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

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The present thesis is a partial fulfilment of the requirements for a Master of Science Degree for International Students (MSc) in Veterinary Medicine, at the Swedish University of Agricultural Sciences (SLU), in the field of Animal Reproduction

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

Abstract

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The oviduct, together with its secretion, forms the environment in which gamete transport, sperm maturation, fertilization as well as early embryo transport and development occur. Morphological aspects, as well as immune cell distribution, have been described only to a limited extent regarding the pig oviduct. To better understand the reproductive physiology, more information is needed regarding morphology and infiltration of immune cells at different stages of the oestrous cycle.

The aim of this study was to investigate the morphological changes and the distribution of leukocytes in the sow endosalpinx throughout the oestrous cycle and at anoestrus. Nineteen crossbred sows (Swedish Landrace × Swedish Yorkshire) at late dioestrus (3), prooestrus (3), oestrus (3), early dioestrus (3), dioestrus (3) and anoestrus (4) were used. Oviductal samples from three different parts (isthmus, ampulla and infundibulum), taken immediately after slaughter, were immersion-fixed, embedded in plastic resin and stained with toluidine blue or stored in a freezer at -70°C until analysed by immunohistochemistry (prooestrus and anoestrus) with an avidin-biotin peroxidase method. Quantitative and qualitative examinations of oviductal epithelial and subepithelial connective tissue layers were performed by light microscopy.

During all stages, a lower degree of morphological changes in the epithelium (pseudostratification, mitosis and secretory granules) was found in the isthmus compared with ampulla and infundibulum. In the ampulla and infundibulum, pseudostratification, mitotic activity and secretory granules were high at prooestrus/oestrus. Cytoplasmic protrusions of epithelial cells with some extruded nuclei were prominent in ampulla and infundibulum at all stages except for oestrus and early dioestrus.

Lymphocytes as well as CD2 and CD3 positive cells were the predominant immune cells in *the epithelial layer*. The numbers of lymphocytes and CD3 positive cells did not differ among segments and stages. Numbers of CD2 positive cells did not differ between prooestrus and anoestrus while the numbers were significantly higher in the infundibulum than in ampulla and isthmus. Neutrophils were only occasionally found and mainly in the infundibulum.

In the subepithelial connective tissue layer, the two most commonly observed immune cell types were lymphocytes and plasma cells. The numbers of lymphocytes as well as CD2 and CD3 positive cells was lower in isthmus than in the other segments ($P \leq 0.001$). Higher numbers of plasma cells ($P \leq 0.001$) were found in infundibulum than in ampula and isthmus. The numbers of lymphocytes and plasma cells were not significantly different between stages of the oestrous cycle. However, the number of neutrophils differed and were highest at prooestrus in ampulla and infundibulum. The numbers of CD2, CD3 and CD79 positive cells did not differ between prooestrus and anoestrus whereas for CD14 and SWC3 positive cells, the numbers were higher at prooestrus ($P \leq 0.05$) than at anoestrus.

In the oviduct, the morphology differed in ampulla and infundibulum with oestrous cycle stages which indicates an effect by ovarian steroid hormones. The immune cell infiltration was less influenced by cyclic changes. However, the immune cell infiltration (in the connective tissue) in the upper part, especially infundibulum, differed significantly from the one in the lower part, isthmus, indicating different immune functions within various parts of the oviduct.

Keywords: Sow, oviduct, endosalpinx, morphology, immune cell and immunohistochemistry

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Contents

General Background

The pig oviduct

The oviducts develop from the the proximal parts of the Müllerian ducts (ductus paramesonephricus) of the embryo. It is a tortuous tube, about 35 cm long in the sow, and attached by mesosalpinx to the broad ligament at the base and side of the pelvis. The oviduct is divided into three main segments: the isthmus, connected caudally (ad uterus) to the uterotubal junction (UTJ); the mid part, ampulla; and the cranial part, infundibulum (Maximow & Bloom, 1945). The isthmus is a narrow and muscular tube. The ampulla is wider and more thin-walled than isthmus and caudally connected to the isthmus by an inconspicuous ampullary-isthmic junction (AIJ). The infundibulum is funnel-shaped and extends toward the ovary. Its cranial part (ad ovarium) is split into many fringes, called fimbriae. Histologically, the oviductal wall is composed of the endosalpinx, a mucous membrane with longitudinal folds; the double-layered myosalpinx, with inner circular and outer longitudinal smooth muscle layers; and the mesosalpinx, the external serous coat. In the infundibulum and ampulla, the myosalpinx is thin and composed of an inner circular layer and a few outer longitudinal bundles of smooth muscle. In the isthmus, the muscle layers are prominent (Priedkalns & Leiser, 1998).

Morphological aspects of the endosalpinx

The endosalpinx, the luminal mucosa, forms longitudinal folds along the tube. The ampulla and infundibulum contain secondary or tertiary folds, but isthmus has only primary folds (Priedkalns & Leiser, 1998). The epithelium of the endosalpinx is simple or pseudostratified columnar. It is separated from the subepithelial lamina propria by a basement membrane. The epithelium is composed of two major cell types: ciliated and secretory cells. The ciliated cells are numerous in the fimbriae, ampulla and isthmus but less abundant in the uterotubal junction (Wu *et al.*, 1976). The cells carry cilia which in ampulla were shown to beat in a downward direction towards AIJ and in a reverse direction in isthmus (Gaddum-Rosse & Blandau, 1973). Nonciliated, secretory cells contain granules. By scanning electron microscopy, marked cyclic changes were observed on the surfaces of epithelial cells in the fimbriae and ampulla. The cells at follicular phase were densely ciliated while at luteal phase, the cilia were concealed by bulbous processes of the secretory cells (Abe & Oikawa, 1992). Few changes were found in the isthmus.

The subepithelial lamina propria consists of a loose connective tissue of which fibroblasts and collagen fibres are the main components.

Functional aspects of the endosalpinx

Endosalpinx, especially the epithelial lining together with its secretions, forms the environment in which gamete transport, sperm maturation, fertilization as well as early embryo transport and development occur. Fertilization takes place in the AIJ

(Hunter, 1974). Buhi *et al*. (1997) showed that ciliated and secretory cells in both ampulla and isthmus of the pig oviduct reached maximal height at oestrus and metoestrus. The cell height decreased with increasing concentrations of progesterone at dioestrus. In the ampulla, the percentage of ciliated cells was greatest at oestrus (80%) and declined at dioestrus with a concomitant increase in nonciliated cells from 20% to 50%. In contrast, in the isthmus, the percentage of ciliated (70-80%) and nonciliated (20-30%) cells did not change throughout the oestrous cycle.

For transport of the ova, adhesive interactions between cumulus cells and the extracellular matrix on the tips of the cilia (Norwood & Anderson, 1980; Talbot *et al*., 1999) act together with the ciliary beating (Gaddum-Rosse & Blandau, 1973). In the pig oviduct, the secretion rate increased during oestrus and decreased during dioestrus (Iritani *et al*., 1974). The oviductal epithelial cells, in response to ovarian steroid hormones, synthesizes and releases proteins into the lumen (Buhi *et al*., 1997). These proteins create a microenvironment important for reproductive events along the different parts of the oviduct. Using *de novo* synthesis, Buhi *et al*. (1989) found that secretion of the pig oviduct-specific protein (pOSP) in the ampulla was greatest during prooestrus and oestrus, while secretion from the isthmus during equivalent periods was low and did not change during the oestrous cycle. In *in vitro* studies, McCauley *et al*. (2003) and Kouba *et al*. (2000) showed that the pOSP decreased polyspermy and enhanced embryo cleavage and blastocyst formation. The importance for fertilization was also shown by Mburu *et al*. (1997) who found that sperms bind to oviductal epithelial cells, in UTJ and isthmus, and maintain intact plasma membranes during the preovulatory period. Hunter *et al*. (1998) also showed that an effective process of sperm capacitation occur in isthmus.

An aseptic milieu must be maintained in the oviduct, *i.e*. free from the microorganisms that may sporadically colonize the upper reproductive tract (Bolin *et al*., 1985; Ellis *et al*., 1986). In general, mucosal surfaces are protected by both unspecific and specific defence mechanisms (Ogra *et al*., 1994). The former includes mechanical barriers such as tight junctions of epithelial cells; non-specific chemicals such as lysozymes, lactoferrine and peroxidases; and non-specific immune cells such as neutrophils and macrophages. The specific mechanisms include immune cells (specifically located in or under the epithelia) that regulate local immune response (T helper lymphocytes), recognize and destroy an infected cell (cytotoxic T lymphocytes) and locally produce antigen-specific antibodies (plasma cells) (Ogra *et al*., 1994). In the human oviductal mucosa, essential functional components of the mucosal immune system, such as immunoglobulin A, antibody secreting cells (Kutteh & Mestecky, 1994) and intraepithelial lymphocytes (Morris *et al*., 1986), have been reported. However, functional immune regulation of the oviduct in supporting the survival of sperms and semiallogenic conceptuses as well as stimulating active immunity against microbial pathogens is still unclear. Cardenas *et al*. (1998) showed that there are gaps in the knowledge about the immune system in the mammalian oviduct as well as interspecies differences which make it essential to further investigate this part of the reproductive tract.

