (Moffett-King, 2002). The $CD16^{-}CD56^{bright}$ NK cells, referred to as uterine NK (uNK) cells, have a weak lytic activity and constitute the main lymphocyte population in the human and murine endometrium (Moffett-King, 2002; Dosiou & Giudice, 2005). The function of uNK cells is not clearly understood but they are suggested to be important at pregnancy (Yu *et al.*, 1993; Engelhardt *et al.*, 2002), at least partly due to their IFN-gamma production (Ashkar & Croy, 2001). Alternatively, their major function is to act in the local maternal immune response to infections (Le Bouteiller & Piccinni, 2008). In sows, Dimova *et al.* (2008) recently showed that the number of endometrial $CD16^{+}$ NK cells significantly declined at the attachment phase of placentation and remained relatively low during the course of placentation. In addition, the number of $CD56^{+}$ lymphocytes was highest at the pre-attachment phase of placentation and dropped at the time of attachment. In contrast to the uterus, the presence of NK cell (lytic or weak lytic) subpopulations in the oviduct has, to my knowledge, never been investigated, either in pigs or in other species, and these cells are therefore obvious candidates for further studies on immune regulation in various segments of the porcine oviduct.

Effects of post-ovulatory insemination

Compared with pre-ovulatory insemination, post-ovulatory insemination (Paper II) resulted in a different distribution of neutrophils in the connective

tissue of the endosalpinx. Neutrophils were found, especially in the infundibulum, in three out of four groups (Ala II, III and V). The presence of neutrophils in the infundibular connective tissue was observed at around 5 h after insemination (group AIa II) compared to at about 40 h after the pre-ovulatory inseminaton (group AIb II). The interval from ovulation to collection of the oviductal tissue (i.e. slaughter time) was the same for both groups AIb II and AIa II (about 15-20 h). One factor involved may be the different hormonal levels at insemination, i.e. before and after ovulation, that indirectly affected the passage of semen. After ovulation, when the progesterone level starts to increase, the UTJ may not limit the amount of semen that enters the oviduct to the same extent as during the pre-ovulatory period. However, the numbers of neutrophils found after the post-ovulatory insemination was lower than after the pre-ovulatory insemination. In addition, neutrophils were found over a more extended period, i.e. also at about 50 h after insemination (group AIa III), compared with the pre-ovulatory inseminated sows (group AIb III). The results indicate that post-ovulatory insemination influences the pattern of neutrophil presence in the oviduct which may affect the local defense mechanism.

Antibody-producing plasma cells in the oviduct

IgA producing plasma cells have been demonstrated in the sow oviduct previously (Hussein *et al.*, 1983a; 1983b). In the present study, the number of plasma cells in the oviduct was approximately five times higher than the amount found in the endometrial connective tissue of the same animals (Kaeoket *et al.*, 2001c, 2003c). These observations suggest that, under physiological conditions, the antibody-producing plasma cells are more important for the oviduct (especially in the upper part) than for the uterus. Neither pre- or postovulatory insemination nor early pregnancy influenced the plasma cell distribution in the oviduct (Paper II). This indicates that spermatozoa and SP in the oviduct did not stimulate antibody-producing cells during the period studied (up to Day 19 of early pregnancy). It is thereby suggested that they have no specific role for reproductive events but, instead, are important for the defense against pathogens reaching the oviduct, as shown by Kutteh et al. (1990). The latter, studying normal and infected human oviductal tissues collected during surgery, found that the numbers of plasma cells were six- to ten-fold higher in infected oviducts than in non-infected ones.

Studies on gilts (Papers III and IV)

The studies in the sow oviduct showed a clear difference in immune cell pattern between the isthmus and infundibulum. Therefore, these segments were selected for further studies on cytokine expression. Gilts were chosen instead of sows for these studies in order to avoid any influence from earlier exposure to semen and/or pregnancy that could affect the results. The time around oestrus (5-6 h and 35-40 h after insemination) was chosen because this is the period when the oviduct has a high activity and fertilization takes place.

Cytokine expression in the gilt oviduct

In the oviductal segments isthmus and infundibulum of gilts, IHC revealed presence of the cytokines IL-6, IL-10 and TGF-β1, primarily in tubal epithelial cells (Paper III, Fig. 2 and Paper IV, Fig. 2). Cytokine mRNA expression was found in both segments, with the level for TGF-β1 being higher than for IL-1β, IL-6 and IL-10, both shortly and later after insemination (Paper III and IV).

