การติดเชื้อในมดลูกสุนัขและการแสดงออกของตัวรับโทล-ไลค์ 2 และ 4 ในมดลูกสุนัข

นางสาวสร้อยสุดา โชติมานุกูล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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CANINE UTERINE INFECTION AND THE EXPRESSION OF TOLL-LIKE RECEPTOR 2 AND 4 IN CANINE UTERUS

Miss Sroisuda Chotimanukul

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…… Dean of the Faculty of Veterinary Science
(Professor Mongkol Techakumphu, D.V.M., Doctorate 3e cycle)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Padet Tummaruk, D.V.M., Ph.D.)

……..……. Thesis Advisor
(Associate Professor Sudson Sirivaidyapong, D.V.M., Ph.D.)

…….. Examiner
(Associate Professor Chainarong Lohachit, D.V.M., Dr. Med. Vet.)

…….. Examiner
(Suppawiwat Ponglowhapan, D.V.M., M.Sc., Ph.D.)

…….. External Examiner
(Associate Professor Kampon Kaeoket, D.V.M., Ph.D.)

สร้อยสุดา โชติมานุกูล : การติดเชื้อในมดลูกสุนัขและการแสดงออกของตัวรับโทล-ไลค์ 2 และ 4 ในมดลูกสุนัข (CANINE UTERINE INFECTION AND THE EXPRESSION OF TOLL-LIKE RECEPTOR 2 AND 4 IN CANINE UTERUS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.น.สพ.ดร.สุดสรร ศิริไวทยพงศ์, 141 หน้า.

การทดลองที่ 1 ศึกษาชนิดของเชื้อแบคทีเรียในช่องคลอดและมดลูกของสุนัขปกติและสุนัขที่มีภาวะมดลูกอักเสบแบบปิดและแบบเปิด ในการศึกษาพบเชื้อแบคทีเรียหลายชนิดในช่องคลอดและมดลูกของสุนัข ซึ่งในสุนัขปกติไม่พบความสัมพันธ์ของชนิดของเชื้อแบคทีเรียในช่องคลอดและมดลูก และในสุนัข 13 ตัวที่มีภาวะมดลูกอักเสบแบบปิดพบว่าสุนัข 10 (76.92%) ตัวไม่พบความสัมพันธ์ของชนิดของเชื้อแบคทีเรียระหว่างช่องคลอดกับมดลูก ในขณะที่สุนัขเพียงแค่ 6 ตัว (30%) จากทั้งหมด 20 ตัวที่มีภาวะมดลูกอักเสบแบบเปิด ไม่พบความสัมพันธ์ระหว่างชนิดของเชื้อแบคทีเรียระหว่างช่องคลอดกับมดลูก ดังนั้น ชนิดของเชื้อแบคทีเรียที่พบในช่องคลอดอาจไม่ได้เป็นสาเหตุเริ่มต้นของการติดเชื้อในมดลูกของสุนัขที่มีภาวะมดลูกอักเสบแบบปิด ในขณะที่ชนิดของเชื้อแบคทีเรียในช่องคลอดของสุนัขที่มีภาวะมดลูกอักเสบแบบเปิดอาจจะเป็นสาเหตุของการติดเชื้อในมดลูกหรืออาจจะไหลมาจากมดลูกในขณะที่ปากช่องคลอดเปิด

การทดลองที่ 2 ศึกษาปริมาณของเม็ดเลือดขาวในปีกมดลูก ตัวมดลูกและคอมดลูกในสุนัขปกติที่ระยะต่างๆ ของวงรอบการเป็นสัด ชนิดของเม็ดเลือดขาวที่พบได้แก่ลิมโฟไซต์ มาโครฟาจ นิวโทรฟิลและพลาสมาเซลล์ โดยส่วนใหญ่แล้วจะพบเม็ดเลือดขาวชนิดลิมโฟไซต์ เม็ดเลือดขาวทั้งหมด ($P<0.05$) และเม็ดเลือดขาวชนิดมาโครฟาจ ($P<0.01$) พบปริมาณมากอย่างมีนัยสำคัญทางสถิติในชั้นใต้เยื่อบุมดลูกของสุนัขที่อยู่ในระยะแอนเอสตรัสเทียบกับระยะอื่นๆและพบว่าปริมาณเม็ดเลือดขาวทั้งหมดในชั้นเยื่อบุปีกมดลูกลดลงอย่างมีนัยสำคัญทางสถิติในระยะเอสตรัส ($P<0.01$) แต่ลดลงอย่างมีนัยสำคัญทางสถิติในระยะแอนเอสตรัส ($P<0.05$) ที่เยื่อบุคอมดลูกเมื่อเทียบกับระยะอื่นๆ จากการศึกษาพบว่าปริมาณของเม็ดเลือดขาวมีความแตกต่างกันในแต่ละชั้นของเนื้อเยื่อมดลูกและในแต่ละบริเวณของมดลูกสุนัขที่ระยะต่างๆ ของวงรอบการเป็นสัด ซึ่งบ่งบอกถึงบทบาทการทำงานที่ต่างกันของระบบภูมิคุ้มกันในการตรวจตราและป้องกันไม่ให้เกิดการติดเชื้อในมดลูกสุนัข

การทดลองที่ 3 ศึกษาการแสดงออกของตัวรับโทล-ไลค์ 2 และ 4 ในมดลูกสุนัขที่ระยะต่างๆ ของวงรอบการเป็นสัดและในสุนัขที่มีภาวะมดลูกอักเสบ พบว่าการแสดงออกของตัวรับโทล-ไลค์ 4 ในชั้นเยื่อบุมดลูกสูงขึ้นอย่างมีนัยสำคัญทางสถิติในสุนัขที่มีภาวะมดลูกอักเสบเทียบกับสุนัขกลุ่มอื่นๆ ($P<0.01$) ในขณะที่การแสดงออกของตัวรับโทล-ไลค์ 2 ในชั้นเยื่อบุตัวมดลูกลดลงอย่างมีนัยสำคัญทางสถิติในสุนัขที่มีภาวะมดลูกอักเสบ ($P<0.05$) และพบการแสดงออกของตัวรับโทล-ไลค์ 2 ในชั้นเยื่อบุมดลูกแต่ไม่พบในชั้นใต้เยื่อบุมดลูก ในสุนัขที่มีภาวะมดลูกอักเสบเมื่อเปรียบเทียบในแต่ละบริเวณของมดลูกพบว่าการแสดงออกของตัวรับโทล-ไลค์ 4 มากขึ้นอย่างมีนัยสำคัญทางสถิติในชั้นเยื่อบุและชั้นใต้เยื่อบุปีกมดลูก ($P<0.01$) เปรียบเทียบกับตัวมดลูกและคอมดลูก แต่ในทางตรงกันข้ามการแสดงออกของตัวรับโทล-ไลค์ 2 ในชั้นเยื่อบุคอมดลูกสูงขึ้นอย่างมีนัยสำคัญทางสถิติ ($P<0.01$) เทียบกับปีกมดลูกและตัวมดลูก จากการศึกษาพบว่าระดับของการแสดงออกของตัวรับโทล-ไลค์ 2 และ 4 ที่แตกต่างกันเกี่ยวข้องกับชนิดของเซลล์ที่อยู่ในมดลูก, เม็ดเลือดขาวและฮอร์โมนเพศ

ภาควิชา สูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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SROISUDA CHOTIMANUKUL: CANINE UTERINE INFECTION AND THE EXPRESSION OF TOLL-LIKE RECEPTOR 2 AND 4 IN CANINE UTERUS. ADVISOR: ASSOC. PROF. SUDSON SIRIVAIDYAPONG, D.V.M., Ph.D., 141 pp.

EXP. 1 aimed to identify the bacterial species in the vagina and uterus healthy bitches and bitches with closed-cervix and opened-cervix pyometra. Various bacterial species were found from the vagina and uterus of bitches in this study. No healthy bitches had a relationship between bacterial species in vagina and uterus and in 13 bitches with closed-cervix pyometra, the bacterial species of 10 bitches (76.92%) were not related between vagina and uterus. Meanwhile, in 20 bitches with opened-cervix pyometra, the bacterial species between vagina and uterus in 6 bitches (30%) had no relationship. Bacteria found in the vagina may not be an initial cause of uterine infection in bitches with closed-cervix pyometra. Bacterial species in the vagina of bitches with opened-cervix pyometra may be a cause of uterine infection or can be the result of uterine drainage.

EXP. 2 aimed to investigate the number of leukocytes in the horn, body and cervix of the uterus in healthy dogs during the estrous cycle. The leukocytes that found from this study were lymphocytes, macrophages, neutrophils, and plasma cells. And lymphocytes were the most common leukocytes found in the uterus. In the endometrial stroma, the total leukocytes ($P < 0.05$) and the number of macrophages ($P < 0.01$) were significantly increased in anestrous dogs compared to other stages. The number of total leukocytes in the surface epithelium of the uterine horn significantly decreased at estrus ($P < 0.01$) but significantly decreased at anestrous stage in the cervix compared to other stages ($P < 0.05$). From this study, the number of leukocytes varied in a different tissue layers and different regions of the uterus at different stages of the estrous cycle which indicated the different role in the uterine immune surveillance protecting the host from the pathogen invasion.

EXP. 3 aimed to investigate the expression of TLR2and TLR4 in canine uterus during the estrous cycle and in pyometra. The expression of TLR4 in the endometrial surface epithelium was significantly higher in dogs with pyometra compared with all other groups ($P < 0.01$). While, the expression of TLR2 in the surface epithelium of the uterine body was significantly decreased in pyometra dogs ($P < 0.05$). Interestingly, TLR2 was expressed in endometrial epithelium but absent in the endometrial stroma of healthy dogs at all stages. In dogs suffering from pyometra, when compared between the uterine regions, the expression of TLR4 was significantly more intensely in the surface epithelium and stroma of the uterine horn compared to the uterine body and the cervix ($P < 0.01$). Conversely, the expression of TLR2 in the surface epithelium of the cervix was significantly higher than the uterine horn and body ($P < 0.01$). From this study, the different levels of TLR2 and TLR4 expression related to distinct cell types of uterus, leukocytes populations and sex hormones.

Department : Obstetrics Gynaecology and Reproduction Student's Signature .....

Field of Study : Theriogenology Advisor's Signature .....