The infiltration of leukocytes in the endosalpinx

Most studies on the immune cell infiltration in endosalpinx have been performed in humans (Morris *et al.*, 1986; Kutteh *et al.*, 1990; Boehme & Donat, 1992; Hagiwara *et al.*, 1998; Suenaga *et al.*, 1998) and rodents (Parr & Parr, 1985; Dalton *et al.*, 1994). In cows, 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 pig, information on leukocyte distribution related to the oestrous cycle is limited to immunoglobulin containing cells (Hussein *et al.*, 1983). Higher numbers of these cells were found at oestrus than at dioestrus. Rodriguez-Martinez *et al*. (1990) showed that neutrophils were absent in the uterotubal junction and the isthmic epithelium, irrespective of the presence of spermatozoa in the lumen.

General aspects of the leukocyte populations

Immune responses can be divided into two types: innate immune response and adaptive immune response (Janeway *et al.*, 2001). The innate immune system, largely involves granulocytes and macrophages (phagocytic cells). They generally are effective without prior exposure to a pathogen. The adaptive immune response is highly specific for a particular pathogen that occurs as an adaptation to infection and with memory to re-infection. Adaptive immune responses depend upon lymphocytes.

Lymphocytes (B, T, and NK cells) are typically small to medium sized cells with densely stained nuclei surrounded by a thin rim of cytoplasm. Both T and B lymphocytes bear highly diverse receptors on their surfaces that allow them to recognize antigen (Janeway *et al.*, 2001). B lymphocytes, when activated, differentiate into plasma cells (Calame, 2001) that secrete antibodies. T lymphocytes are functionally divided into two main classes: cytotoxic T cells, which destroys for instance virus infected cells, and helper T cells, which activate macrophages and B cells. In all cases, T cells recognize their targets by detecting peptide fragments (foreign proteins). The molecules that display peptide antigen to T cells are *major histocompatibility complex (MHC) molecules*. There are two types of MHC molecules, called MHC class I and MHC class II. MHC class I molecules present peptides derived from proteins synthesized in the cytoplasm, *e.g.* virus proteins, to cytotoxic T cells. MHC class II molecules present peptides (derived from antigen internalized by phagocytic cells or B cells) to T helper cells.

A third class of lymphocytes, called natural killer (NK) cells, lack antigen-specific receptors and are therefore part of the innate immune system. In the pig, these cells are activated during placental development and may be important in early interactions between the conceptus and the maternal immune system (Croy *et al.*, 1998).

Plasma cells produce antibodies and are large cells with an eccentric round or oval nucleus. The chromatin is coarsely clumped in a characteristic ‘cartwheel’ or ‘clock face’ pattern (Young & Wheath, 2000). By light microscopy, the cytoplasm appears purple due to its large content of ribosomal RNA and protein which are stained by both acidophilic and basophilic dyes (amphophilic).

Neutrophil granulocytes contain highly lobulated nuclei lightly stippled with purplish granules in the cytoplasm (Young & Wheath, 2000). They are short-lived, surviving only a few hours after leaving the bone marrow (Janeway *et al.*, 2001). Neutrophils are triggered to leave the blood vessels as a result of the endothelial cells expressing adhesive proteins that bind the neutrophils to the walls of small blood vessels. They are then squeezed out and attracted to the site of infection. Neutrophils are the earliest phagocytic cells to be recruited. They engulf pathogens, destroy it and then die after a short time (Male, 2002).

Circulating monocytes migrate into tissues where they mature to macrophages. *Macrophages* are long-living cells, can proliferate locally, and may survive for months in the tissues. Most of the macrophages are attracted into the site of infection after neutrophils (Janeway *et al.*, 2001). 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 (Junqueira & Carneiro, 2003). Macrophages, as professional antigen-presenting cells, are also important in the induction of the adaptive immune response (Janeway *et al.*, 2001).

Mast cells are long-living cells found in connective tissue where they are able to proliferate (Young & Wheath, 2000). The cell can be recognised by its content of metachromatic granules when appropriately fixed and stained with metachromatic dyes such as toluidine blue. Histamine, the major active substance found in mast cells, is a vasoactive amine which promotes increased vascularization and permeability and causes contraction of smooth muscle (Junqueira & Carneiro, 2003). A role of mast cells as potential regulatory linkers between innate and adaptive immunity has also been suggested (Frossi *et al.*, 2004).

The eosinophil is larger than the neutrophil and easily recognised by its large specific granules which stain bright red with eosin (Young & Wheath, 2000). Eosinophils develop in the bone marrow but reside in large numbers in peripheral tissues (Costa *et al.*, 1997), especially mucosal connective tissue. Although their precise life span is not known, eosinophils live longer than neutrophils and may survive for weeks within tissues. Eosinophils play an important role in parasite infestation and are involved in hypersensitivity reactions (Gleich & Loegering, 1984).

Characteristics of leukocyte surface molecules

Leukocytes bear particular combinations of cell-surface proteins that are involved directly in the function of the cell (Janeway *et al.*, 2001). Such surface molecules were originally called *differentiation antigens*. When groups of monoclonal antibodies were found to recognize the same differentiation antigen, they were said to define a *cluster of differentiation*, abbreviated to *CD*, followed by an arbitrarily assigned number. At international immunology workshops the pattern of monoclonal antibody binding are determinded, *e.g.* Haverson *et al.* (2001) and Saalmüller *et al.* (2001). For instance, the characteristics of porcine T-lymphocytes differ significantly from other species (review Saalmüller *et al.*, 2002).

Importance of the oviduct at reproductive disturbances

Lambert *et al*. (1991) found that substantial proportions of prenatal loss, about 25%, including fertilization failure, takes place before day 10 of pregnancy. Embryonic loss is greater in sows than in gilts and increase with higher ovulation rates (Meredith, 1995). Furthermore, it has been shown that asynchrony between uterine changes and embryonic development is an important factor for embryonic loss, *i.e.* that less-developed embryos succumb when more advanced embryos trigger changes in the uterine environment (Pope *et al.*, 1990). A negative effect of post-ovulatory food deprivation on numbers of accessory spermatozoa in zona pellucida and cleavage rate of the recovered embryos (Mburu *et al.*, 1998) as well as on isthmic pressure (Mwanza *et al.*, 2000) have been shown. Changes in oviductal functions may result in a disruption of the normal development and transportation of ova through the oviduct with fertilized ova being out of synchrony with the uterine environment. Foxcroft *et al*. (1999) found that the critical time during which progesterone-mediated effects of nutrition (or any other factor) become established, is likely to be the first 3 to 4 days of gestation. Therefore, changes in the normal oviduct function, such as immunological reaction against an immunogen, or hormonal unbalance caused by stress or improper nutrition, may cause an unsatisfactory oviductal environment for gametes, resulting in a decrease in litter size or increase in the numbers of sows returning to oestrus.

Introduction to the Research Report

Endosalpinx forms the environment in which gamete transport, sperm maturation and fertilization as well as early embryonic development and transport take place. A substantial proportion of embryonic loss (including fertilization failure) takes place before day 10 of pregnancy (Lambert *et al.*, 1991) and parts of that may be caused by disturbed oviductal functions.

In gilts/sows, some cyclic changes of oviductal epithelial morphology have been reported (Nayak & Zimmerman, 1971; Wu *et al.*, 1976; Abe & Oikawa, 1992). However, differences between the segments of the oviduct in relation to different oestrous cycle stages have not been fully elucidated.

Functional immune regulation of the oviduct in supporting the survival of sperms and semiallogenic conceptuses as well as stimulating active immunity against microbial pathogens is still unclear. In the pig oviduct, information on leukocyte distribution related to different stages of the oestrous cycle is limited. Immunoglobulin containing cells (Hussein *et al.*, 1983) were found in higher numbers at oestrus than at dioestrus.

Regarding effects by insemination, Rodriguez-Martinez *et al*. (1990) showed that neutrophils were absent in the uterotubal junction and the isthmic epithelium, irrespective of the presence of spermatozoa in the lumen. That is different compared to the uterus. Artificial insemination/mating causes a physiological, acute inflammatory reaction in the sow endometrium (*e.g*. Lovell & Getty, 1968; Bischof *et al.*, 1995; Rozeboom *et al.*, 1998; Kaeoket *et al.*, 2003) and within 30 minutes after mating, neutrophils are found in the uterine lumen (Lovell & Getty, 1968).