It has been reported that human oviductal epithelial cells can produce several cytokines (Fahey *et al.*, 2005), and IL-6, IL-10 and TGF-β have been found previously at high levels in the oviductal fluid of women (Srivastava et al., 1996). In the present studies of the gilt oviduct (Papers III and IV), endogenous production of the cytokines was indicated by the expression of their mRNAs and was further supported by the flushing samples being positive for IL-10, TGF-β1 and Il-6 in the control gilts, i.e. those not inseminated (catheter group, Paper IV).

Effects of different inseminated components

No difference between treatments, i.e. insemination with semen components compared with BTS alone (control), was found for any of the cytokines studied shortly after insemination (Paper III). However, at 35-40 h after insemination (Paper IV), the mRNA TGF-β1 level was lower when only a catheter was inserted (control) than in the inseminated groups (with fluid infusion) but at the same level as in all groups included in Paper III, i.e. 5-6 h after insemination. Taken together, these results show that insemination with SP, with spermatozoa in BTS or with BTS alone, appears to up-regulate the TGF-β1 mRNA expression at 35-40 h after insemination compared with the control. The fact that a similar up-regulation was caused by BTS as by semen components indicates an effect of inseminated fluid irrespective of any of the contents tested in the present studies.

In accordance with these results, Taylor et al. (2008) found that not only SP but also a commercial semen extender (Androhep™) induced TGF-β mRNA expression in porcine endometrial cells at 3 h after insemination. This lack of difference between treatments could be due to several reasons and from the results obtained in Paper IV it can not be excluded that the introduction of fluid itself, i.e. intra-luminal pressure, up-regulated the TGF-β1 expression. Furthermore, it cannot be ruled out that up-regulation of TGF-β1 in the oviduct could be indirect via signaling processes started in the uterus. For example, Kusharski et al (2008) showed that exogenous cytokines administered into the uterus could be absorbed and found in the oviductal tissue.

There were no consistent differences in IHC-labelling of the cytokines in relation to different treatments (Papers III and IV), except for IL-6 being slightly higher in the infundibular epithelium of the SP group, and IL-10 in infundibular connective tissue being higher in the SP and sperm groups at 35-40 h after insemination (Paper IV). More apparent effects by SP could have been expected since the fluid has been reported to exert both suppressive (Stanek *et al.*, 1985; Veselsky *et al.*, 1991; Rozeboom *et al.*, 1999; 2001) and stimulatory (Leshin *et al.*, 1998; Yang *et al.*, 1998; Rodriguez-Martinez *et al.*, 2005) effects on immune cell reactivity. Consequently, the presence of immunoregulatory cytokines was examined in samples of boar semen, using commercially available ELISAs for porcine IL-6, IL-10 and TGF-β1 (Paper IV).

Seminal plasma cytokines

The level of TGF-β1 in the pooled SP used for insemination of the gilts was surprisingly low (18 pg/ml) although a high variation was observed in ejaculates from the individual boars. A plausible explanation for the low TGF-β1 concentration in the pooled SP used in these experiments may be that the first 10 ml of sperm-rich fraction was excluded for use in another study on sperm viability at cryo-preservation (Saravia *et al.*, 2008). In boars, the content of SP proteins in the first fractions of an ejaculate (mainly from the epididymides) is higher compared to later fractions (mainly from seminal vesicles) (Lavon & Boursnell, 1975). A possible impact of semen fraction on the concentration of TGF-β1 therefore deserves further clarification.

In contrast to TGF-β1 and IL-10 that were found in all samples, IL-6 was detected in SP from only two out of the five boars and in only two out of three samples (at low levels) in each of these two boars. IL-6 and IL-10 concentrations in boar SP have not been reported before, but a similar low level of IL-6 as found in the present study (Paper IV) has been found in