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LIST OF ABBREVIATION

CD	Cluster of differentiation
CEH	Cystic glandular hyperplasia
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBD	Human beta-defensin
IFN-γ	Interferon-gamma
IL	Interleukin
Ig	Immunoglobulin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
Muc-1	Mucin-1
NAP	Natural Antimicrobial peptide
NK cell	Natural killer cell
PAMP	Pathogen associated molecular pattern
PBL	Peripheral blood leukocyte
PMN	Polymorphonuclear leukocyte
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
SLPI	Secretory leukocyte protease inhibitor
Th cell	T helper cell
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The female reproductive tract is expose to the outside environment and must have sufficient immunological defense mechanisms to avoid infection (Abrahams and Mor, 2005; Aflatoonian and Fazeli, 2008) while it must tolerate allogeneic sperm and permit implantation and fetal development. To accommodate these disparate functions and protection against potential pathogens, the female reproductive tract relies upon both innate and adaptive immune systems (Medzhitov, 2001; Pioli et al., 2004). However, the innate immune system is the most universal, the most rapidly acting and by some appraisals, the most important type of immunity (Beutler, 2004). Mucosal epithelial cells are the first line of defense against potentially pathogenic microorganisms by the development of the innate immune mechanisms that can immediately inhibit microorganism growth (Hecht, 1999; Fahey et al., 2005). Furthermore, in the female reproductive tract, epithelial cells are in constant contact with the normal flora and must differentiate between commensal microorganisms and pathogens in order to generate protective immune responses. Therefore, control of normal flora along with rapidly recognition of pathogens by cells of the innate immune system is important to the survival of the host (Soboll et al., 2006). Rapid innate immune defense mechanisms against infection involved the recognition of invading pathogens by pattern recognition receptors (PRRs) attributed to the Toll-like receptors (TLRs). The member of the TLR family recognize distinct pathogen-associated molecular patterns (PAMPs) produced by various bacterial, viral and fungal pathogens (Medzhitov and Janeway, 2000; Janeway and Medzhitov, 2002; Aflatoonian et al., 2007) and play the important role in activating intracellular signaling pathways after PAMP engagement (Werling and Coffey, 2007). TLRs signals initiate acute inflammatory responses by induction of antimicrobial genes, inflammatory cytokines and chemokines which resulting in the recruitment of neutrophils and activation of macrophages, lead to direct killing of pathogens (Takeda

et al., 2003; Pasare and Medzhitov, 2004). Many cases of inflammatory disease in the reproductive tract of dogs and cats are considered to be caused by infectious agents (Schultheiss et al., 1999). In the bitch, the most important pathological conditions of the uterus due to uterine infection is pyometra (chronic uterine inflammation with accumulation of pus in the uterus) (Ishiguro et al., 2007). Uterine pathology due to cystic endometrial hyperplasia (CEH)/pyometra complex can be also responsible for pregnancy loss that has been related to infertility (inability of the embryos to implant) in bitches. Even though the association between diestrus and pyometra has been well established, the precise mechanism is still unclear (Verstegen et al., 2008). However, Niskanen and Thrusfield (1998) indicated that no significant effect of progesterone treatment was found. However, if the effect of progesterone present, the risk seems to be low whereas, nulliparous dogs had a moderately higher risk of developing pyometra. In general principle, CEH-associated degenerative changes within the uterine tissue are suggested to supply appropriate conditions for establishment of uterine infection. Nevertheless, while general principle indicates that CEH commonly exists prior to pyometra development, it is also apparent that CEH does not develop to pyometra in all dogs. On the other hand, pyometra can develop in young dogs which do not have previous pathological or clinical sign of CEH (Verstegen et al., 2008). The classical model of progesterone leading to CEH and consequently, CEH to pyometra may not be true. The sequence may be inverted with bacteria being the primary cause. The subclinical uterine infection may first occur during the first half of diestrus or at the end of estrus or, supplying the stimulus for an inordinate endometrial hyperplasia or hypertrophy which leading to the increase in surface epithelial cells secretions and uterine glandular proliferation that can stimulate the progression of pyometra (Koguchi et al., 1995; Nomura, 1994, 1995, 1996a, 1996b, 1997; Nomura and Makino, 1997; Nomura and Nishida, 1998; Verstegen et al., 2008). Nevertheless, this concept was questioned by De Bosschere et al. (2001), which can also be supported by clinical observations of pyometra in young dogs that CEH is absent. Therefore, at present, pyometra is seemed to be a result from the interplay of pathogenic bacteria and progesterone primed uterus, in a sequence which still need to be further investigated (Verstegen et al., 2008). The hormonal levels fluctuate during the estrous cycle which may influence uterine immunity and the susceptibility to the

uterine infection. Thus, sex hormones play a very important role in controlling host immune system in the reproductive tract, discriminating this from other mucosal organs (Entrican and Wheelhouse, 2006). The difference of TLRs expression in the uterus may be related to the different levels of sex hormones (Hirata et al., 2007). Moreover, of the 13 TLRs identified, TLR2 and TLR4are the best understood in terms of responses to Gram-positive bacteria and Gram-negative bacteria respectively (Darville et al., 2003).

1.2 Literature review

1.2.1 Canine uterine infection

The female reproductive tract is frequently exposed to pathogens. The mucosal surfaces are the primary sites of pathogen entry in the body (Mowat, 2003). In normal dogs, bacteria are usually found in the uterus at proestrus and estrus, but rarely at other stages of the estrous cycle (Watts et al., 1996; Kida et al., 2006). This may be due to bacteria invade into the uterus through the cervical opening at estrus (Baba et al., 1983; Kida et al., 2006). Canine uterine infections are associated with bacteria (Baba et al., 1983), which can be occurred in many circumstances and cause a detrimental effect to the bitch. Uterine pathology and endometritis of bitches are usually a result of uterine bacterial infection that can be leading to illness, infertility and pregnancy losses (Verstegen et al., 2008). The commonest form of endometritis in the dog is the condition loosely described as pyometra (Dow, 1958). Pyometra is the most common infectious disease in the uterus of bitches (Sugiura et al., 2004). Pyometra is the accumulation of purulent material within the uterine lumen of intact dogs (Pretzer, 2008). The clinical disease varies greatly in severity and is influenced by a number of factors. The commonest lesion is cystic glandular hyperplasia of endometrium (Dow, 1958). Because of this association between cystic glandular hyperplasia and endometritis, Dow (1958) have been described the entire range of lesions under the broad term of CEH/pyometra complex. The series has been divided into 4 major groups on a histological basis. Group 1, cystic glandular hyperplasia of the endometrium without any superimposed inflammatory reaction, an increase in the

number of glandular elements in all layers of the endometrium is found. Group 2, diffuse plasma cell infiltration of the endometrium superimposed on cystic glandular hyperplasia, the uterus reveals a marked cystic glandular hyperplasia and diffuse infiltration of the endometrium by plasma cells. Group 3, acute inflammatory reaction in endometrium exhibiting a variable degree of cystic glandular hyperplasia, which can be characterized by congestion, edema and infiltration by neutrophils. Group 4 is chronic endometritis in the series. These were divided into two distinct sub-groups according to the degree of patency of the cervix. The closed-cervix reveals marked atrophy of the endometrium so that in many parts, only a few collagen fibers separate the myometrium from the surface epithelium of the endometrium. Glands are rare thought a few may be seen penetrating the myometrium. The endometrium shows infiltration by lymphocytes and a few plasma cells. In the opened-cervix, there is a commonly considerable myometrial hypertrophy and fibrosis. The endometrium is atrophy and infiltrated by lymphocytes and occasional plasma cells (Dow, 1958). Pyometra is considered as a disease of diestrus, even if some anestrous dogs could be diagnosed with pyometra (Noakes et al., 2001; Verstegen et al., 2008). Furthermore, pyometra in the bitch is also considered to result from bacterial and hormonal interaction (Hardy, 1980; Arthur et al., 1989; Zaragoza et al., 2004). The importance of progesterone in the pathogenesis of this disease is related to its inhibition of immune responses, stimulation of uterine glandular secretions which provide a suitable environment for bacterial development, functional of the cervical closure which suppresses the drainage of exudates from the uterus and the mediation of CEH. By the way, even though bacteria are present, pyometra rarely occurs in the period of proestrus or estrus. The uterus of dog might thus have some defense mechanisms from bacterial infection in these periods and these mechanisms may disappear in the first half of diestrus (Kida et al., 2006).

1.2.2 Innate immunity in female reproductive tract

The female reproductive tract has developed immune systems to defense against pathogens (Wira et al., 2005). The immune system has been classified into innate and adaptive immune system, each with a different role and function. The adaptive immune system is organized into two types of specialized lymphocytes, T cells and B cells, each of that demonstrate a specific receptor with a unique antigen, leading to a diverse and large repertoire of antigen receptors within the T or B cell population. This diversity is essential to certify that each individual cell can encounter its unique antigen, hence inducing proliferation, maturation and activation of these cells. The process itself is relatively delay and always taking days before enough effecter cells and the products of these cells can be produced. This delay supplies the majority of microorganisms that invade into the body with time to attach and completely replicate in the body. Conversely, the effecter systems of innate immune mechanisms are activated after infection immediately to control the replication of invading microorganisms at the area of entry (Werling and Coffey, 2007). Innate immunity is composed of the sensing or afferent arm and the effecter or efferent arm. Each arm of innate immune system may further be classified into cellular and humoral components. It is largely determined that any true immune system, nevertheless primitive or advanced, must be able to do three things: firstly, recognition of a various invading microorganisms; secondly, killing microorganisms once they are recognized; and thirdly, sparing the host tissues. In vertebrates, innate immune system is widely dependent upon myeloid cells, professional immune cells that can engulf and destroy invading microorganisms. These cells have stand-alone abilities, however they have developed the best function in combination with proteins and cells of the adaptive immune system. The mononuclear cells and polymorphonuclear cells are the myeloid cells. The mononuclear phagocytes include macrophages and dendritic cells. However, dendritic cells exist as a small amount among the mononuclear cells. While, the polymorphonuclear cells, that compose of neutrophils, eosinophils and basophils are important key protecting the host from infection (Beutler, 2004).

The epithelium forms the lining of the cavities of reproductive mucosa by forming impenetrable tight junctions coated with mucous that act as a mechanical barrier in preventing invading pathogen from gaining access to the interior of the body (Pitman and Blumberg, 2000). The uterine defense mechanisms against invading pathogens are maintained in several ways: chemically, by mucus secretions from the uterine glands; anatomically, by the simple or pseudostratified columnar epithelium covering the uterus; immunologically, by the action of polymorphonuclear cells and humoral antibodies (Asbury et al., 1980; Dhaliwal et al., 2001). The innate immune mechanisms of the female reproductive tract is a dynamic and complex system including specific barriers, commensal microorganisms, cellular effectors and bactericidal peptides and proteins (Quayle, 2002; Russell and Mestecky, 2002; King et al., 2003; Mak et al., 2004). The innate immune defense mechanisms of the female reproductive tract has developed to eradicate sexually transmitted infection whereas maintaining the capability to adjust specialized physiological functions which contain fertilization, implantation, pregnancy, parturition and menstruation (Horne et al., 2008). The innate immune system incorporates more rapid and fundamental reactions to infection than adaptive immune system, such as surface defenses, phagocytic responses, complement activation and cytokine elaboration (Janeway and Medzhitov, 2002; Tosi, 2005; Horne et al., 2008). Hence, the innate immune system plays an important role in the recognition of invading pathogens (Takeda et al., 2003).

1.2.3 Toll-like receptors (TLRs) and their ligands

The key mediators of the innate immune system are natural antimicrobial peptides (NAPs) and pattern recognition receptors (PRRs) (Wira et al., 2005; Horne et al., 2008). TLRs are the major family of PRRs (Akira et al., 2001; Takeda and Akira, 2005; Aflatoonian and Fazeli, 2008), and they are expressed by cells as in the first line of defense such as mucosal epithelial cells, macrophages, neutrophils and dendritic cells (Aflatoonian et al., 2007). To date, 13 murine TLRs and 11 human TLRs have been described (Lin et al., 2011). TLRs recognized conserved pathogen-associated molecular patterns (PAMPs) produced by pathogens including bacteria,

viruses, fungi and parasites as well as endogenous ligands involved with cell damage such as heat-shock protein 60 (Nasu and Narahara, 2010) as shown in table 1.