A review by Cardenas *et al.* (1998) showed that there are gaps in the knowledge about the immune system in the mammalian oviduct as well as interspecies differences. To better understand the reproductive physiology of the pig oviduct, further investigations are needed regarding morphological changes and the infiltration of immune cells at different stages of the oestrous cycle.

Aims of the Study

The aims of the present study on the sow endosalpinx (isthmus, ampulla and infundibulum) were to:

1. describe the general morphology at different stages of the oestrous cycle and at anoestrus,
2. describe changes regarding the leukocyte infiltration (quantitative and qualitative) at different stages of the oestrous cycle and at anoestrus.

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Research Report

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

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Abstract

The aim of this study was to investigate the morphological changes of the sow endosalpinx and the distribution of leukocytes throughout the oestrous cycle and at anoestrus. Nineteen crossbred sows (Swedish Landrace × Swedish Yorkshire) at late dioestrus (3), prooestrus (3), oestrus (3), early dioestrus (3), dioestrus (3) and anoestrus (4) were used. Oviductal samples from three different parts (isthmus, ampulla and infundibulum), taken immediately after slaughter, were fixed, embedded in plastic resin and stained with toluidine blue or stored in a freezer at -70°C until analysed by immunohistochemistry (prooestrus and anoestrus) with an avidin-biotin peroxidase method. Quantitative and qualitative examinations of oviductal epithelium and subepithelial connective tissue were performed by light microscopy.

During all stages, a lower degree of morphological changes (pseudostratification, mitosis and secretory granules) was found in the isthmus compared with ampulla and infundibulum. In ampulla and infundibulum, pseudostratification, mitotic activity and secretory granules of the epithelium were high at prooestrus/oestrus. Cytoplasmic protrusions of epithelial cells with some extruded nuclei were prominent in ampulla and infundibulum at all stages except for oestrus and early dioestrus.

Lymphocytes as well as CD2 and CD3 positive cells were the predominant immune cells in *the epithelial layer*. The numbers of lymphocytes and CD3 positive cells did not differ among segments and stages. Numbers of CD2 positive cells did not differ between prooestrus and anoestrus while the numbers were significantly higher in the infundibulum than in ampulla and isthmus. Neutrophils were only occasionally found and mainly in the infundibulum.

In the subepithelial connective tissue layer, the two most commonly observed immune cell types were lymphocytes and plasma cells. The numbers of lymphocytes as well as CD2 and CD3 positive cells was lower in isthmus than in

the other segments ($P \leq 0.001$). Higher numbers of plasma cells ($P \leq 0.001$) were found in infundibulum than in ampulla and isthmus. The numbers of lymphocytes and plasma cells were not significantly different between stages of the oestrous cycle. However, the number of neutrophils differed and were highest at prooestrus in ampulla and infundibulum. The numbers of CD2, CD3 and CD79 positive cells did not differ between prooestrus and anoestrus whereas for CD14 and SWC3 positive cells, the numbers were higher at prooestrus ($P \leq 0.05$) than at anoestrus.

In the oviduct, the morphology differed in ampulla and infundibulum with oestrous cycle stages which indicates an effect by ovarian steroid hormones. The immune cell infiltration was less influenced by cyclic changes. However, the immune cell infiltration (in the connective tissue) in the upper part, especially infundibulum, differed significantly from the one in the lower part, isthmus, indicating different immune functions within various parts of the oviduct.

Keywords: Sow, oviduct, endosalpinx, morphology, immune cells, and immunohistochemistry

Introduction

In the oviduct, important reproductive events take place, such as capacitation of spermatozoa, fertilization and early embryonic development (Hunter 1998; Rodriguez-Martinez et al. 2001). Oviductal fluid is crucial for these processes and consists of transudate from serum together with specific compounds synthesized by the luminal epithelium. The pattern of oviductal secretion coincides with changes of the oestrous cycle. The highest volume was measured at, or shortly after, ovulation and the lowest volume was found during the luteal phase of the cycle (Hunter 1998). Cyclic changes of oviductal epithelial morphology have been reported in farm animals such as the goat (Abe et al. 1993), ewe (Hollis et al. 1984), cow (Nayak and Ellington 1977; Abe and Oikawa 1993) and sow/gilt (Nayak and Zimmerman 1971b; Wu et al. 1976; Abe and Oikawa 1992). For instance, Abe and Oikawa, (1992) by scanning electron microscopy, observed marked cyclic changes on the surfaces of cells in fimbriae (of infundibulum) and ampulla. In the follicular phase the cells were more densely ciliated, while in the luteal phase, the cilia were concealed by bulbous processes of the secretory cells. Few changes were found in isthmus and the uterotubal junction. However, differences between the segments of the oviduct in relation to different oestrous cycle stages have not been fully elucidated.

In the oviduct, an aseptic *milieu* must be maintained, *i.e.* free from the microorganisms that sporadically may colonize the upper reproductive tract (Bolin et al. 1985; Ellis et al. 1986). In general, mucosal surfaces are protected by both unspecific and specific defence mechanisms (Ogra et al. 1994). The former includes mechanical barriers such as tight junctions of epithelial cells; non-specific chemicals such as lysozymes, lactoferrine and peroxidases; and non-specific immune cells such as neutrophils and macrophages. The specific mechanisms include immune cells (specifically located in or under the epithelia) that regulate local immune response (T helper lymphocytes), recognize and destroy an infected cell (cytotoxic T lymphocytes) and locally produce antigen-specific antibodies

(plasma cells) (Ogra et al. 1994). In the human oviductal mucosa, essential functional components of the mucosal immune system, such as secretory immunoglobulin A, antibody secreting cells (Kutteh and Mestecky 1994) and intraepithelial lymphocytes (Morris et al. 1986), have been reported. However, the oviduct must also support the survival of sperms and semi-allogenic conceptuses (Rodriguez-Martinez et al. 2001). For instance, in newly inseminated gilts, Rodriguez-Martinez et al. (1990) did not find any neutrophils in the uterotubal junction or in the isthmic epithelium. A review by Cardenas et al. (1998) showed that there are gaps in the knowledge about the immune system in the mammalian oviduct as well as interspecies differences which make it essential to further investigate this part of the reproductive tract.

In the pig uterus, the distribution of immune cells at different oestrous cycle stages vary (Bischof et al. 1994; Kaeoket et al. 2001a, b; Kaeoket et al. 2001c). For instance, in both gilts (Bischof et al. 1994) and sows (Kaeoket et al. 2001c) high numbers of neutrophils infiltrated the endometrial subepithelial stroma at prooestrus and oestrus. Correlations between plasma levels of oestradiol-17β (positive) or progesterone (negative) and the number of neutrophils were shown by Kaeoket et al. (2001c). However, in the oviduct, studies of human (van Bogaert et al. 1978; Kutteh et al. 1990; Boehme and Donat 1992) and mice (Parr and Parr 1985) showed that the number of immune cells did not differ depending on stage of the oestrous cycle. On the other hand, in sows, Hussein et al.(1983) reported that IgA-, IgG- and IgM-containing cells in the ampulla were found to be in higher numbers at oestrus than at dioestrus and to our knowledge, this is the only study about Ig-subclasses of plasma cells of the pig oviduct. To better understand the reproductive physiology of the pig oviduct, more information is needed regarding the infiltration of immune cells at different stages of the oestrous cycle.

The aim of the present study was to examine the morphological changes of the sow endosalpinx (isthmus, ampulla and infundibulum) and the quantitative and qualitative distribution of immune cells during different stages of the oestrous cycle and at anoestrus.

Materials and methods

Animals and general management

Nineteen crossbred sows (Swedish Landrace × Swedish Yorkshire) with an average parity number of 3.4 ± 0.7 (mean ± SD, range 2-5) were purchased from a commercial herd and brought to the Department of Obstetrics and Gynaecology, Swedish University of Agricultural Sciences, directly after weaning. Prior to the study, the sows had shown a normal reproductive performance. Body weights at weaning ranged from 185 to 268 kg and the average age was around 2 years. Four sows with a lactation period of 4 weeks were slaughtered at the day of weaning. Fifteen sows, with a lactation period of 5 weeks, were after weaning kept in individual pens. Boars were housed separately in the same stable throughout the experimental period. The sows were fed with the Swedish standard diet for dry sows (Simonsson 1994) (barley-based sow diet, 14.5% protein and 12.5 MJ/kg

metabolizable energy), 5 kg per day until the first oestrus after weaning, and thereafter, they were fed about 2 to 2.5 kg per day. Water was available ad libitum.

Detection of oestrus and ovulation

Twice daily, oestrus was controlled by observation of the vulva, for reddening and swelling, and by checking the standing reflex in the presence of a boar, every 4 hours from the onset of prooestrus.