human seminal plasma (Gutsche *et al.*, 2003; Basu *et al.*, 2004; von Wolff *et al.*, 2007). Relatively high concentrations of IL-10 were found in the present study (both individual and pooled SP, Paper IV) compared to the level reported for human SP (Gutsche *et al.*, 2003; Basu *et al.*, 2004). Due to the high within boar variation of IL-10 and TGF-β1, no significant differences between boars were found. A high concentration of TGF-β1 has previously been found in boar SP (O'Leary *et al.*, 2002) and TGF-β1 has been suggested to be an important mediator of maternal immune tolerance, both in the pig uterus (see review Robertson *et al.*, 2002) and at the human feto-maternal interface during normal pregnancy (see reviews Rutella *et al.*, 2006; Blois *et al.*, 2007). The level of TGF-β1 found by O'Leary *et al.* (2002) in boar SP was, however, much higher (about 100 times) compared with the present result. The divergent results might be due to methodological differences in the analysis or in the sampling techniques. Thus, it could be valuable to expand the studies on factors influencing the presence of cytokines in boar seminal plasma, as well as to study their role for oviductal immune regulation.

Studies on sows (Papers I and II) and gilts (Papers III and IV)

Immune cell pattern and cytokine expression in different segments of the oviduct

Several findings in the present thesis show apparent differences between the segments isthmus and infundibulum of the porcine oviduct, illustrating that the two opposing ends of the oviduct have different roles in the reproductive process (i.e. the infundibulum receives and transports the oocytes, while the isthmus has to support the presence of both spermatozoa and early embryos). Consequently, also the immune regulation may vary along the oviduct.

In the sub-epithelial connective tissue of non-pregnant cyclic sows (Paper I) as well as in sows after pre- or post-ovulatory insemination (Paper II), the numbers of lymphocytes (including CD2 and CD3 subpopulations) and plasma cells differed significantly between oviductal segments. The lowest numbers of lymphocytes and plasma cells, as well as very low number of neutrophils, were found in the isthmus.

In the studies on cytokine responses after insemination in gilts (Paper III and IV), the mRNA expression of IL-6, IL-10 and TGF-β1 (tendency) was higher in the isthmus than in the infundibulum, which indicates different regulatory roles between segments. In addition, higher levels of the

immunosuppressive cytokines (IL-10 and TGF-β1) were found compared to the pro-inflammatory ones (IL-1β and IL-6). Taken together, these results indicate a more suppressive status in the isthmus. In addition, the IHC-labelling for TGF-β1 demonstrated both shortly (Paper III) and later (Paper IV) after treatments, a stronger intensity of epithelial cilia in the isthmus than in the infundibulum. In contrast, labelling of the pro-inflammatory IL-6 appeared to be stronger on the cilia of the infundibulum than of the isthmus. These differences in expression of the two cytokines may be related to a need for a local immune defense in the infundibulum, whereas the higher TGF-β1 in the isthmus may illustrate a down regulation of the immune reactivity at a site where the spermatozoa and early embryos need to be protected against rejection.

IL-1β mRNA expression was very low in both segments of the porcine oviduct (Paper III, Fig. 5 and IV, Fig. 4). IL-1 is generally known as a potent pro-inflammatory cytokine (Dinarello, 1996). Its important role in infection has been shown in the human oviduct by Hvid *et al.* (2007) who, in an *in vitro* study, showed that oviductal epithelial cells produced IL-1 after infection with *Chlamydia trachomatis* and that IL-1, in turn, could induce IL-8, a neutrophil attractant.

In gilts, the numbers of CD8-positive cells along the epithelium and in the connective tissue area were lower in the isthmus than in the infundibulum, both shortly (at 5-6 h, Paper III, Fig. 4) and later (at 35-40 h, Paper IV, Fig. 3) after treatment. One reason for this result is that CD8 may be related to the higher expression of TGF-β1 mRNA in the isthmus, since a negative effect of TGF-β1 on CD8-expressing leukocytes has been shown *in vitro* (Ouellette *et al.*, 1999). In addition, the limited number of cells expressing the receptor for IL-2 (CD25-positive cells, Paper III and IV), a marker for lymphocyte activation (Bailey *et al.*, 1992), found in these areas indicated a low cell-mediated immune reactivity.

As mentioned before, studies on oviducts from both cyclic and inseminated/pregnant sows (Papers I and II), showed higher numbers of lymphocytes and plasma cells in the upper part (infundibulum) than in the lower part (isthmus). In contrast, the expression of both pro-inflammatory (IL-1β and IL-6 mRNA) and suppressive (IL-10 and TGF-β mRNA) cytokines was higher in the isthmus than in the infundibulum. The opposing patterns of immune cell numbers versus levels of cytokine mRNAs in the isthmus and infundibulum emphasize that cytokines may also have other roles in reproductive physiology than those strictly related to immune functions.