TLRs	Ligands
TLR1	Triacyl lipopeptides
TLR2	Peptidoglycan, lipoteichoic acid,
	lipoprotein, lipopeptides (Gram-positive bacteria)
	Zymozan (fungi)
	Glycolipids (protozoa)
TLR3	dsRNA (virus)
	Poly (I : C) (synthetic dsRNA)
TLR4	LPS (Gram-negative bacteria)
	Taxol (plant)
	Envelope protein , fusion protein (virus)
	Heat-shock protein 60
TLR5	Flagellin (bacteria)
TLR6	Diacyl lipopeptide (Mycoplasma)
TLR7	ssRNA (virus)
	Imidazoquinoline (synthetic antiviral compound)
TLR8	ssRNA (virus)
TLR9	Hemozoin (protozoa)
	CpG-ODN (synthetic CpG-rich oligonucleotide)
TLR10	Unknown
TLR11	Profilin-like protein (*Toxoplasma gondii*)
TLR12	Unknown
TLR13	Unknown

Table 1 TLRs and their ligands (modified from Lin et al., 2011)

1.2.4 Role of TLRs

TLRs are the cellular components of the afferent arm of the innate immune system (Beutler, 2004). Thereby, various members of TLR family which expressed on various cell types appears to mediate signal transduction to a range of antigenic stimuli by binding with specific ligands leading to the production of different proinflammatory cytokines, chemokines and effecter molecule depend on the type of cell that is stimulated (Aflatoonian et al., 2007). Recognition of pathogens by TLRs also triggers activation of adaptive immune system, making this receptor family an important link between innate and adaptive immune system (Takeda et al., 2003; Werling and Jungi, 2003; Linde et al., 2007). The signals for stimulation of adaptive immune system are widely supplied by dendritic cells. In the periphery, immature dendritic cells have a high capacity for endocytosis that allows antigen uptake. These cells are stimulated by different pathogen components to undergo maturation and express many of TLRs. Furthermore to regulating the evolvement of adaptive immune system, TLRs activation may be directly related to induction of antimicrobial function. TLRs are likely to activate the secretion of antimicrobial peptides, consequently controlling the direct killing of pathogens at the surface epithelium (Takeda et al., 2003). Besides, Macrophages infected with invading pathogens undergo apoptosis (Zychlinsky et al., 1992; Takeda et al., 2003). The induction of apoptosis may restrict the expansion of pathogens by localizing cell death at the area of infection. Many pathogen components such as lipopolysaccharide or lipoprotein trigger apoptosis of macrophages and endothelial cells. TLR2 confers lipoprotein-induced apoptosis of macrophage suggesting the plausible influence of TLRs in infection-induced cell death (Aliprantis et al., 1999; Takeda et al., 2003). In addition, TLRs are complicit in not only immune responses but also more general cellular homeostasis (Takeda and Akira, 2004; Hopkins and Sriskandan, 2005; Linde et al., 2007).

1.2.5 TLRs in female reproductive tract

In female reproductive tract, receptors that recognize conserved PAMPs present on microorganisms are expressed in epithelial cells (Lea and Sandra, 2007). the female reproductive tract express TLRs 1-10 (Darville et al., 2003; Pioli et al., 2004; Aflatonian et al., 2007; Horne et al., 2008). While, the first reported of mRNA for TLR1, 2, 3, 5, and 6 are found to be expressed in cell lines of epithelium from the lower part of human female reproductive tract (endocervix, ectocervix, vagina) (Fichorova et al., 2002; Yu et al., 2009). There is also demonstrating that TLR2 and TLR4 are found in cell lines of human vaginal epithelium (Pivarcsi et al., 2005; Yu et al., 2009). Furthermore, endometrial epithelial cell lines, primary endometrial cells and primary decidual cell cultures express TLR1-9 which indicate the capability of these receptors to recognize a wide range of pathogens (Schaefer et al., 2004, 2005; Krikun et al., 2007; Horne et al., 2008), however each cell line appears to show various profiles of TLR expression (Young et al., 2004; Yu et al., 2009). The immunohistochemical study in vivo confirmed that TLR1, 2, 3, 5, and 6 are present in the epithelium of various parts of the female reproductive tract in human. Interestingly, TLR2 is expressed dominantly in cervical tissues and fallopian tubes, followed by ectocervix and endometrium. Conversely, TLR4 is expressed dominantly in the upper parts of the female reproductive tract (uterine tube, uterus) and the lack of this receptor is found in ectocervix and vagina (Fazeli et al., 2005; Yu et al., 2009). These results indicate that TLRs are differentially expressed in distinct compartments of the female reproductive tract. It is probably that the distribution of TLR in the female reproductive tract indicates the immunotolerance to the commensal microorganisms in lower parts of the female reproductive tract and the intolerance to commensal microorganisms in the upper part of the female reproductive tract (Pioli et al., 2004; Fazeli et al., 2005; Yu et al., 2009). Nevertheless, the expression of some TLRs in the distinct part of the human female reproductive tract alters slightly according to the studies from various laboratories (Yu et al., 2009). Both mRNA and protein for TLR2 and TLR4 have been detected in the epithelium of vagina in another study (Pivarcsi et al., 2005; Yu et al., 2009). Moreover, Yu et al. (2009) found the expression of TLR4 in squamous epithelium of cervix using immunohistochemistry.

The expression of TLR4 mRNA and protein has been studied in epithelial cells and stromal cells of human endometrium (Hirata et al., 2005; Yu et al., 2009) and stromal fibroblasts of fallopian tube, but not in epithelial cells of fallopian tube (Itoh et al., 2006; Yu et al., 2009). Besides to TLR1-6, the expression of TLR9 in endometrial tissue was detected (Young et al., 2004; Yu et al., 2009). In contrast to this study, except for these TLRs, the expression of TLR7, 8, and 10 was also observed in endometrial tissue of human by immunohistochemical staining (Aflatoonian et al., 2007; Yu et al., 2009). The differences of the results may be due to the difference of specimen, techniques and physiological status. Consequently, the signaling of TLR that mediated inflammatory cells and antimicrobial peptides may play important functions in immune system in order to eliminate invading pathogens in the female reproductive tract (Yu et al., 2009).

1.2.6 TLRs and sex hormones regulation

In human, reproductive cycle changes under the control of sex hormones influence a wide range of genes in the uterus and ovary, that act to prevent these tissues against pathogens invasion whereas concurrently preparing them for ovulation, menstruation and implantation by regulate the anatomical and histological characteristics of the uterus. Sex hormones are also related to the influx and localization of leukocytes in the uterus (Spornitz, 1992; Yeaman et al., 1997; Aflatoonian and Fazeli, 2008). Estradiol has proinflammatory functions in the uterus and has been related to an influx of leukocytes in the mouse at estrus (De and Wood, 1990; Lea and Sandra, 2007). In rodents, estradiol may play a role in the recruitment of leukocytes as more macrophage infiltrates the endometrium when estradiol concentration is higher (Herath et al., 2006b; Khan et al., 2009). Besides, estradiol changes the nature of the endometrial epithelial cells, decreasing the attachment of pathogens (Nishikawa, 1985; Nishikawa and Baba, 1985; Sugiura et al., 2004) and increasing the secretion of bactericidal lactoferrin (Teng et al., 2002a, 2002b; Sugiura et al., 2004). In rat uterus, estradiol stimulates epithelial cells to produce factors with broad-spectrum anti-bacterial function (Fahey et al., 2005). Conversely, progesterone has been related to anti-inflammatory function (Lea and Sandra, 2007) by inhibits the

immune response to make the uterus more susceptible to spontaneous infection (Olson et al, 1984; Lewis, 2003; Herath et al., 2006a). Elevated blood progesterone concentrations inhibit production of chemokines in uterus for influx of monocytes and neutrophils (Critchley et al., 2001; Sugiura et al., 2004), and inhibit both peripheral blood and uterine neutrophil phagocytic functions (Vandeplassche, 1981; Dhaliwal et al., 2001). Moreover, in human, cattle and rodents, progesterone inhibits uterine immune response by decreasing the proliferative function of lymphocytes. (Herath et al., 2006b; Khan et al., 2009). Consequently, the antigen-specific immunity changed by sex hormones appears to play an important role in protecting the uterus from infectious (Sugiura et al., 2004). While, in the period of increased uterine receptivity, epithelial cells exhibit increased expression of TLR and changed production of specific antimicrobial peptides, therefore improving the capability to both recognize and respond to PAMPs on microorganisms. Ovulation, implantation and menstruation are widely characterized by alters in the type and distribution of both immune and non-immune cells in the uterus and/or ovary (Lea and Sandra, 2007). Aflatoonian et al. (2007) found that the lowest amount of TLR2-6, 9 and 10 genes in the endometrial tissue is expressed at proliferative and menstrual phase of the reproductive cycle. Estrogen levels are higher at proliferative phase compared to secretory phase. Meanwhile, the level progesterone is relatively higher at secretory phase compared to proliferative phase. This may suggest the suppressing effect of estrogen and/or a stimulating effect of progesterone on TLR expression in the endometrium. In addition, Hirata et al. (2007) found that TLR2-4, 9 in the human endometrium being high in the perimenstrual phase and low in the periovulatory phase. It has been observed that the proliferative phase of the reproductive cycle, particularly day 1 to 7, is the major risk factor for ascending infection by pathogens (Korn et al., 1998; Eckert et al., 2002; Hirata et al., 2007). In human, menstrual blood and shed endometrium could be a favorable environment for bacterial growth. Hence, increased TLRs expression in menstrual phase might be a possible immune defense mechanism of the uterus (Hirata et al., 2007). The menstrual phase is a stage when numerous leukocytes are recruited in the endometrium (Salamonsen and Woolley, 1999; Hirata et al., 2007). Leukocytes are the major source of interferon gamma in the endometrium (Yeaman et al., 1998; Hirata et al., 2007), and interferon gamma upregulates TLR4

expression in the stromal cells of endometrium (Hirata et al., 2005, 2007). Accordingly, TLRs and leukocytes may coordinately prevent the uterus from pathogen invasion (Hirata et al., 2007). In contrast, decreased expression of TLRs in periovulatory phase might protect inappropriate inflammatory response of the uterus evoked by pathogens contaminants with upcoming sperm (Friberg et al., 1987; Svenstrup et al., 2003; Hirata et al., 2007). From the previous studies indicate that sex hormones may control the expression and function of TLRs in the female reproductive tract, and consequently regulate or influence innate and adaptive immune system to prevent against pathogens whereas supplying a suitable environment which supports the fetus (Aflatoonian and Fazeli, 2008).

1.2.7 TLRs in domestic animals

In the last few years, studies on TLRs has been made in identifying TLRs in different domestic animal species which compose of cattle, buffalo, sheep, goat, horse, pig, chicken, cat and also dog (Kannaki et al., 2011). In dogs, the differential expression of lactoferrin, a family of antimicrobial peptides (Kida et al., 2006) and Muc-1, an anti-adhesive molecule (Ishiguro et al., 2007) have been reported in the uterus. However, in dog, only little is studied about TLRs functions, and most reports are restricted to mRNA levels (Bazzocchi et al., 2005; Swerdlow et al., 2006; Linde et al., 2007; House et al., 2008; McMahon et al., 2010). Recently, gene transcriptions of TLR2 and TLR4 by real-time PCR have been reported in endometrium of diestrous dogs and dogs with pyometra (Silva et al., 2010). TLR2 mRNA is found in mononuclear cells of blood, lymph node, lung, liver, spleen, bladder, pancreas, small intestine, large intestine and skin of the dog. Since TLR2 is expressed in wide range organs, TLR2 would be important in the immune system in different organs of the host (Ishii et al., 2006). While, TLR4 mRNA is expressed dominantly in peripheral blood leukocytes (PBL), moderately in spleen, stomach and small intestine, and low levels in liver, with absent in skin, kidney and large intestine (Asahina et al., 2003). Nevertheless, in canine reproductive tract, it has never been reported about TLRs in a different tissue layer. Thus, the study of TLR2 and TLR4 in the canine female

reproductive tract may reflect the immunological response in the uterus of both healthy bitches and bitches with uterine bacterial infection or pyometra

1.3 Objectives of the thesis

1. To characterize the types and relationship of bacteria in vagina and uterus of healthy bitches and bitches with closed-cervix and opened-cervix pyometra
2. To determine the uterine leukocytes infiltration of healthy bitches at different stages of the estrous cycle
3. To evaluate the roles of TLR2 and TLR4 in the uterus of healthy and pyometra bitches

1.4 Hypothesis

1. The types of bacteria in the vagina and uterus are different between healthy and pyometra bitches and also varies in closed-cervix and opened-cervix pyometra**.**
2. The leukocytes infiltration in the uterus are varies depend on the stages of the estrous cycle and the number of the total leukocytes are different in distinct compartments of the uterus.
3. TLR2 and TLR4 have cycle-dependent expressions in the uterus of healthy bitches and highly express in pyometra bitches compare to healthy bitches.
4. The expressions of TLR2 and TLR4 are different in tissue layers and distinct compartments.

1.5 Keywords: uterus, innate immunity, Toll-like receptor

1.6 Research merits:

1. The information about the influence of factors including sex hormones, leukocytes and innate immunity on the presence of bacterial species in canine reproductive tract.