Ovarian follicle development and ovulation were followed in each sow by transrectal ultrasonography (Kaeoket et al. 2001c). Scanning was performed once a day from the onset of prooestrus. From 16 hours after the start of the standing oestrus until completed ovulation, the sows were scanned every 4 hours.

Blood collection and hormone assays

One hour prior to slaughter, a blood sample was collected from the jugular vein on a restrained animal, using vacutainer heparin-tubes. The blood samples were centrifuged at 3000 rpm, for 10 minutes immediately after collection, and the plasma was stored in plastic tubes at -20°C until analysed at the Department of Clinical Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

The plasma oestradiol-17β (E_2) level was determined by radioimmunoassay (double antibody oestradiol, Diagnostic Products Corporation, Los Angeles, USA) as previously described for analysis of bovine plasma (Duchens et al. 1994) and validated for oestradiol- 17β analyses in the pig (Mwanza et al. 2000).

The progesterone (P_4) level was determined by a luminescence immunoassay (Amerlite, Kodak Clinical Diagnostics Ltd., Amersham, England). The kit was used according to the manufacturer's instructions and the method had earlier been validated for progesterone analyses in the pig (Rojkittikhun et al. 1993).

Tissue collection

Oviductal samples were collected from sows at weaning (n = 4, anoestrus stage) and at five different stages of the oestrous cycle (Day 1 was the first day of *standing oestrus*). The cyclic sows were slaughtered at: *dioestrus* (Day 11 to 12 of the first oestrous cycle after weaning, n = 3); *late dioestrus* (Day 17 of the first oestrous cycle after weaning, n = 3); *prooestrus* (Day 19 of the first oestrous cycle after weaning, n = 3); *oestrus* (11.5 to 17.5 h after the start of standing oestrus in the second oestrous cycle after weaning, n = 3) and *early dioestrus* (70.5 to 71.0 h after ovulation of the second oestrous cycle after weaning, n = 3). Immediately after slaughter, the genital organs were collected and examined for normality and ovarian status. Oviductal samples from three different parts (isthmus, ampulla and infundibulum) were fixed in 3% glutaraldehyde in 0.067 M sodium cacodylate buffer (pH 7.4) for light microscopy (LM) and transmission electron microscopy (TEM). For immunohistochemical staining, oviductal specimens were immediately frozen by plunging into liquid nitrogen. The frozen samples were stored in a

freezer at -70°C until immunohistochemistry was carried out. Specimens of lymph nodes (located in the mesometrium) were also collected to be used as control.

Preparation of oviductal samples for histological examination

The tissue samples were trimmed, dehydrated and embedded in water soluble methylmetacrylate (Historesin®, LKB, Bromma, Sweden) for LM or post-fixed in osmium tetroxide, dehydrated by exposure to graded concentrations of ethanol and propylene oxide and embedded in Agar 100® plastic resin (Agar Aids, Essex, England, UK) for TEM. For LM examination, semi-thin sections (3-4 µm) were obtained with a Leica RM 2165®, placed on glass slides, and stained with buffered toluidine blue. Photographs were taken from selected sections with a Nikon-FXA photomicroscope. For TEM, ultrathin sections (1-2 µm) were cut with a diamond knife (LKB Ultratome®) from selected areas of the Agar 100® embedded material. The ultrathin sections were picked up onto uncoated copper grids, counterstained with uranyl acetate and lead citrate and examined as well as photographed in a Philips EM 420 electron microscope at 60 or 80 kV.

Monoclonal antibodies

In order to characterise leukocyte subpopulations in the oviduct, mouse monoclonal antibodies to CD2, CD3, CD79, CD14, SWC3 and MHC class II were used (Table 1).

Immunohistochemistry

Due to technical reasons, oviductal cryostat samples from only prooestrus and anoestrus could be subject to immunohistochemical staining. ABC-Elite mouse kit (Vector laboratories Inc., Burlingame, CA, USA) was used. Cryostat sections were cut at 7 µm and placed on SuperFrost® Plus (Histolab Products AB, Göteborg, Sweden) glass slides. Prior to staining, the sections were dried at room temperature for 24 hours and fixed in chilled acetone (+4°C) for 10 minutes. Tris-buffered saline (TBS), 0.05 M, pH 7.6 was used for all dilutions and washings. Non-specific protein binding was diminished by incubation with 10% normal horse serum (NHS) 2×30 minutes. The tissue sections were then overlaid with 50 µl of primary antibodies, and incubated in a humid chamber overnight at +4°C. The secondary biotinylated antibody (horse anti-mouse, 1:1000, Vector laboratories Inc.) was applied for 30 minutes at room temperature. Diaminobenzidine (DAB, Dakopatts Ab, Älvsjö, Sweden) was used as chromogen, and the sections were counterstained with Mayer's haematoxylin followed by mounting in glycerine-gelatin. Negative controls were performed on tissue sections of porcine oviduct and lymph node using normal mouse IgG (Santa Cruz, California). The porcine lymph node was also used as positive control.

Morphological and immune cell evaluation

Microscopic examination of the samples was done by the same person who was unaware of the identity of the sows, *i.e.* all slides were coded before examination. In all nineteen sows, oviduct samples of three parts (isthmus, ampulla and

infundibulum) were evaluated in toluidine blue stained sections; and in 7 of the sows (4 anoestrus and 3 prooestrus) also in sections subject to immunohistochemistry. A light microscope was used with objective ×40 and eyepieces ×10. Cell counts were performed by using an ocular reticule with 100 small squares placed in one eyepiece of the light microscope. With 400× magnification, the length of 100 small squares of the reticule corresponded to 0.25 mm of real tissue length and the area of 100 small squares of the reticule correspond to 0.0625 mm^2 of real tissue area. Only optimal sections (free of artefacts and with correct orientation) were accepted for counting. In each section, counts were performed by movement of the entire area in a non-overlapping manner. Each section was divided into two compartments for counting - epithelium and subepithelial connective tissue.

Counting was carried out along the length of the epithelial layer and in as large areas as possible of the subepithelial connective tissue. For the epithelial layer length, at least 180 small squares of isthmus and 340 small squares of ampulla and infundibulum were examined. For the connective tissue area, at least 120 small squares of isthmus and 210 small squares of ampulla and infundibulum were examined.

In toluidine blue stained sections, counting was made for the following parameters:

-numbers of immune cells as lymphocytes, plasma cells, mast cells, eosinophils, neutrophils and macrophages;

-numbers of vessels in cross-sections (expressed as small size ~12.5 μ; medium size ~ 12.5-50 μ and large size ≥ 50 μ of outer diameter, estimated by the ocular reticule);

-numbers of mitotic figures in epithelial cells.

In immunohistochemical sections, counts were made for numbers of positive cells of CD2, CD3, CD79, CD14 and SWC3. MHC class II sections were evaluated under the light microscope but positive cells were not counted.

For analysis and presentation of the results, the data were recalculated into 100 small squares to have the same unit of all samples. For toluidine blue stained sections, the immune cells with typically small to medium sized, round to oval and densely stained nuclei surrounded by a thin rim of cytoplasm as described by Peters (1986) were counted as lymphocytes. Cells with phagosome-like bodies in the cytoplasm were counted as macrophages. Some mononuclear unidentified cells with abundant cytoplasm and spherical euchromatic nuclei were not included in the counting but described with electron microscopy. Small, medium and large sized vessels refer to capillaries, arterioles or venules and small veins or small arteries, respectively.

The morphological changes occurring in the endosalpinx were evaluated by a scoring system with respect to:

-pseudostratification of the epithelium as 1 = low columnar with nuclei appearing on one level, 2 = tall columnar with nuclei appearing on two levels and 3 = highest columnar with nuclei appearing on three levels;

-cytoplasmic protrusions and secretory granules of the non-ciliated epithelial cells as 0 = none; 1 = few; 2 = moderate and 3 = high numbers;

-density of fibroblasts in the subepithelial connective tissue by comparison of pictures taken under the light microscope of various stages and segments as 1 = low; 2 = moderate; 3 = high; and 4 = very high density.

Statistical analyses

Data were handled and statistically analysed using the SAS statistical package (Version 8, SAS Institute Inc. 1998, Cary, NC, USA). Normal distribution of residuals from the statistical models was tested using the UNIVARIATE procedure option NORMAL. Homoscedasticity was investigated using Bartlett test in the GLM procedure. A natural log transformation of CD3, CD14, SWC3 positive cell density and lymphocyte density was applied to achieve the assumption required for analysis of variance. Differences in mean numbers of lymphocytes, plasma cells and CD2, CD3, CD14, CD79, SWC3 positive cells were tested using analysis of variance (Proc MIXED). The statistic model included the fixed effects of stage (5 groups: prooestrus, oestrus, early dioestrus, dioestrus, late dioestrus and anoestrus) and segment (3 segments: isthmus, ampulla and infundibulum); the interaction between stage and segment; and the random effect of sow nested within stage. Bonferroni *t*-test was used to compare least squares means between groups when overall significance for that effect was found. Other variables, such as mast cell, eosinophil, neutrophil, macrophage, vessel and mitotic cell density, in which the distribution was far from normal were analysed using NPAR1WAY procedure (Wilcoxon's rank-sum test) with the effect of stage and segment included monofactorially. Because of few samples for each stage (3-4 sows), pairwise comparison between stages or segments was not performed. A *P*-value ≤ 0.05 was considered statistically significant.