Cytokine expressions in the mesometrial lymph nodes

For mucosal immune responses to an antigen, recruitment of the appropriate immune cells from regional lymph nodes is mostly determined by the cytokines/chemokines present in the peripheral tissue and later on in the lymph nodes (Mowat, 2003; Blois *et al.*, 2007; Kaiko *et al.*, 2008). In the mesometrial lymph nodes included in the studies of material from gilts, the relative expressions of mRNAs for IL-10 and TGF-β1 was higher than that of mRNAs for IL-6 and IL-1β (Paper III, Fig. 6 and Paper IV, Fig. 5), but no differences were observed either between experimental groups or at different times after insemination. In addition, the abundant labelling for IL-6, IL-10 and TGF-β1 (IL-1β not analyzed) (Paper III Fig. 1 and Paper IV, Fig. 1) in the mesometrial lymph nodes indicates the importance for a T_H type-2 dominance in the regional lymph node. Accordingly, down-regulation of a T_H type 1 response is important to allow alloantigen to survive, e.g. during graft implantation (Mariotti *et al.*, 2008) and for pregnancy maintenance (Raghupathy, 1997).

Summary of major findings

- The morphology (pseudostratification, mitotic cells and secretory granules) of the oviductal epithelial cells in the ampulla and the infundibulum varied depending on oestrous cycle stage, indicating an influence of ovarian hormones. The most marked morphological activity was observed at oestrus. The cytoplasmic protrusions of oviductal epithelial cells, found at all stages, are suggested to be a process during which dying epithelial cells are eliminated, since the number of phagocytes (neutrophils and macrophages) in the epithelium was low.
- The numbers of lymphocytes, plasma cells and neutrophils in the upper part of the oviduct, especially the infundibulum, always exceeded those in the lower part, the isthmus. The only change in occurrence observed during the oestrous cycle was for the number of neutrophils in the infundibular connective tissue (higher at pro-oestrus), indicating a low general influence of ovarian hormones on the infiltration of immune cells in the oviduct.
- Following pre-ovulatory (normal) insemination, the highest number of neutrophils was found in the infundibular connective tissue about 40 h after insemination. In early pregnancy the number of intra-epithelial lymphocytes increased. Following post-ovulatory (late) insemination, the neutrophil pattern differed from that observed after pre-ovulatory insemination. The numbers were lower but the neutrophils were present for a longer period.
- The immunohistochemical labelling for IL-6, IL-10 and TGF-β1 was apparent, especially in the epithelial cells as well as in some cells in the connective tissue of the isthmus and the infundibulum, and also in cells of the regional lymph nodes.

- No effect of seminal plasma and spermatozoa in extender (BTS) compared with the extender alone (BTS) was found on the mRNA expression of cytokines studied (IL-1β, IL-6, IL-10 and TGF-β1) shortly (5-6 h) after insemination. However, later (at 35-40 h after treatment), the TGF-β1 mRNA appeared to be up-regulated by the inseminated fluid irrespective of content as compared to the control (only catheter introduced).
- Endogenous production of the cytokines TGF-β1, IL-6, IL-10 and IL-1β in the isthmus and the infundibulum was indicated by mRNA expression. This assumption was further supported by the samples of oviductal flushing fluid being positive for TGF-β1, IL-10 and IL-6 in the control group. An endogenous production suggests that these cytokines have a physiological role in the oviduct.
- The expression of mRNA for IL-1β, IL-6, IL-10 and TGF-β1 mRNA was higher in the isthmus than in the infundibulum.
- The mRNA expression of TGF-β1 was higher than the expression of IL-10, IL-6 and IL-1β, indicating that TGF-β1 has a role in the isthmus for tolerance to paternal antigens.
- IL-10 and TGF-β1 and, to a lesser extent IL-6, were detected in boar SP. However, the cytokine concentrations varied markedly between ejaculates within boar.

Concluding remarks and future aspects

The studies of the porcine oviduct presented in this thesis include a number of different parameters, which were assumed to interact. The effects of reproductive stage (oestrous cycle and early pregnancy) and insemination at different times and with different semen components on the presence of immune cells and cytokines were studied in different segments of the oviduct. In contrast to most other studies of the oviduct, the present investigations include the infundibulum. The studies were conducted *in vivo/ex vivo*, and no induction of oestrus or ovulation was performed. The reproductive stages included were judged to be of special interest with regard to hormonal levels and the effects from insemination. The results consist of a multitude of findings and their interpretation answers some questions but also poses many new ones.