2. Better understanding in roles of leukocytes and innate immunity (TLR2 and TLR4) in normal and infected canine uterus.
3. The knowledge about TLR2 and TLR4 in canine uterus could be the basis information for further study into mechanisms of innate immunity for canine uterine pathogenicity and may be useful for the development of new efficient drugs for treatment of canine uterine infection in the future.

CHAPTER II

THE RELATIONSHIP OF BACTERIA IN VAGINA AND UTERUS OF HEALTHY AND PYOMETRA BITCHES

2.1 Abstract

Identification of bacterial species was performed in the vagina and uterus of 25 healthy bitches, 13 bitches with closed-cervix pyometra and 20 bitches with opened-cervix pyometra. The ages of the bitches were 1-13 years. Various bacterial species were found from the vagina and uterus of bitches in this study. No healthy bitches had a relationship between bacterial species in vagina and uterus and in 13 bitches with closed-cervix pyometra, the bacterial species of 10 bitches (76.92%) were not related between vagina and uterus. Meanwhile, in 20 bitches with opened-cervix pyometra, the bacterial species between vagina and uterus in 6 bitches (30%) had no relationship. Bacteria found in the vagina may not be an initial cause of uterine infection in bitches with closed-cervix pyometra. On the other hand, bacterial species in the vagina of bitches with opened-cervix pyometra may be a cause of uterine infection or can be the result of uterine drainage. Therefore, for the eradication of bacteria in canine pyometra, bacterial swabs should be taken from the infection site in order to choose a proper antimicrobial agent.

2.2 Introduction

The female reproductive tract is frequently exposed to pathogens. The mucosal surfaces are the primary sites of pathogen entry into the body (Mowat, 2003). The bacterial flora of the genital mucous membranes is composed of microorganisms that usually exist in a state of balance with the host and with one another and may protect the host from infection (Bjurström and Linde-Forsberg, 1992). Canine uterine infections are associated with bacteria (Baba et al., 1983) and have a detrimental effect on the bitch. The most common uterine pathological condition of dog is pyometra (chronic purulent endometritis) (Kida et al., 2006). Pyometra is the

accumulation of purulent discharge in the uterus of dogs (Pretzer et al., 2008). Pyometra is considered a diestrous disease, even though some anestrous dogs can be diagnosed with pyometra (Verstegen et al., 2008). Furthermore, pyometra in a dog is also considered to result from bacterial and hormonal interaction (Zaragoza et al., 2004). The importance of progesterone in the pathogenesis of this disease is related to its suppression of immune functions, activation of uterine glandular secretions that supply an appropriate environment for bacterial development, function of cervical closure that obstructs drainage of exudates from the uterus and mediates of pyometra (Kida et al., 2006).

Organisms in the uterus in the normal estrous cycle rarely cause pathologic changes. Although bacteria in vaginal usually flow into the uterus, but rarely cause uterine infection (Baba et al., 1983). In the normal female reproductive tract, mucosal surfaces are the first line of defense against pathogens (Fahey et al., 2005). Mucosal epithelial cells have developed innate immune functions as the first line of defense that can immediately suppress bacterial growth (Hecht, 1999; Fahey et al., 2005). Furthermore, Bactericidal activity in the female reproductive tract has also been found to be affected by sex hormones (Fahey et al., 2005). Various factors may be involved in inducing uterine infection (Baba et al., 1983). Disorder of sexual hormones may be one of the factors contributing to uterine infections (Dow, 1958; Dow, 1959; Baba et al., 1983). Pyometra in dogs is frequently found in the first half of diestrus, when the blood concentration of progesterone is highest, while the estradiol is lowest. Conversely, the incidence of pyometra is relatively decreased at estrus, when blood concentration of estradiol is highest, whereas the progesterone is lowest (Concannon et al., 1989; Sugiura et al., 2004). The vagina of dog may contain exudates originating from a several causes such as vaginitis, metritis and post-partum infections of the uterus. Treatment by antimicrobial drugs is recommended for use in such conditions (Hirsh and Wiger, 1977). Canine vaginitis is a common disease in prepubertal and postpubertal bitches (Osbaldiston, 1968; Olson and Mather, 1978). To form a basis for the reasonable use of antimicrobial drugs, vaginal cultures are often taken in an attempt to define the cause of the clinical condition (Hirsh and Wiger, 1977) and characterize the vaginal flora associated with juvenile and post pubertal vaginitis, neonatal septicemia or infertility (Olson and Mather, 1978).

Nowadays, antimicrobial agents are used in routine treatment but bacteria also develop antimicrobial-resistance that may cause problems for bitches. To reduce antimicrobial resistance, antimicrobial treatments should be used restrictively. In selected cases, bacteriological culture and determination of antimicrobial resistance patterns allows selection of appropriate antimicrobial treatment. The purpose of this study is to investigate the presence and relationship of vaginal and uterine bacterial species from clinical cases of healthy bitches at various stages of the estrous cycle and bitches with closed-cervix and opened-cervix pyometra.

2.3 Materials and methods

2.3.1 Animals

In total, 58 nulliparous bitches, ranging in age from 1-13 years, were submitted for ovariohysterectomy at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. All bitches were at puberty and none of bitches had received hormonal treatment. The bitches were allocated into three groups. Group 1 had healthy bitches (n = 25). Group 2 and 3 had bitches with pyometra; in group 2, bitches presented with closed-cervix pyometra (n = 13); in group 3, bitches presented with opened-cervix pyometra (n = 20). All healthy bitches were examined for vulva swelling, estrous behaviour and blood collection. Stages of the estrous cycle were confirmed by vaginal cytology, gross aspect of the ovaries and serum progesterone levels, none of bitches presented abnormal clinical signs and the blood profile was normal (Lumsden et al., 1979). In bitches with pyometra, the diagnosis of pathology is based on clinical signs, blood collection and usually confirmed by radiography and/or ultrasonography (Sandholm et al., 1975).

2.3.2 Hormonal analysis

A blood sample was collected from the cephalic vein of the healthy bitches before ovariohysterectomy. For serum production, blood samples were centrifuged for 5 min at 2500 × *g*. Peripheral blood serum progesterone concentrations were measured using chemiluminescent assay (Chapwanya et al., 2008).

2.3.3 Estrous cycle stage determination

The stage of the estrous cycle of healthy bitches was confirmed by vaginal cytology (proestrus: mixed types of epithelial cells, red blood cells and white blood cells may be present in early to midproestrus; estrus: > 90% cornified cells with fewer red blood cells than proestrus, few to no white blood cells; diestrus: > 50% parabasal and intermediate cells on first day of diestrus, white blood cells may be present with fewer red blood cells than proestrus; anestrus: > 90% parabasal and intermediate cells with few white blood cells, fewer bacteria), by gross aspect of the ovaries (proestrus and estrus: presence of follicles; diestrus: large corpora lutea; anestrus: regressed corpora lutea), and by serum progesterone concentration (anestrus: ≤ 0.5 ng/ml; proestrus: < 1 ng/ml; estrus: 1-15 ng/ml; diestrus: > 1 ng/ml) (Van Cruchten et al., 2004; Kida et al., 2010).

2.3.4 Vaginal and uterine bacterial culture

The vaginal swabs were obtained by spreading the lips of the vulva and inserting a sterile swab then withdrawing it after rolling it on the entire surface. For the uterine swabs, the incision was made into the dorsal wall of the uterine body. The sterile cotton swab was inserted into the incision and rotated 360 degrees (Olson and Mather, 1978). The vaginal and uterine swabs were collected into transport media and submitted for aerobic bacterial culture and identification. The samples were cultured on 10% sheep blood agar at 37 °C for 24 hour. Bacteria were identified by family, genus or species according to the manual for the identification of medical bacteria (Barrow and Feltham, 1993).

2.3.5 Statistical analysis

Frequency analysis was conducted for bacterial identification follow by r × k contingency table. The differences of bacterial identification between groups were evaluated using Fisher's exact test.

2.3.6 Experimental design

To characterized the types of bacteria in vagina and uterus of healthy bitches and bitches with closed-cervix and opened cervix pyometra. The bacteria were swabbed from the vagina and uterus of bitches before and after ovariohysterectomy respectively. The bacterial swabs were collected into transport media and then culture in order to indentify the types of bacteria.

2.4 Results

Bacterial species	**Number of isolates**		**%**	
	Vagina	**Uterus**	**Vagina**	**Uterus**
Gram-negative bacteria				
Escherichia coli	4	3	16	12
Proteus spp.	3	0	12	0
Enterobacter spp.	2	1	8	4
Klebsiella spp.	1	0	4	0
Citrobacter diversus	1	0	4	0
Pseudomonas spp.	0	2	0	8
Gram positive bacteria				
Staphylococcus spp.	7	1	28	4
Streptococcus spp.	5	2	20	8
Bacillus spp.	1	0	4	0
No growth	4	16	16	64

Table 2 Bacterial species and the number of bacterial isolates in vagina (n = 28) and uterus (n = 25) from healthy bitches (n = 25)

Stages of estrous cycle	Bacterial species	
	Vagina	**Uterus**
Proestrus (n = 2)	*Staphylococcus spp.* (2/2)	*Streptococcus spp.* (1/2) No growth (1/2)
Estrus (n = 5)	*Streptococcus spp.* (2/5) *Citrobacter diversus* (1/5) *Klebsiella spp.* (1/5) *Proteus spp.* (1/5)	*Escherichia coli* (1/5) *Pseudomonas spp.* (1/5) *Staphylococcus spp.* (1/5) No growth (2/5)
Diestrus (n = 7)	*Proteus spp.* (2/7) *Enterobacter spp.* (1/7) *Escherichia coli* (1/7) *Staphylococcus spp.* (1/7) *Streptococcus spp.* (1/7) No growth (2/7)	*Escherichia coli* (2/7) *Pseudomonas spp.* (14.29) *Streptococcus spp. (*14.29) No growth (42.86)
Anestrus (n = 11)	*Staphylococcus spp.* (4/11) *Escherichia coli* (3/11) *Streptococcus spp.* (2/11) *Bacillus spp.* (1/11) *Enterobacter spp.* (1/11) No growth (2/11)	*Enterobacter spp.* (1/11) No growth (10/11)

Table 3 Bacterial species and the number of bacterial isolates in vagina (n = 28) and uterus (n = 25) from healthy bitches (n = 25) during estrous cycle

The bacterial species of healthy bitches during estrous cycle found from this study are presented in Table 2 and Table 3 respectively. In the group of healthy bitches, no healthy bitch had a relationship between bacterial species in vagina and uterus. In the vagina, *Staphylococcus spp.* was found significantly higher than the uterus ($P < 0.05$).

Bacterial species	Number of isolates		%	
	Vagina	Uterus	Vagina	Uterus
Gram-negative bacteria				
Escherichia coli	5	5	38.5	38.5
Enterobacter spp.	2	1	15.4	7.7
Klebsiella spp.	2	0	15.4	0
Pseudomonas spp.	1	0	7.7	0
Providencia rettgeri	1	0	7.7	0
Citrobacter diversus	0	1	0	7.7
Gram positive bacteria				
Staphylococcus spp.	1	4	7.7	30.78
Streptococcus spp.	0	1	0	7.7
Bacillus spp.	0	1	0	7.7
No growth	2	0	15.4	0

Table 4 Bacterial species and the number of isolates in vagina (n = 14) and uterus (n = 13) from bitches with closed-cervix pyometra (n = 13)

The bacterial species of bitches with closed-cervix and opened-cervix pyometra are presented in Table 4 and Table 5 respectively. Of the vaginas of 13 bitches with closed-cervix pyometra, 11 (84.62%) were found positive on bacterial culture. And 10 bitches were found to have one bacterial species in each bitch and in one bitch were two bacterial species isolated. Gram-negative bacteria in the vaginas from 10 bitches out of 13 bitches (76.92%) were found, while, Gram-positive bacteria were found in 1 bitch (7.69%). Of 13 uterine swabs, all of samples were positive on bacterial culture and in each sample only one bacterial species was isolated. Seven bitches (53.8%) were recognized for Gram-negative bacteria whereas Gram-positive bacteria were detected in 6 bitches (46.2%).