Results

Clinical observations and macroscopic findings

For the 15 cyclic sows, ovarian follicular development and ovulation time as well as formation of corpora lutea were within normal ranges (Kaeoket et al. 2001c). Small multiple follicles (≤ 5 mm in diameter) and regressed corpora lutea of pregnancy were found in the ovaries of sows at anoestrus. The plasma levels of oestradiol-17β and progesterone at the day of slaughter are presented in Table 2.

Morphological evaluation

In the ampulla and infundibulum, the epithelia had the same pattern of pseudostratification being high columnar at oestrus (2-3 nuclei levels) and low columnar at dioestrus, late dioestrus and anoestrus (~1 nuclei level) (Fig. 1a). The epithelial cells of isthmus were consistently low columnar in all stages and with only slightly increased height at prooestrus.

Cytoplasmic protrusions of epithelial cells including some extruded nuclei were observed in the ampulla and the infundibulum (Figs. 2 and 3) (not evaluated in isthmus because of lack of free luminal cell area). It was prominent at all stages (including anoestrus) except for oestrus and early dioestrus (Fig. 1b).

Mitotic activity in the oviductal epithelium was found mainly at prooestrus and the highest mitotic activity was observed in infundibulum at that stage (Fig. 1c). Secretory granules in epithelial cells, located at supranuclear level, were found nearly solely at oestrus with the highest amount in the ampulla which was significantly different from isthmus (Fig. 1d).

The density of small sized vessels (capillaries) was comparatively low at prooestrus and oestrus (Fig. 1e). The highest density of small sized vessels was found at anoestrus in all segments, however, only in isthmus and infundibulum an overall significant difference among stages was found ($P \leq 0.05$). Medium (arterioles and venules) and large (small veins and arterioles) sized vessels were found mainly in ampulla and infundibulum. Numbers of medium and large sized vessels were low at anoestrus, however, overall significant differences between stages were found for the large sized vessels in the infundibulum ($P \leq 0.05$).

The semiquantitative estimation of fibroblast density showed that it was markedly higher at anoestrus than during the oestrous cycle. Overall significant difference between stages was found in all segments ($P \leq 0.05$) (Fig. 1f).

By electron microscopy, three types of mononuclear cells (differing regarding cytoplasmic and nuclear characteristic) with a basal location in the oviductal epithelium were observed as shown in Fig.3. Some protruding and sloughed non-ciliated cells were also observed in the oviductal lumen.

Leukocyte evaluation

Epithelium

Lymphocytes were the predominant immune cells in the epithelia of all oviductal segments and stages. For lymphocytes, macrophages and neutrophils, the percentages were 94, 5.4 and 0.6 respectively. The cells were found mainly in a basal position of the epithelium (Fig. 2 and 3). The numbers of intraepithelial lymphocytes (IELs) and macrophages did not differ (N.S.) among segments and stages (Fig. 4a and 4b). High variation in the numbers of lymphocytes was, however, found between individual sows. Furthermore, the numbers of macrophages was low. Neutrophils were found only in infundibulum of two sows, one at prooestrus and the other one at oestrus.

Connective tissue

The two most commonly observed immune cell types in the connective tissue were lymphocytes and plasma cells, while very low numbers of mast cells, eosinophils, neutrophils and macrophages were found (Fig. 5a and 5b).

Significant differences among the segments of the oviduct were found for lymphocytes, plasma cells and neutrophils (Fig. 5a). Higher numbers of lymphocytes were found in the infundibulum and ampulla than in the isthmus ($P \leq 0.001$). A significantly higher numbers of plasma cells were found in the infundibulum than in ampulla and isthmus ($P \leq 0.001$). The lowest number of lymphocytes was found at oestrus (Fig. 5b), and it differ significantly from the lymphocyte number at anoestrus ($P \leq 0.05$). No significant difference among

stages was found for the numbers of plasma cells, mast cells, eosinophils and macrophages. Only occasional neutrophils (altogether 2 cells) were found in isthmus. Some neutrophils were found in ampulla and infundibulum but in few animals. Most of these neutrophils were found close to the basal lamina of the epithelium (Fig. 2). Neutrophils were found mainly at prooestrus and an overall significant difference among stages within segment was found in ampulla and infundibulum ($P \leq 0.05$). At prooestrus, the number of neutrophils was significantly higher in infundibulum than in ampulla ($P \leq 0.05$).

Immunohistochemical evaluation

Cell markers of CD2, CD3, CD79, CD14, SWC3 and MHC class II were analyzed in sows at prooestrus (3 sows) and anoestrus (4 sows).

In the immunohistochemical analyses, isotype controls were negative on sections of lymph node and oviduct. Sections of the lymph node were used as positive controls and showed labelling appropriate for each antibody.

The distribution of CD2 (Fig. 6a and 6b) and CD3 (Fig. 6c and 6d) positive cells was similar in both epithelium and connective tissue with higher numbers of CD2 positive cells than CD3 positive cells. The numbers of both cell markers was lower in the epithelial layer than in the connective tissue. In the connective tissue (Figs. 6a and 6c), the numbers of CD2 and CD3 positive cells was higher (NS) at prooestrus than at anoestrus. The numbers of CD2 positive cells in the infundibular epithelium was significantly higher than those in ampulla and isthmus ($P \leq 0.05$). Equal amounts of CD2 as well as CD3 positive cells were found in the ampulla and infundibular connective tissue, but the amounts in isthmus were significantly lower ($P \leq 0.05$) (Fig. 6b and 6d).

No CD79 positive cells could be detected in the epithelium of the oviduct (Fig. 6e and 6f). In the connective tissue, the numbers of CD79 positive cells was higher at anoestrus than at prooestrus and higher in infundibulum than ampulla and isthmus, however, the differences were not significant due to high individual variation.

CD14 and SWC3 positive cells were found mainly subepithelially in the connective tissue. Few cells were observed in the epithelial layer (Fig. 6g, 6h and 6i).

In the connective tissue of all segments, the CD14 positive cells were significantly higher at prooestrus than at anoestrus ($P \leq 0.05$) and, for both stages together, significantly higher in the ampulla and infundibulum than in isthmus ($P \leq 0.05$) (Fig. 6g and 6h). For the number of SWC3 positive cells, however, there was an interaction between segments and stages. The difference between each combination of segment and stage is shown in Fig. 6i. The number of SWC3 positive cells, found at prooestrus in infundibulum, was significantly higher than the numbers at anoestrus of all segments and at prooestrus of isthmus ($P \leq 0.05$).

In the epithelial layer, very few cells were positively stained with the MHC class II cell marker. In the connective tissue, in all segments and both at anoestrus and prooestrus, high numbers of cells were positively stained. In general, all

endothelial cells were positively marked for MHC class II. No counting was made because it was difficult to distinguish individual leukocytes from endothelial cells of capillaries.

Discussion

In this study on the sow oviduct, morphological differences were found, both between oestrous cycle stages and between segments of the oviduct. Pseudostratification of the epithelial cell layer in ampulla and infundibulum was high at prooestrus and oestrus, *i.e.* the stages when also the oestradiol plasma level was high. Nayak and Zimmerman (1971a) found that in the ampulla of ovariectomized gilts, oestradiol treatment increased the height of the epithelium while progesterone treatment resulted in prominent cytoplasmic protrusions. In this study, cytoplasmic protrusions in ampulla and infundibulum were found to be high at stages with progesterone levels being high (at dioestrus) and medium (at late dioestrus) as well as low (at prooestrus and anoestrus). Palmer et al. (1965) found high numbers of cell protrusions in the ampulla of lactating anoestrus sows with low progesterone levels. On the other hand, in gilts during the luteal phase, *i.e.* high progesterone, Abe and Oikawa (1992) observed bulbous processes of the secretory cells in ampulla and fimbriae of infundibulum by scanning electron microscopy. The cause of cytoplasmic protrusions has been explained in different ways. Mburu et al. (1996), studying isthmus of sows, suggested it to be an artefact as a result of luminal flushing prior to fixation. In ewes, Murray (1995) suggested that shedding of extruded cells into the oviductal lumen was a process of cell death. Yet another explanation of cell protrusion was given by Hollis et al. (1984). In ewes, they found that secretory granules were released by exocytosis into the oviductal lumen during the luteal phase accompanied with nucleated apical protrusions of these cells. In the present study, very few macrophages (which normally take care of dead cells) were found in the epithelium. Therefore, the apical protrusions shown in the present study are suggested to be a part of the process in which dead epithelial cells are eliminated.