The studies show that cytokines of endogenous origin are present in the endosalpinx. The role of the different cytokines, the interaction between them and their potential effect on the local immune cell reactivity however needs to be studied further in more methodical experimental models.

From *in vitro* studies, e.g. on murine and human endometrial cells, it is known that oestradiol can influence cytokine expression. In the present study, cytokine expression was investigated during two "windows", 5-6 h and 35-40 h after insemination at oestrus, and with only minor changes in hormonal levels. Thus, the effects of other reproductive stages, e.g. with progesterone influence, on oviductal cytokine expression, need to be investigated. In addition, the basic expression of cytokines, with no manipulation or treatment, should be included.

When comparing the upper and lower parts of the oviduct (infundibulum and isthmus, respectively) notable differences regarding the presence of immune cells and cytokine expression were found. The single most important role of the oviduct is to provide an environment in which

fertilization can occur successfully. One prerequisite is that the spermatozoa are not damaged by the local immune defense. This is achieved by a local tolerance to paternal antigens in the isthmus. That need could be related to a local production/accumulation of suppressive cytokines such as TGF-β1 in the oviduct and consequently the influence of internal and external factors on immune regulation in the porcine oviduct need further clarification.

The oviduct must provide protection against pathogens introduced during insemination/natural mating. More neutrophils found in the endosalpinx of the infundibulum indicate that a higher protective level is carried out by the upper part of the oviduct than by the lower part. In the present studies, inseminations were performed with an extender (BTS) containing antibiotics and consequently the oviduct was not challenged with bacterial pathogens.

When comparing pre- and post-ovulatory inseminations, a marked difference in the neutrophil distribution was seen. This difference may be the related to the hormonal status of the female and/or speed and time of semen passage through the oviduct. The proper insemination time in relation to ovulation is important for fertility but the present findings indicate that it is also important for the defense mechanism of the oviduct.

Seminal plasma (SP) can induce both suppressive and stimulatory effects on the uterine immune response. One aim of the present studies was to investigate if this was valid also for the oviduct. This is of practical importance, since SP is removed during the cryopreservation process to produce frozen boar semen for insemination. It was unexpected to find that there were no clear differences in cytokine expression after insemination with SP, with sperm in extender, or with extender alone.

The cytokines TGF-β1, IL-10 and IL-6 were detected at the analyses of the pooled seminal plasma. The analyses of SP from individual boars showed that the concentration of these cytokines varies between ejaculates. In addition, variations in cytokine concentration between different ejaculate fractions cannot be excluded and should be further investigated.

For the present study, much time and effort was invested in the establishment of the methods (IHC and quantitative real-time RT-PCR) for analysis of immune cells and cytokines in porcine oviductal tissue, generating a "tool-box" for future research. Additional cytokines of interest are for example granulocyte-macrophage colony stimulating factor, GM-CSF (e.g. suggested to be a central mediator for leukocyte trafficking in the porcine endometrium), innate immunity mediators (e.g. IL-17 and IL-8) and T_H type 1 cytokines (e.g. IFN-γ and IL-12).

Dendritic cells are a heterogeneous group of potent antigen-presenting cells with the capacity to prime naïve T-cell responses. The important role of dendritric cells both for innate and for adaptive immune response in pigs has recently been given special attention. Because these cells are important regulators of the development of immunity or tolerance to foreign substances, the existence and nature of DC in the porcine oviduct needs to be examined.

It would be interesting to study the NK cell subpopulation since, in the present study, the number of CD2-positive cells was higher than the number of CD3-positive cells, indicating the presence of NK cells. Another subpopulation with regulatory function worthy of further investigation is the CD8-positive cell. For continued studies on subpopulation of T-regulatory cells in the endosalpinx, IHC – double/triple-labelling should be performed.

For the analyses of mRNA cytokine expressions, a mixture of cells representing the mean of all cell types from endosalpinx and myosalpinx was used. However, for a closer tissue-related illustration of cytokine mRNA expression (qualitative and quantitative), other methods, such as *in situ* hybridization, are needed. In addition, the use of confocal microscopy could be applied to visualize the localization of various cell types and their products.

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