Bacterial species	Number of isolates		%	
	Vagina	Uterus	Vagina	Uterus
Gram-negative bacteria				
Escherichia coli	6	8	30	40
Enterobacter spp.	3	2	15	10
Klebsiella spp.	2	3	10	15
Pseudomonas spp.	2	1	10	5
Proteus spp.	2	1	10	5
Citrobacter diversus	1	1	5	5
Gram positive bacteria				
Staphylococcus spp.	4	1	20	5
Streptococcus spp.	2	2	10	10
No growth	0	2	0	10

Table 5 The number and percentage of bacterial isolates in vagina (n = 22) and uterus (n = 21) from bitches with opened-cervix pyometra (n = 20)

All of the bitches with opened-cervix pyometra had bacterial species in the vagina. From 19 samples (90.5%) one bacterial species was isolated in each bitch and from 2 samples two bacterial species were isolated. Gram-negative bacteria were found in 15 samples (71.4%) while 7 samples (33.3%) were detected for Gram-positive bacteria (one bitch was found with both Gram-negative and Gram-positive bacteria). The bacterial species isolated from the uterus were positive for 19 bitches (90.5%), meanwhile, another 2 bitches (9.5%) were not found with bacteria in uterus. Out of 19 samples, 17 samples were found with one bacterial species in each bitch and only one sample was found with two bacterial species. 16 bitches (84.2%) were found with Gram-negative bacteria while 3 bitches (15.8%) were found with Gram-positive bacteria. Interestingly, in 13 bitches with closed-cervix pyometra found that the bacterial species of 10 bitches (76.92%) were not related between vagina and uterus. Meanwhile, in 20 bitches with opened-cervix pyometra, the bacterial species between vagina and uterus in 6 bitches (30%) had no relationship.

2.5 Discussion

In this study, no healthy bitches had a relationship between bacterial species in the vagina and uterus and this may indicated that the immune surveillance in the female reproductive tract was critically influenced by the distinct microenvironment in each compartment of the female reproductive tract, which may be influenced by the interplay of sex hormones and resident leukocyte populations (Hart et al., 2009). Furthermore, it was possible that the presence of bacteria in vagina and uterus of healthy bitches was also influenced by the estrous cycle. In this study, all of bitches in proestrus and estrus found bacteria in vagina. In accordance with the previous study that the total number of vaginal bacteria during estrus was higher than other stages of the estrous cycle in dogs, mice, rats and hamsters (Noguchi et al., 2003). In estrus, the glands in the cervix of the uterus secreted mucus and reached maximum at that time (Centola, 1978; Holderegger, 1980; King, 1983; Corbeil et al., 1985; Noguchi et al., 2003). Thus, the proliferation of bacteria in vagina may caused by an increased of mucus secretion from the uterus, since mucopolysaccharides served as a kind of culture medium (Savage, 1972; Noguchi, 2003). And in estrus, 3 out of 5 bitches from this study found bacteria in the uterus. At this stage the bacteria may enter the uterus through the more patent cervical canal (Baba et al., 1983; Watts et al., 1996; Kida et al., 2006) and the presence of uterine fluid with blood at this stage may provide a suitable medium for bacterial growth (Watts et al., 1996). Meanwhile, one bitch at proestrus and 2 out of 5 bitches at estrus bacterial species could not be isolated from the uterus, this may be due to the role of sex hormone regulation of the immune functions. For instance, uterine fluids from rats in proestrus, when estrogen levels were known to be that their highest, had significantly higher anti-bacterial activity relative to other stages of the estrous cycle because estradiol stimulated epithelial cells from the rat uterus to produce factors with broad-spectrum anti-bacterial activity. Therefore, bactericidal activity in the female reproductive tract has also been shown to be influenced by sex hormones. From the stages of estradiol dominance, estradiol acts directly on uterine epithelial cells to decrease transepithelial resistance, a measure of tight junction formation. Besides, estradiol alters the nature of the endometrial epithelium, reducing the adherence of organisms (Nishikawa, 1985; Nishikawa and

Baba, 1985; Sugiura et al., 2004). Furthermore, estrogen seems to be a negative regulator of myelopoiesis in bone marrow but promotes recruitment of macrophages and neutrophils into the uterus as a pro-inflammatory activity, which is antagonized by progesterone. And, estrogen appears to directly activate immune cells including T cells and B cells (Sugiura et al., 2004).

In diestrus, 4 out of 7 bitches (57.14%) bacterial species could be detected in the uterus, this may have been due to progesterone suppression of the immune response which made the uterus more susceptible to spontaneous bacterial infection (Herath et al., 2006a). Thus, the endometrium was more susceptible to infection under progesterone than estrogen dominance. During the estrogen phase of the ovarian cycle there was increased blood flow to the uterus and intensified polymorphonuclear leukocyte (PMN) activity conversely, in the luteal phase, there was delayed leukocyte stimulation and absented of detoxifying agents in the uterine secretions (Dhaliwal et al., 2001). Immune functions measured as lymphocyte proliferation and white blood cell changes were up-regulated at estrus and down-regulated during the luteal phase (Wulster-Radcliffe et al., 2003). Progesterone, while not inducing anti-bacterial activity, reversed the stimulating effect of estradiol (Fahey et al., 2005). Elevated blood progesterone concentrations inhibited production of chemokines in the uterus for an influx of neutrophils and monocytes (Sugiura et al., 2004), and inhibited both uterine and peripheral blood neutrophil phagocytic activities (Dhaliwal et al., 2001). Furthermore, progesterone appeared to inhibit the generation and activation of lymphocytes. Progesterone directly inhibited the development of Th1 cells. The increased incidence of pyometra in the early phase of diestrus results partially from the suppression of antigen-specific Th1 cell responses and cellular immunity by progesterone during the period in which the concentration in the peripheral blood peaks (Sugiura et al., 2004). Therefore, the antigen-specific immunity altered by ovarian hormones seemed to play an important role in preventing infectious disease in uterus (Sugiura et al., 2004). However, the bacterial species in the uterus could not be isolated from 3 out of 7 bitches (42.86%) during this stage. This may also indicate that there may have been different processes involved in this uterine clearance. The presence of many white blood cells in the vaginal smear of early diestrus was well documented and may reflect an inflammatory response of the uterus. On the other

hand, the lack of microorganisms during diestrus was important given the sensitivity of the canine uterus to damage at this stage and cystic endometrial hyperplasia or uterine infection are a common sequel when the uterus is exposed to the trauma of bacterial invasion when progesterone levels are high (Watts et al., 1996).

The bacterial species could be isolated from the uterus of only one of healthy bitches during anestrus. The result from this study was accordance with the previous studied that the uterus was usually free of microorganisms, despite being more susceptible to bacterial invasion at this stage when the epithelium was being replaced. It may be possible that microbes can enter the uterus at this time and be removed once the endometrium is repaired during late anestrus (Watts et al., 1996) or some of the uterine immune mechanisms may be involved in the clearance of bacteria at this stage.

In addition, when compared between vagina and uterus of healthy bitches, *Staphylococcus spp.* was found in vagina higher than the uterus. Bacterial species isolated from vagina in this study were similar to those from those previous studied which reported that the predominant organisms in canine vaginal microflora were *Staphylococcus spp.*, *Streptococcus spp.* and *Escherichia coli* (Olson and Mather, 1978; Ling and Ruby, 1978; Baba et al., 1983). Meanwhile, *Escherichia coli*, *Staphylococcus intermedius* and β-hemolytic streptococci were also the species most frequently isolated in pure cultures from bitches with vaginitis (Bjurström, 1993).

The uterine bacterial species frequently isolated from this study was *Escherichia coli* which is in accordance with previous study (Watts et al., 1996). While, *Pseudomonas spp.* and *Streptococcus spp*. were also frequently found second to *Escherichia coli* in the uterus of healthy bitches in accordance with previous studies which have reported that Streptococcus species were the common bacterial group found in healthy uteri (Baba et al., 1983; Watts et al., 1996; Hagman and Kühn, 2002). From the previous studies, *Escherichia coli, Streptococcus spp. Pseudomonas spp.* and *Staphylococcus spp*. were the common bacteria isolated from bitches with pyometra (Grindlay et al., 1973; Sandlhom et al, 1975; Nomura, 1984; Bjurström, 1993; Wadås et al., 1996; Fransson et al., 1997; Hagman and Kühn, 2002). This may indicate that there is no direct correlation between these bacteria and clinical illness from uterine bacterial infection. Other types of organisms such as viruses and

mycoplasms may also have contributed to the infectious processes and should be considered as possible etiologic agents (Clemetson and Ward, 1990). In addition, the types of bacteria isolated from normal animals and animals with vaginitis were frequently the same even though, the relative numbers of a particular type of bacteria were often increased with disease (Hirsh and Wiger, 1977; Clemetson and Ward, 1990).

In previous study, common bacteria isolated from bitches with pyometra were *Streptococcus spp.*, *Klebsiella spp.*, *Staphylococcus spp.*, *Pasteurella spp.*, *Proteus spp.* and *Pseudomonas spp.* (Hagman and Kühn, 2002) which is in accordance with this study except for *Pasteurella spp.* that was not found from this study. Similarly, the bacterial species in the vaginas of bitches found in this study were in accordance with previous study (Bjurström, 1993) except for *Pasteurella spp.* that was not found in this study. In bitches with closed-cervix pyometra, 3 out of 13 bitches had the same type of bacteria in the vagina and uterus. In other cases with closed-cervix pyometra, a different bacterial species from the vagina and uterus of the same dog was found and this was in which most of cases. This may be related to the different existence of microorganisms in the vagina and uterus and the many different processes involved in the clearance of bacteria (Watts et al., 1996). Other factors possibly involved in the presence of bacteria in the vagina and uterus, such as a primary hormonal imbalance or abnormal response to normal hormone levels affect the epithelial cells of the uterus and facilitate bacterial adherence, colonization and growth (Hagman and Kühn, 2002). The innate immune defense system of the female reproductive tract is a complex and dynamic system comprising specific physical or physicochemical barriers, commensal microflora, cellular effectors as well as bactericidal proteins and peptides (Mak et al., 2004) that may also be a reflex to the presence of bacteria in vagina and uterus. In the bitches with opened-cervix pyometra, different bacterial species between vagina and uterus were found in only 6 cases from 20 cases. Whereas, in most cases the same type of bacterial species was found between the vagina and uterus. This finding may be due to the uterine infection leading to drain the purulent discharge from the uterus to vagina by the opening of cervix. Thus, the bacteria from uterus may contaminate the vagina. In contrast, it may be due to the increasing amount of bacteria in the vagina that may flow through the cervical

opening to the uterus. In addition, *Escherichia coli* strains found in the disease may originate from urinary tract infection (Hagman and Kühn, 2002). In bitches with opened-cervix pyometra, the bacterial species could not be isolated from the uterus in 2 cases (10%) similar to the previous studies that reported that bacterial species could not be isolated from the uterus in 15% (Osbaldiston, 1978) and 17.6% (Dhaliwal et al., 1998) of cases. It has been indicated that in such cases the bacteria that were initially involved in the pathogenesis of pyometra may be killed by the uterine defense mechanisms (Dhaliwal et al., 1998).

The nature and course of uterine infections also depends on the type of invading bacteria and the immune status of the host (Singh et al., 2008). The immune system has traditionally been divided into innate and adaptive immunity. However, the innate immunity is the most universal, the most rapidly acting, and by some appraisals, the most important type of immunity (Beutler, 2004). The innate immune system is generally considered to be the first line of defense against infection (Yu et al., 2009). In addition, innate immunity is principally responsible for the elimination of bacterial contamination of the uterus after parturition. If the anatomical barriers are breached, the presence of invading bacteria is quickly detected by specialized immune cells and cells of the endometrium which are armed with Toll-like receptors (TLRs) for the detection of bacterial ligands, such as lipopolysaccharide (LPS) of Gram-negative bacteria and peptidoglycans of Gram-positive bacteria (Singh et al., 2008). Interestingly, when the types of bacteria were classified into Gram-negative and Gram-positive bacteria, the dominance of Gram-negative bacteria in both healthy and pyometra bitches were found. In agreement with the previous reports on humans, TLR4 which recognizes Gram-negative bacteria was observed mainly in the upper part of the female reproductive tract (uterus and uterine tubes) (Fazeli et al., 2005; Yu et al., 2009). However, Gram-positive bacteria were found mainly in vagina of healthy bitches at proestrus and anestrus. Thus, innate immunity seems related to the presence of bacterial species in canine endometrium.