This study showed that during all stages of the oestrous cycle, a lower degree of epithelial morphological changes (pseudostratification, mitosis and secretory granules) occurred in the isthmus than in ampulla and infundibulum. Most secretory granules were found at oestrus, which is in accordance with Nayak and Zimmerman (1971b) who, by electron microscopy of gilt ampullae and fimbriae, observed secretory granules at all stages but more abundant during oestrus. Similar results were found in ewes by Hollis et al. (1984). They observed that synthesis of secretory granules occurred in cells of the ampulla during the follicular and early luteal phases. *In vitro*, Buhi et al. (1989) showed that porcine epithelial cells of the ampulla had a significantly higher rate of secretory protein synthesis than cells of the isthmus. In this study, the mitosis observed at prooestrus and the secretory granules at oestrus, can be interpreted as signs on approaching higher activity. Especially during prooestrus and oestrus, functional changes take place in the oviduct which are very important for sperm capacitation and oocyte development (review Rodriguez-Martinez et al. 2001).

In this study, the degree of subepithelial connective tissue oedema could be indirectly estimated by scoring of fibroblast density. By this indirect scoring, oedema was estimated to be highest at oestrus, when also the plasma oestradiol-17β level was high, and lowest at anoestrus, particularly in ampulla and infundibulum. A positive influence of oestrogen on the degree of oviductal oedema was shown by Overstrom et al. (1980) who found in ovariectomized rabbits, by measuring the total water loss, that tissue water content increased in all regions of the oviduct after oestradiol-17β treatment.

The density of subepithelial connective tissue blood vessels found in this study, particularly the small sized vessels, was low at oestrus and prooestrus and higher at the other stages, especially at anoestrus. These results may be explained by the effect of a high tissue oedema at oestrus and a low degree of oedema at other stages, especially at anoestrus.

The results of the present study clearly showed that the most common immune cell type in the epithelial layer of all segments was the lymphocyte (intraepithelial lymphocytes: IELs) which is in agreement with observations in humans (van Bogaert et al. 1978; Morris et al. 1986). Lymphocytes and plasma cells were the major immune cells of the connective tissue in the present study. Lymphocytes were found in numbers approximately twofold higher than plasma cells, which is in accordance with a study on the human oviduct (Kutteh et al. 1990).

Lymphocyte subpopulations in the oviductal mucosa have been described in humans (Morris et al. 1986; Peters 1986; Boehme and Donat 1992). However, to our knowledge, the present study is one of the first to describe leukocyte subpopulations in the pig oviduct. Among those investigated in this study, we found that CD2 positive cells (marker for T-cells, B-cells and NK cells) were the most common lymphocyte subpopulation in both epithelium and connective tissue layers. T cells, as determined by a CD3 marker, were probably the major part of the CD2 population in both epithelium and connective tissue. B cells, as identified by CD79 positive cells were observed only in the subepithelial connective tissue layer. Our results agree with Boehme and Donat (1992) who also found, in human oviductal mucosa, that CD3 positive cells were the predominant cell type and that few B cells could be detected.

The numbers of the major immune cell types in the oviductal epithelium, IELs, and in connective tissue, lymphocytes and plasma cells, of the present study did not differ significantly between *stages of the oestrous cycle* except for neutrophils in the connective tissue of ampulla and infundibulum. However, the number of neutrophils was low. Also the distribution of leukocyte markers was similar for the prooestrus and anoestrus stages except for SWC3 and CD14 positive cells, which was higher in the connective tissue at prooestrus. These results suggest that the immune cell infiltration in the oviduct of sows, except for neutrophils in ampulla and infundibulum, is of less influence by ovarian steroid hormones. This is in contrast to a previous study of the endometrium from the same cyclic sows. The infiltration in the connective tissue of lymphocytes, neutrophils, eosinophils, and plasma cells (Kaeoket et al. 2001c) as well as T lymphocyte subpopulations (Kaeoket et al. 2001b) varied significantly in the endometrium during different stages of the oestrous cycle.

In this study, very few neutrophils were found in the epithelium, and there was no difference in numbers of IELs and macrophages between the epithelial *segments*. In contrast, in the subepithelial layer of the connective tissue, the numbers of lymphocytes and plasma cells as well as the numbers of CD2 and CD3 (at prooestrus and anoestrus) positive cells differed significantly between the segments, the lowest numbers were found in isthmus. Fertilization of oocytes takes place in the junction between ampulla and isthmus, AIJ (Hunter 1974). Therefore, in the isthmus, the immune cell reaction and phagocytic activities have to be low because in isthmus both sperms that move from UTJ to AIJ, and, after fertilization, the semi-allogenic conceptuses, must be able to survive. This is illustrated by the result in a study by Rodriguez-Martinez et al. (1990), who did not find any neutrophils in isthmus after insemination. However, further studies of the immune cell distribution of different segments, particularly in sows after insemination, is needed.

The higher numbers of CD2 than CD3 positive cells shown in the present study may suggest the presence of NK cells in the oviduct (Pescovitz et al. 1988). Studies of lymphocyte subpopulations in pig blood and lymphoid tissue showed that a large proportion of non-T-non-B lymphocytes with $CD2^{+}3^{-}4^{-}8^{lo}$ phenotype contained natural killer (NK) activity (Yang and Parkhouse 1996). In the present study, lymphocyte cell markers were analyzed only at prooestrus and anoestrus due to technical reasons. Therefore, further studies with analyses of different cell markers are needed in order to better understand the immune cell functions in the oviductal tissue of cyclic as well as inseminated sow.

In the present study, subepithelial neutrophils were found especially at prooestrus, and mainly in the infundibulum, *i.e.* the highest amount was found in the upper part of the oviduct. This is in accordance with our immunohistochemical results of SWC3 positive cells, which are the granulocyte and monocyte families. This indicates a local immune defence mechanism in the upper part of the oviduct that has a potential to respond quickly against microorganisms.

The numbers of plasma cells in the subepithelial layer of infundibulum in this study was approximately five times higher than previously reported for the endometrial subepithelial connective tissue of the same animals, cyclic groups, (Kaeoket et al. 2001c). Significantly higher density of plasma cells was found in infundibulum than in the other segments. Locally, subepithelial plasma cells produce immunoglobulins which are secreted into the oviductal lumen (Kutteh et al. 1990). This indicates that plasma cells may have a significant role in the early defence against the invasion of micro-organisms at the mucosal surfaces of the infundibulum as suggested being the case for intestinal mucosa (Kroese et al. 1994; Golby and Spencer 2002).

In this study, very few intraepithelial and subepithelial macrophages were found in the endosalpinx. Immunohistochemical staining with the CD14 marker, labelling monocytes and macrophages, confirmed the low infiltration of macrophages. This suggests that macrophage functions are of minor importance for immune response and tissue reorganisation in the oviduct as compared *e.g.* with the endometrium (Kaeoket et al. 2001c). The same can apply for eosinophils.

Mast cells were also found in low numbers but may still be of importance because of their vasoactive substances, *e.g.* histamine (Dynarowicz et al. 1988).

In the present study, high numbers of cells, particularly endothelial cells, were immunolabelled for MHC class II in the connective tissue. This indicates an antigen-presenting environment that is capable of providing strong stimuli to local T cells. Studies on pig intestinal cells have shown that endothelial cells express MHC class II (Wilson et al. 1996). However, functional studies of the role of endothelial cells as antigen-presenting cells in the pig oviduct need to be further assessed.

In the present study, very few MHC class II marked cells were detected in the oviductal epithelium. Other studies on human oviduct have reported that the oviductal epithelium strongly expressed MHC class II (Edelstam et al. 1992; Imarai et al. 1998). The divergent results may be due to species differences.

Conclusion

In the oviduct, the morphology differed in ampulla and infundibulum with oestrous cycle stages which indicates an effect by ovarian steroid hormones. The immune cell infiltration was less influenced by cyclic changes. However, the immune cell infiltration (in the connective tissue) in the upper part, especially infundibulum, differed significantly from the lower part, isthmus, indicating different immune functions within various parts of the oviduct.

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Table 1. *Monoclonal antibodies used for the immunohistochemical staining*

Antibody	Clone Number	Specificity	Source	Dilution
CD2	MSA4	T cells, NK cells and some B cells	VMRD (Pullman, Washington, USA)	1:100
CD3	PPT3	T cells	ImmunKemi F&D (Järfälla, Sweden)	1:1000
CD14	Mil-2	Monocytes, macrophages and weakly on granulocytes	Haverson and Watson*	1:20
CD79a	11E3	B cells	Novocastra Laboratories Ltd. (Newcastle, United Kingdom)	1:50
SWC3	74-22-15	Monocyte and granulocyte families	Dr. A. Saalmüller* (Tübingen, Germany)	1:200
MHC Class II	H42A	B cells, T cells, monocytes and macrophages and endothelial cells	VMRD (Pullman, Washington, USA)	1:1500

* = Kindly donated

Table 2. *Plasma levels of oestradiol-17β and progesterone (mean ± SD) of sows during 6 different stages; samples taken 1 h before slaughter*

Stages	progesterone (nmol/l)	oestradiol-17β (pmol/l)
Late dioestrus	22.0 ± 14.4	10.3 ± 2.1
Prooestrus	0.8 ± 0.4	62.0 ± 19.1
Oestrus	1.2 ± 0.8	69.0 ± 51.5
Early dioestrus	30.8 ± 4.0	11.0 ± 1.0
Dioestrus	86.6 ± 18.9	13.3 ± 4.1
Anoestrus	0.6 ± 0.4	11.5 ± 4.8

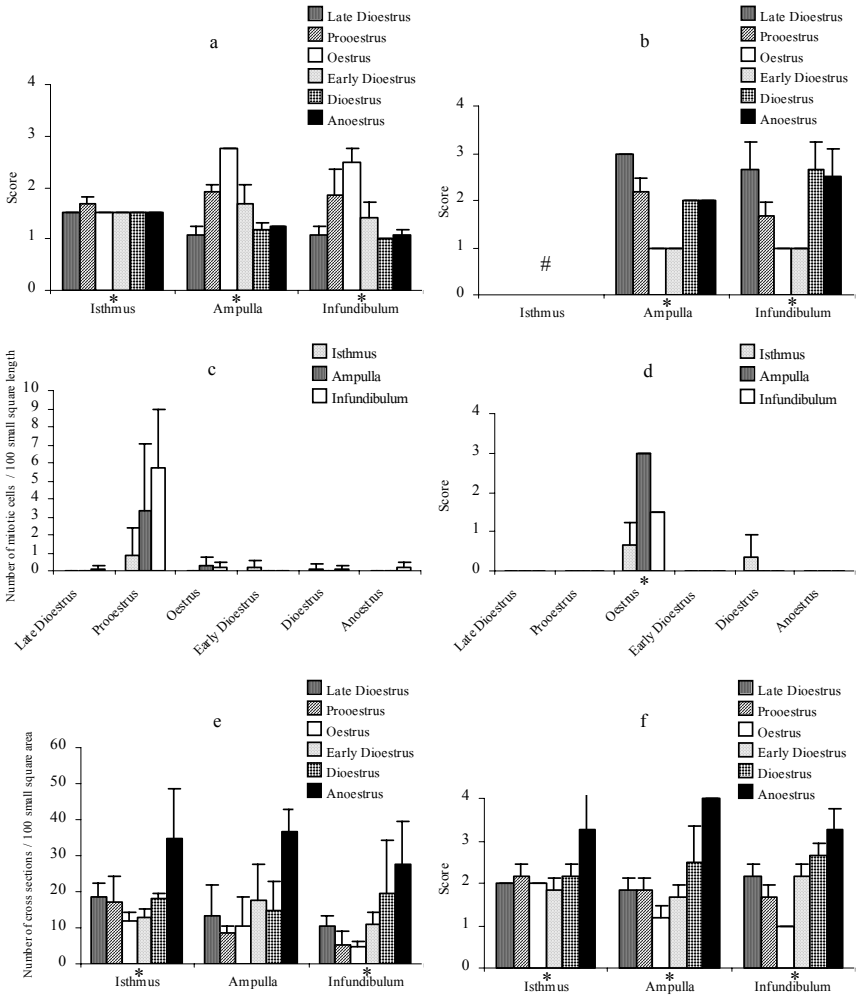

Fig. 1. Estimation of (a) pseudostratification, (b) cytoplasmic protrusions, (c) mitotic figures, and (d) secretory granules of the epithelial cells as well as the numbers of (e) small sized vessels, and (f) degree of fibroblast density in the connective tissue from different stages and segments of the oviduct presented as bars (mean ± SD). * = overall significant difference found among bars. # = not evaluated due to lack of free luminal cell area *i.e.* potential protrusions could not be observed.

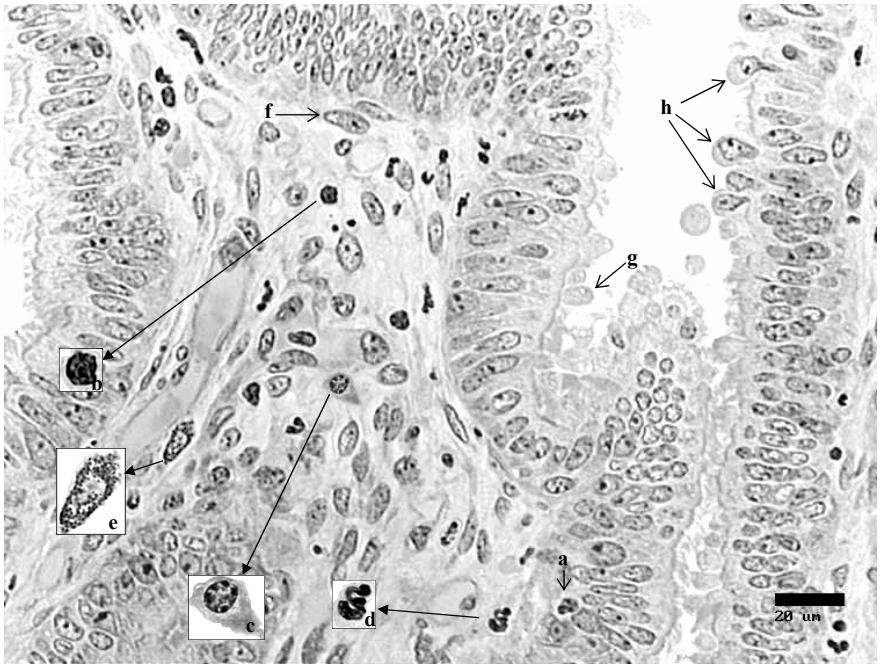

Fig. 2. Morphology of the porcine oviductal mucosa by light microscopy (infundibulum at prooestrus). a, IEL; b, lymphocyte; c, plasma cell; d, neutrophil; e, mast cell; f, fibroblast; g, cytoplasmic protrusion; and h, nucleated protruding cells.

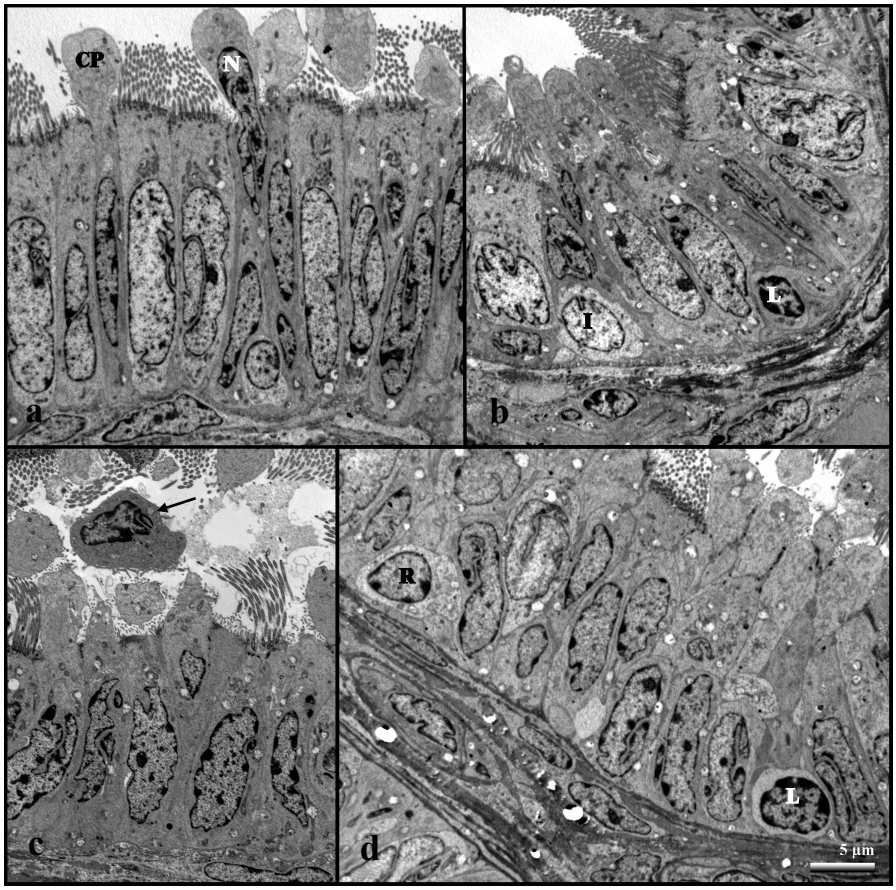

Fig. 3. Morphology of the porcine endosalpinx by electron microscopy (infundibulum at dioestrus). Showing (a) cytoplasmic (CP) and nucleated (N) protrusions, (b) sloughed epithelial cell in the oviductal lumen (arrow), (c) and (d) three types of mononuclear cells basally in the epithelium: round (R) or irregular (I) shaped nucleus with light cytoplasm and small amonts of heterochromatin clumped along the nuclear envelope, and lymphocyte-like cells (L) with a thin rim of cytoplasm and dense nuclear chromatin.