In conclusion, the bacteria found in the vagina may not be an initial cause of uterine infection in bitches with closed-cervix pyometra. Bacterial species in the vaginas of bitches with opened-cervix pyometra may a cause of uterine infection or they may be found as a result of uterine drainage at the same time. For the eradication

of bacteria in canine pyometra, bacterial swabs should be taken at infection site in order to choose a proper antimicrobial agent. Nevertheless, factors other than the type of bacteria, such as uterine immunity influence the susceptibility to canine uterine infection. Therefore, the immune cells infiltration and TLR (the key mediators of innate immunity) in canine uterus are subjects of further investigation.

CHAPTER III

THE INVESTIGATION OF UTERINE LEUKOCYTES INFILTRATION OF HEALTHY BITCHES AT DIFFERENT STAGES OF THE ESTROUS CYCLE

3.1 Abstract

The number of leukocytes in horn, body and cervix of the uterus in healthy bitches during the estrous cycle was investigated in this study. The leukocytes that found from this study were lymphocytes, macrophages, neutrophils and plasma cells. And lymphocytes were the most common leukocytes found in the uterus. In the endometrial stroma, the number of total leukocytes ($P < 0.05$) and macrophages ($P < 0.01$) were increased in anestrous dogs compared to other stages. While, the endometrial glandular epithelium found an increased number of total leukocytes and lymphocytes in proestrous and estrous dogs compared to anestrous dogs ($P < 0.05$). The number of total leukocytes in the surface epithelium of the uterine horn significantly decreased at estrus ($P < 0.01$) but significantly decreased at anestrous stage in the cervix compared to other stages ($P < 0.05$). In addition, the leukocytes were dominantly in the stroma when compared to other tissue layers. However, the trend of the number of leukocytes was decreased in the lower part of the reproductive tract. From this study, the number of leukocytes varied in a different tissue layers and different regions of the uterus at different stages of the estrous cycle which indicated the different role in the uterine immune surveillance protecting the host from the pathogen invasion.

3.2 Introduction

The uterine defense mechanisms against contaminant microorganisms are maintained in several ways by anatomical, chemical and immunological mechanisms (Asbury et al., 1980; Dhaliwal et al., 2001). Immune surveillance of the female reproductive tract is critically affected by the interaction of many factors including resident leukocyte populations, sex hormones and the distinct microenvironment of

each component of the reproductive tract (Hart et al., 2009). Immune cells in the female reproductive tract maintain a critical balance that allow for a response to pathogenic challenge while being supportive to allogeneic spermatozoa and development of a semi-allogeneic fetus (Ochiel et al., 2008; Basu et al., 2009). Prevention against potential microorganisms in the female reproductive tract is provided by the innate and adaptive immune system. The innate immune system composed of neutrophils, macrophages, dendritic cells and natural killer cells. The innate immunity is responsible for recognizing and responding to the existence of microorganisms and is dependent on leukocytes (Baumann and Gauldie, 1994; Beutler et al., 2003; Herath et al., 2006a). Subsequent to pathogen recognition, leukocytes release pro-inflammatory components including interleukin (IL), tumor necrosis factor-α (TNF-α) and nitric oxide (Baumann and Gauldie, 1994; Herath et al., 2006a). These components support the recruitment and activation of more leukocytes (Herath et al., 2006a). IL-1β enhances T cell differentiation at the mucosal surfaces and involves in the production of IgA by stimulation of cytokine (Finkelman et al., 1990; Cohen and Pollard, 1996; Vandermolen and Gu, 1996; Franklin and Kutteh, 1999). IL-8 is a novel potent chemotactic cytokine for neutrophils (Matsushima et al., 1992; Ito et al., 1994) and T cells (Larsen et al., 1989a; Ito et al., 1994). Monocytes and macrophages are known to produce IL-8 (Matsushima et al., 1992; Strieter et al., 1989; Thornton et al., 1990; Larsen et al., 1989b; Yasumoto et al., 1992; Ito et al., 1994). IL-12 potentially enhances cellular immunity mediated by Th1 (Sugiura et al., 2004). TNF increases the production of nitric oxide in order to promote bacteria-killing activity of macrophages (Sugiura et al., 2004). And, nitric oxide has strong antimicrobial function against wide range of pathogens (Bogdan, 2001; Witkin et al., 2007). While, in the adaptive immune system, the antigen from pathogen is processed by antigen presenting cells and this pathogen is presented to T cells, consequently inducing T-cell stimulation. Lymphocytes effecter functions including cytokine production, cytotoxicity and antibody synthesis are stimulated subsequent to antigen presentation. Prevention is mediated by specific antibodies produced by B cells or the specific pathogen elimination by T cells (Wira et al., 2005). Furthermore, cytokines secreated by Th cells also influence proliferation of T and B cells which migrate to the endometrium as effector of the immune response

(Singh et al., 2008). A variety of innate and adaptive immune mechanisms are affected by sex hormones (Wira and Kaushic, 1996; Fahey et al., 2005). For example, sex hormones may be related to the functional activity of PMNs migrating into the uterus (Dhaliwal et al., 2001). Besides, the stage of the reproductive cycle or the treatment of exogenous sex hormones may promote resistance or enhance susceptibility to exposure of pathogens (Hawk et al., 1955; Fahey et al., 2005).

The nature and cause of uterine infection also depends on the type of invading bacteria and the immune status of the host (Singh et al., 2008). However, the study of uterine immunity in dogs is still unclear. And, the uterine immunity may different in a distinct microenvironment. Thus, to clarify the role of immune system in the canine female reproductive tract, the immune cells in the horn, body and cervix of canine uterus have been investigated during the estrous cycle.

3.3 Materials and methods

3.3.1 Animals

In total, 44 nulliparous bitches, ranging in age from 1-13 years, were submitted for ovariohysterectomy at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. All bitches were at puberty and none of bitches had received hormonal treatment. The healthy bitches were classified into four groups depending on the stage of the estrous cycle; group1, bitches in proestrus (n = 7); group 2, bitches in estrus (n = 10); group 3, bitches in diestrus (n = 16); group 4, bitches in anestrus (n = 11). All healthy bitches were examined for vulva swelling, estrous behaviour and blood collection. Stages of the estrous cycle were confirmed by vaginal cytology, gross aspect of the ovaries and serum progesterone levels, none of bitches presented abnormal clinical signs and the blood profile was normal (Lumsden et al., 1979).

3.3.2 Hormonal analysis

A blood sample was collected from the cephalic vein of bitches before ovariohysterectomy. For serum production, blood samples were centrifuged for 5 min at 2500 × *g*. Peripheral blood serum progesterone concentrations were measured using chemiluminescent assay (Chapwanya et al., 2008).

3.3.3 Tissue collection

Uterine tissues were collected from each group of bitches undergoing ovariohysterectomy. Each tissue sample was divided into horn, body and cervix of the uterus. Tissue collected from the uterine horn in all samples was collected from the left horn and the middle portion. Full thickness segments of the uterus, approximately 1 cm in length were removed. Tissue samples were fixed in 4% paraformaldehyde for histological examination.

3.3.4 Estrous cycle stage determination

The stage of the estrous cycle of healthy bitches was confirmed by vaginal cytology (proestrus: mixed types of epithelial cells, red blood cells and white blood cells may be present in early to midproestrus; estrus: > 90% cornified cells with fewer red blood cells than proestrus, few to no white blood cells; diestrus: > 50% parabasal and intermediate cells on first day of diestrus, white blood cells may be present with fewer red blood cells than proestrus; anestrus: > 90% parabasal and intermediate cells with few white blood cells, fewer bacteria), by gross aspect of the ovaries (proestrus and estrus: presence of follicles; diestrus: large corpora lutea; anestrus: regressed corpora lutea), and by serum progesterone concentration (anestrus: ≤ 0.5 ng/ml; proestrus: < 1 ng/ml; estrus: 1-15 ng/ml; diestrus: > 1 ng/ml) (Van Cruchten et al., 2004; Kida et al., 2010).

3.3.5 Histological examination

The uterine tissue was paraffin-embedded, sectioned at 4 μm thickness and stained with hematoxylin and eosin. The sections of horn and body of the uterine endometrium were divided into three layers; surface epithelium, stroma (define as the area from the basal lamina of the surface epithelium and compose predominantly of connective tissue) and glandular epithelium (define as the area from stroma and consist mainly of glands). The sections of cervix were divided in to two layers; surface epithelium and stroma. The sections of horn, body and cervix were examined for the number of total leukocytes (neutrophils, macrophages and lymphocytes) (Chu et al., 2006). Other leukocytes (plasma cells, eosinophils and mast cells) were examined if present as reported in swine (Kaeoket et al., 2002a).

Neutrophils define as leukocytes that have an elongated, segmented nucleus with three to five lobes, cytoplasm is light blue-grey but the cytoplasmic granules present do not stain. Macrophages are readily identified by size (3-12 times the diameter of a red blood cell), an oval or bean-shaped nucleus and may contain numerous vacuoles and/or phagocytosed cellular material. Lymphocytes have a round nucleus with condensed, smudged chromatin and a narrow rim of basophilic cytoplasm. Plasma cells define as leukocytes that have a round nucleus, eccentrically locate, and contain variable quantity of chromatin giving most nuclei a typical cartwheel appearance (Haug and Heyeraas, 2005). Eosinophils define as numerous, prominent and pink cytoplasmic granules (Villiers, 2005). Mast cells are round cells with a central round to oval nucleus, red-to-purple granules in the cytoplasm (Blackwood, 2005).

In surface epithelium, stroma and glandular epithelium of each layer were quantified under light microscope (×400). The ocular micrometer with 25 squares corresponding to 15,625 μm^2 of real tissue was used for counting the number of immune cells. For each section and each layer, 20 microscopic fields were arbitrarily selected for investigation (Tummaruk et al., 2009).

3.3.6 Statistical analysis

Data was expressed as mean ± SEM. The number of cells was presented as a number of cells per 20 ocular fields area (312,300 μm^2). Multiple analysis of variance using SAS was used to compare the number of leukocytes between groups (proestrus, estrus, diestrus, anestrus) and tissue layers (surface epithelium, stroma, glandular epithelium). Differences with $P < 0.05$ were regarded as statistically significant, $P < 0.01$ as highly statistically significant.

3.3.7 Experimental design

The types and the number of leukocytes in horn, body and cervix of the uterus of healthy dogs during estrous cycle was investigate. The tissues were collected from the uterus of dogs after ovariohysterectomy and embedded in the paraffin block. The tissue sections were stained with hematoxylin and eosin. The number of leukocytes was counted by using ocular micrometer under light microscope (×400). The number of leukocytes in horn and body of the uterus were counted from surface epithelium, glandular epithelium and stroma. While, the number of leukocytes in cervix was counted from surface epithelium and stroma. In each layer, the number of leukocytes was counted in 20 microscopic fields.

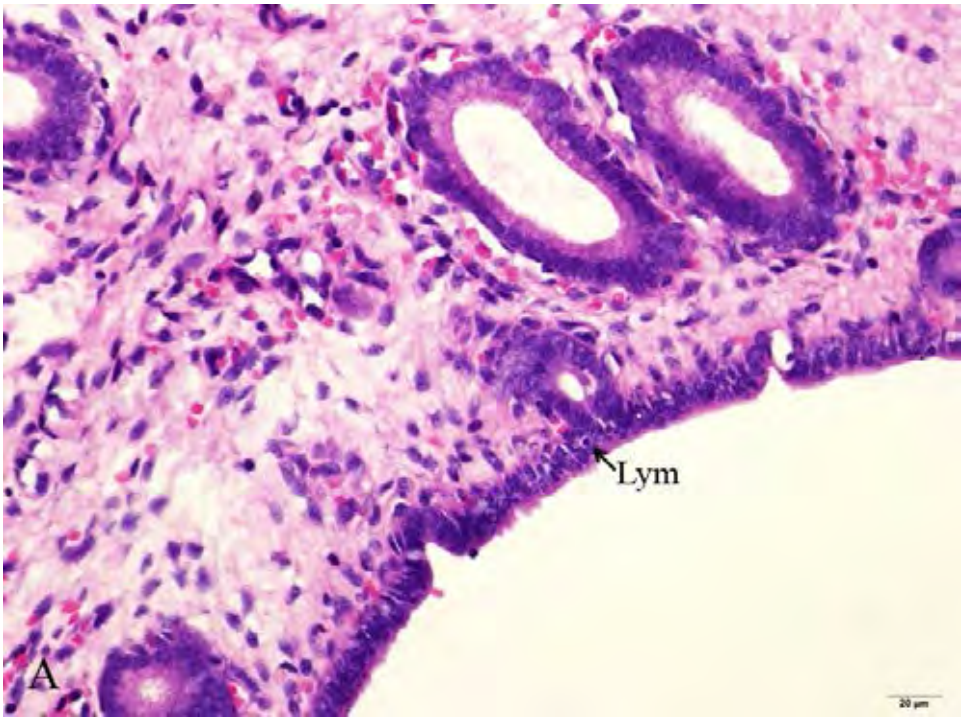
3.4 Results

3.4.1 Leukocytes in the endometrial tissues

3.4.1.1 The part of horn

In this study, no pathological changes of uterine samples were found from all healthy dogs. And, the mean of progesterone concentration in each stage of the estrous cycle were 1.07 ng/ml (proestrus), 8.04 ng/ml (estrus), 25.93 ng/ml (diestrus), 0.48 ng/ml (anestrus). The number of lymphocytes and total leukocytes in the surface epithelium significantly decreased at estrus compared with other stages of estrous cycle ($P < 0.01$) (Figure 1). The glandular epithelium found an increased number of

lymphocytes and total leukocytes in proestrous and estrous dogs compared to anestrous dogs ($P < 0.05$). In the stroma, the number of macrophages ($P < 0.01$) and the total leukocytes ($P < 0.05$) were significantly increased in anestrous dogs compared to other stages of the estrous cycle. And the number of lymphocytes was significantly higher in anestrus than diestrus ($P < 0.05$) (Figure 2). Moreover, the number of macrophages was also significantly increased in the surface epithelium of anestrous dogs compared with dogs at other stages ($P < 0.05$). Neutrophils were found in the stroma which significantly higher at proestrus and estrus compared with anestrus ($P < 0.01$). And, the number of plasma cells in the stroma at proestrus was significantly higher than anestrus ($P < 0.05$) in this study. The different types of immune cells and total leukocytes in all groups and layers were presented in Figure 3 and 4.



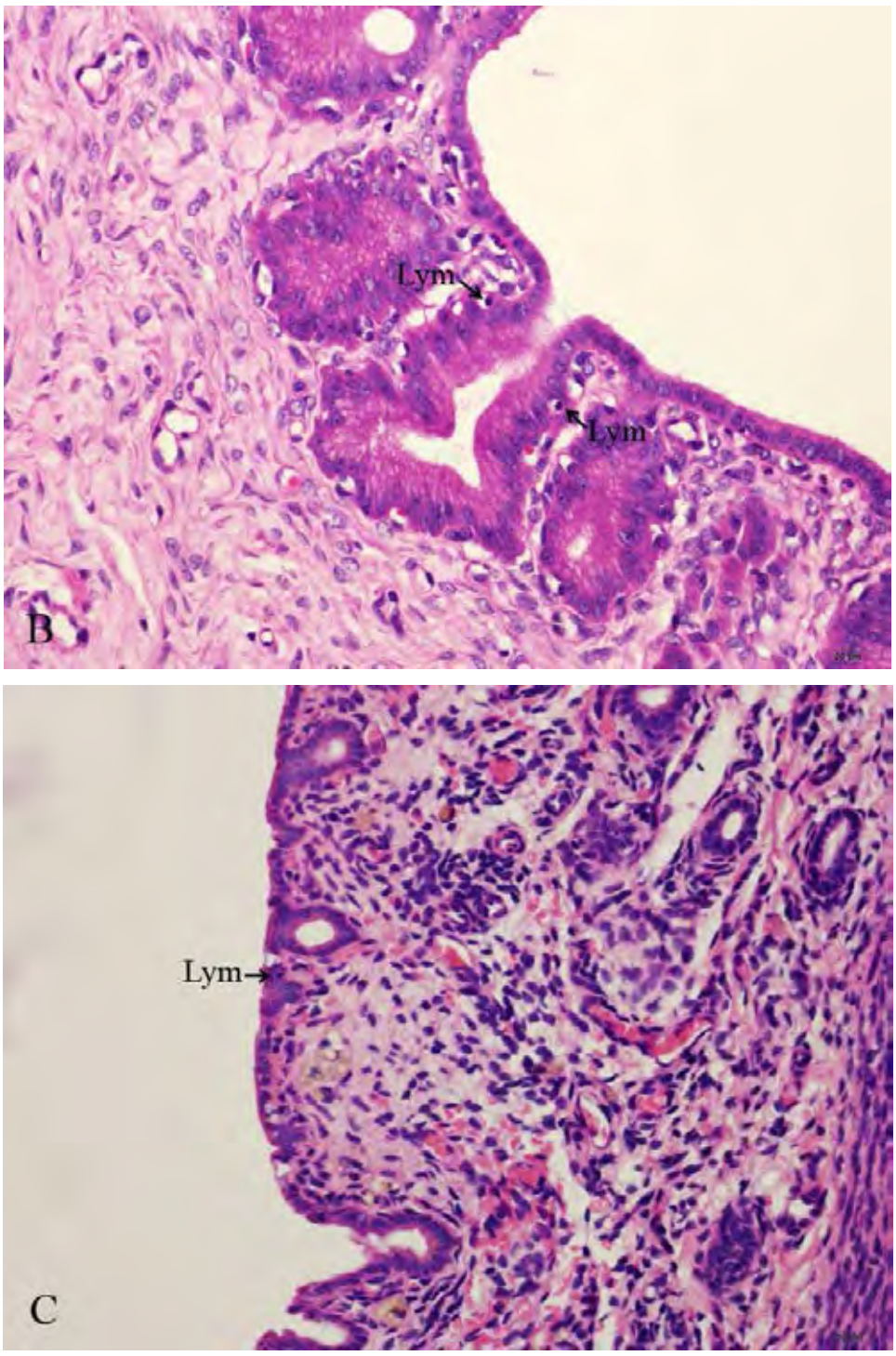
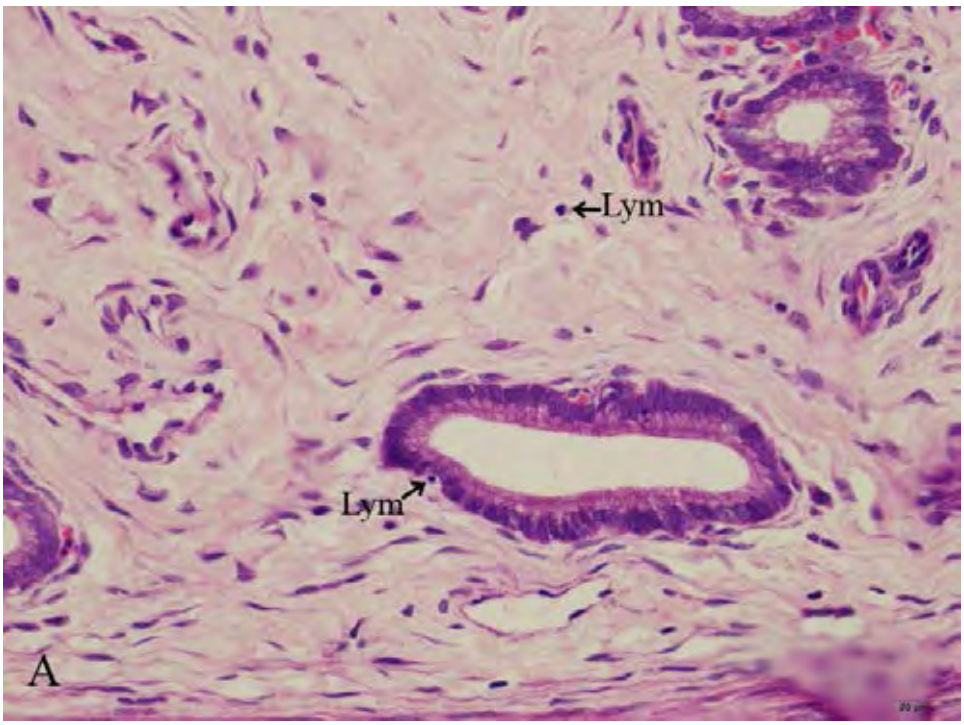
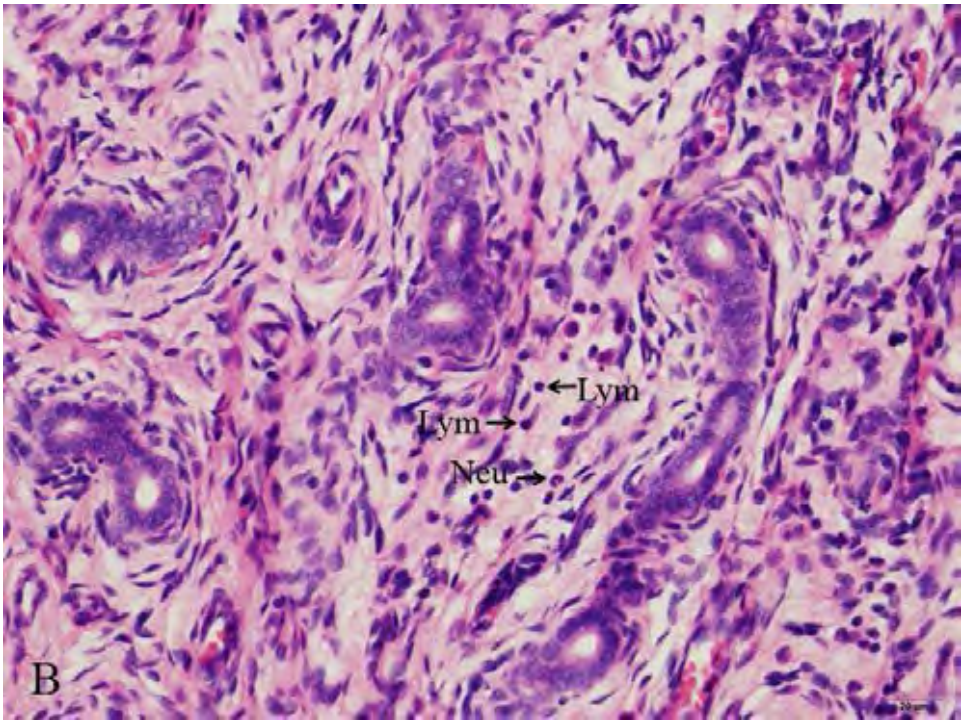


Figure 1 Hematoxylin and eosin staining of uterine horn showing lymphocytes (Lym) in surface epithelium at proestrus (A), diestrus (B) and anestrus (C).