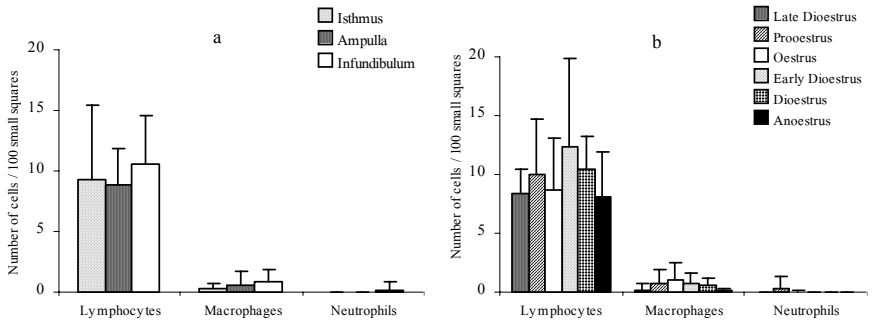

Fig. 4. Distribution of lymphocytes, macrophages and neutrophils in the epithelial layer of the sow oviduct presented as bars (mean ± SD). (a) In different segments of the oviduct. (b) At different stages of the oestrus cycle and at anoestrus.

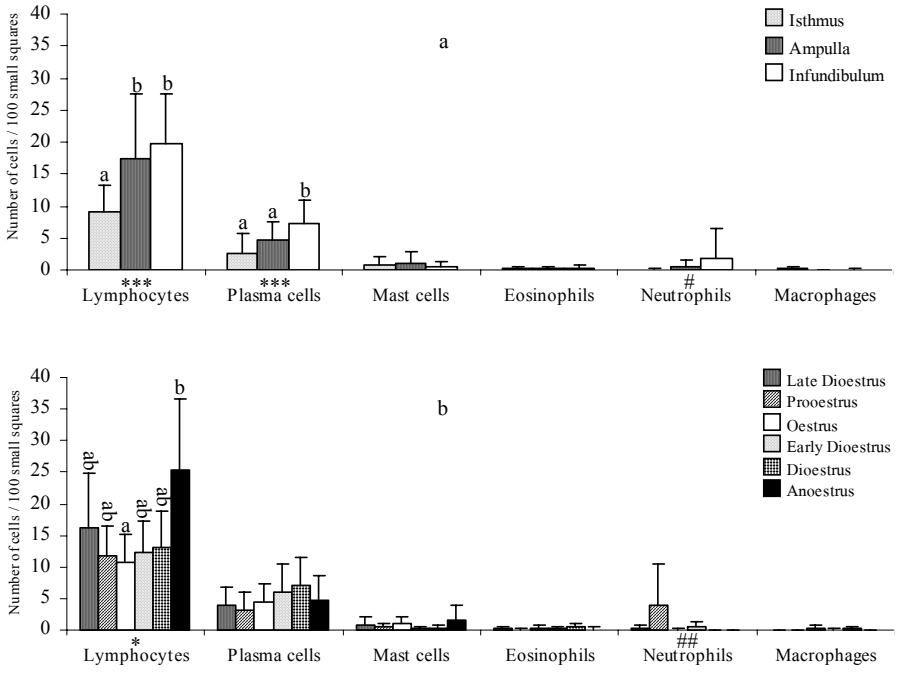

Fig. 5. Distribution of immune cells in the connective tissue of isthmus, ampulla and infundibulum presented as bars (mean ± SD). (a) In different segments of the oviduct, (b) A different stages of the oestrus cycle and at anoestrus. * and *** = overall significant difference found among bars with $P \leq 0.05$ and $P \leq 0.001$, respectively. Bars for the same type of positive cell marked by different letters are significantly different ($P \leq 0.05$). # = overall significant difference found at prooestrus. ## = overall significant difference found in ampulla and infundibulum.

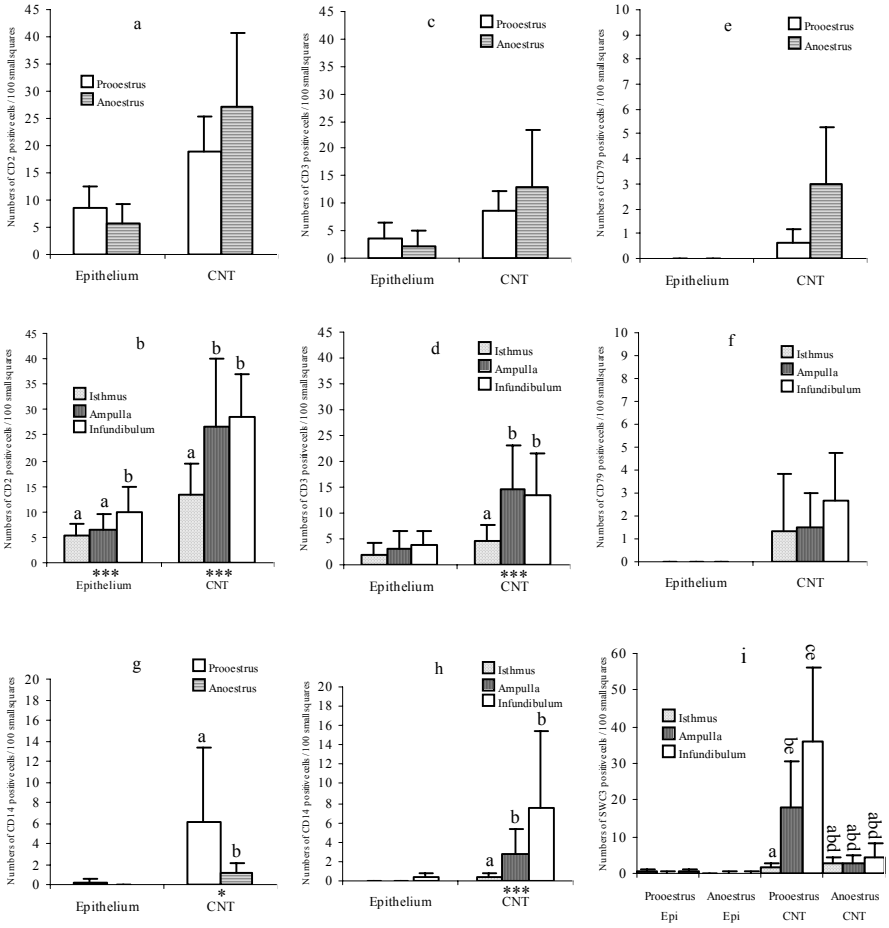

Fig. 6. Distribution (mean ± SD) of the (a and b) CD2, (c and d) CD3, and (e and f) CD79 and (g and h) CD14 positive cells within the epithelium and subepithelial connective tissue of the sow oviduct comparing differences between (a, c, e and g) stages (prooestrus and anoestrus) and (b, d, f and h) segments (isthmus, ampulla and infundibulum) and (i) SWC3 positive cells comparing the difference between stages and segments within the epithelium and subepithelial connective tissue. * and *** = overall significant difference found among bars with $P \leq 0.05$ and $P \leq 0.001$, respectively. Bars for the same type of positive cell marked by different letters are significantly different ($P \leq 0.05$). *Please note different scales.*

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Additional Comments and Perspectives

Few animals per group together with the individual variations observed in the present study should be considered. In spite of that, some interesting findings raise new questions about oviductal physiology. For example, apoptosis/cell death as a possible process in tissue regeneration should be further investigated. Perfusion fixed tissues would give better preservation for ultrastructural studies and immunohisto- and -cytochemistry would be valuable tools to study both cell death and epithelial leukocytes.

In the present study of normal cyclic sows, the density of immune cells was low in the isthmus and higher towards the upper part of the oviduct. Interesting would be to find out if the introduction of spermatozoa as foreign antigens or semen would change the immune cell pattern in the oviduct.

Further studies on the lymphocyte subpopulations are important for better understanding of the immunological control in the oviduct, especially identification of T cells and NK cells.

In the present study, the numbers of plasma cells were comparatively higher than in the previous study on the endometrium of the same sows. This indicates a functional importance of plasma cells in this part of the reproductive tract and the distribution of plasma cell isotypes should be further investigated.

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