Lym
Lym
A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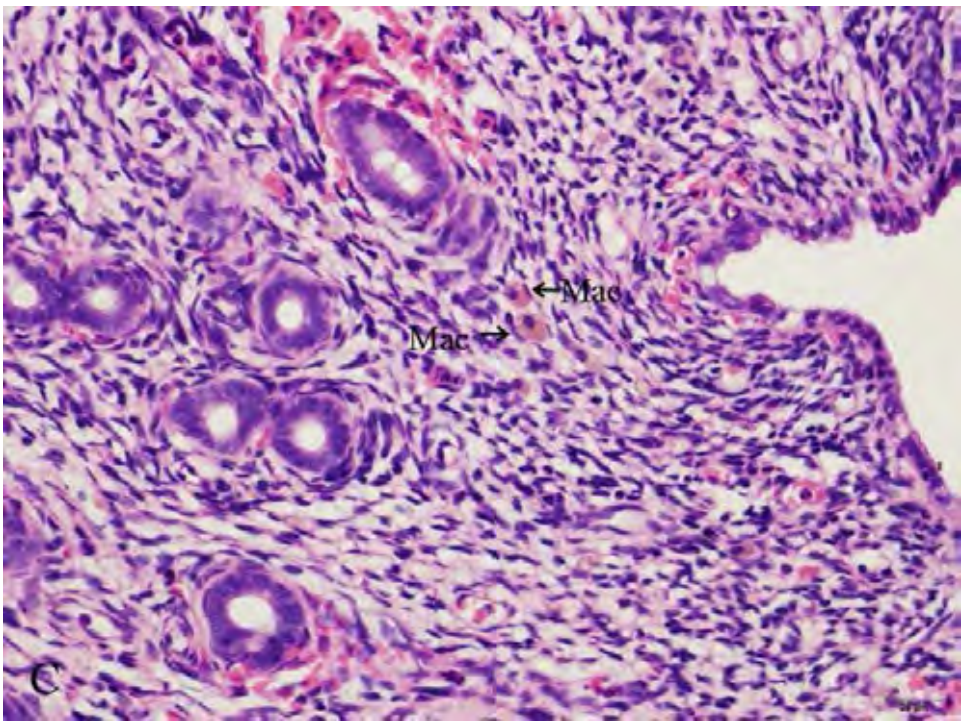
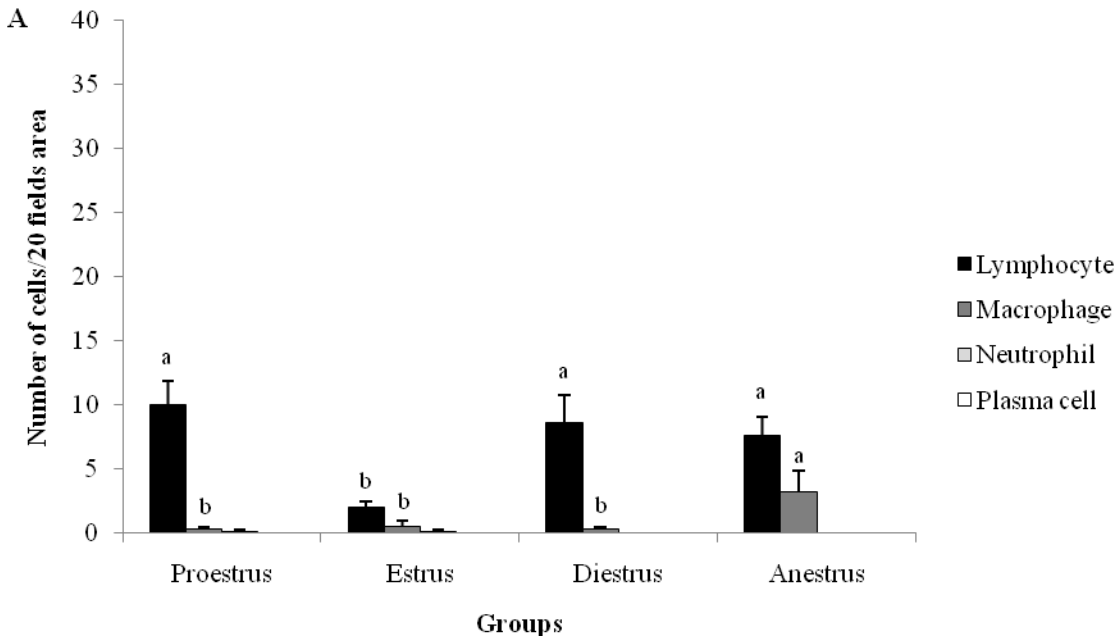


Figure 2 Hematoxylin and eosin staining of uterine horn showing lymphocytes (Lym) in glandular epithelium and stroma at proestrus (A), anestrus (B) and neutrophil (Neu) (B), macrophages (Mac) in stroma at anestrus (C).



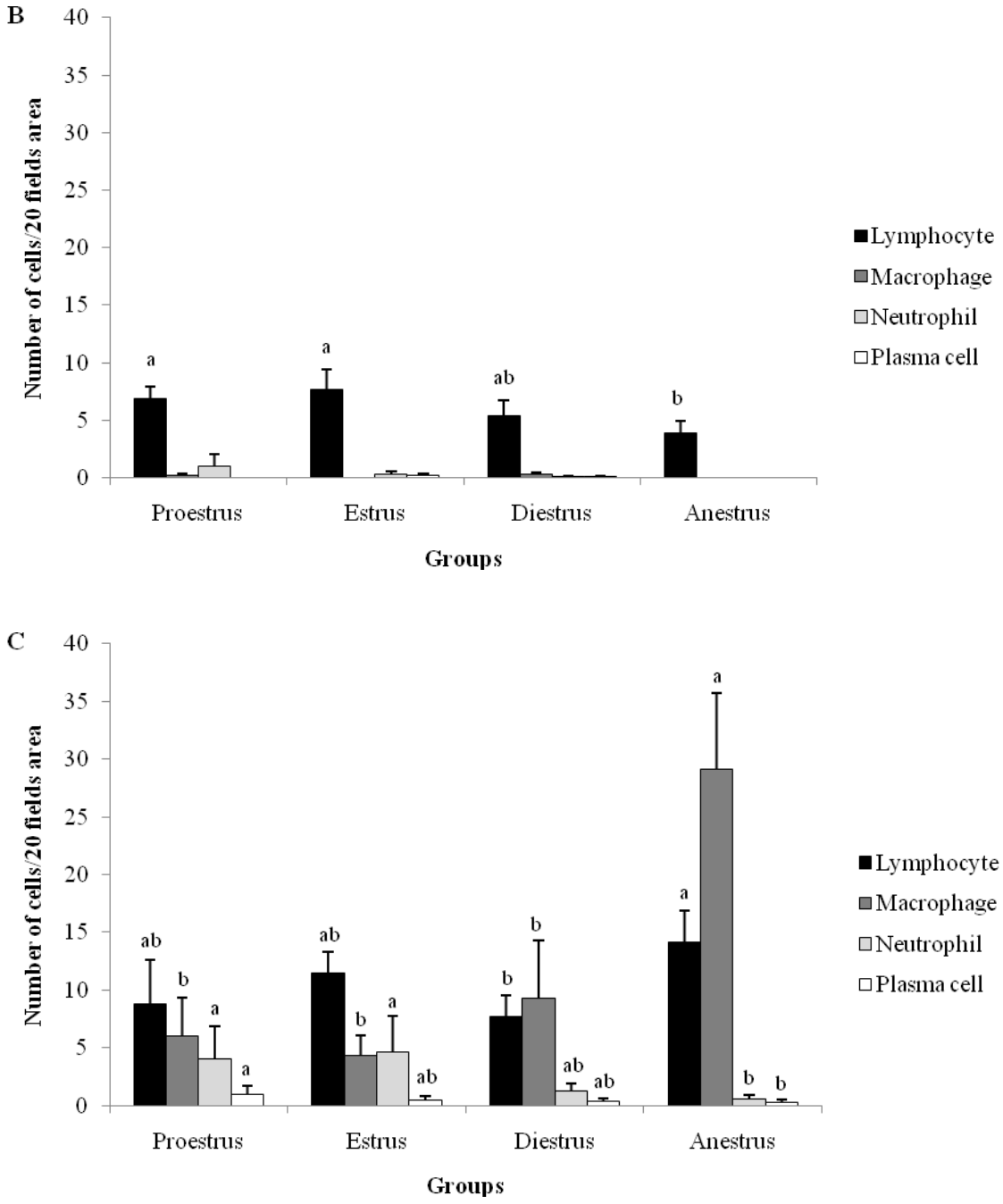


Figure 3 Number (Mean ± SEM) of different types of immune cells (lymphocyte, macrophage, neutrophil, plasma cell) in the surface epithelium (A), glandular epithelium (B) and stroma (C) of the endometrium (horn part) during the estrous cycle. The letters "a" and "b" indicate differences in the number between groups within a similar type of immune cells. Different letters indicate a significant difference ($P < 0.05$).

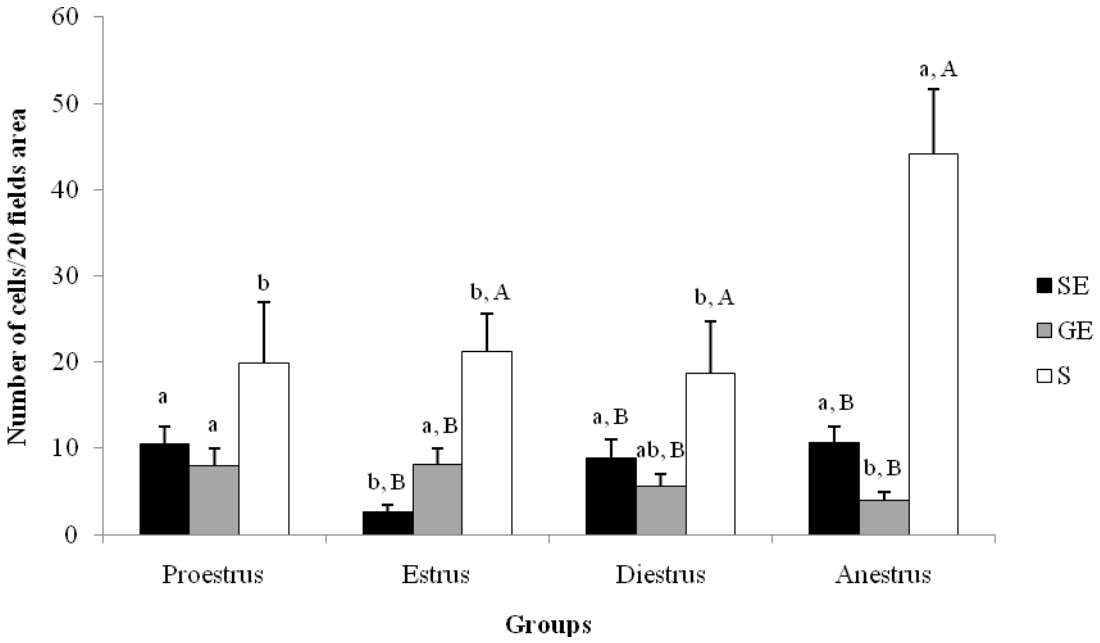


Figure 4 Number of total leukocytes (Mean ± SEM) in tissue layers (SE; surface epithelium, GE; glandular epithelium, S; stroma) of the endometrium (horn part) during the estrous cycle. The letters "a" and "b" indicate differences in the number between groups within a similar tissue layer. The letters "A" and "B" indicate differences in the number between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

3.4.1.2 The part of body

The glandular epithelium found an increased number of lymphocytes and total leukocytes in proestrous and estrous dogs compared to anestrous dogs ($P < 0.01$). In the stroma, the number of macrophages was significantly increased in anestrous dogs compared to other stages of the estrous cycle ($P < 0.01$) (Figure 5) and the total leukocytes also significantly higher at anestrus compared to estrus and diestrus ($P < 0.05$). Furthermore, neutrophils also significantly higher in the stroma at proestrus compared to diestrus ($P < 0.05$). And, the number of plasma cells in the stroma at estrus was significantly increased compared to diestrus ($P < 0.01$) in this part (Figure 6). The different types of immune cells and total leukocytes in all groups and layers were shown in Figure 7 and 8.

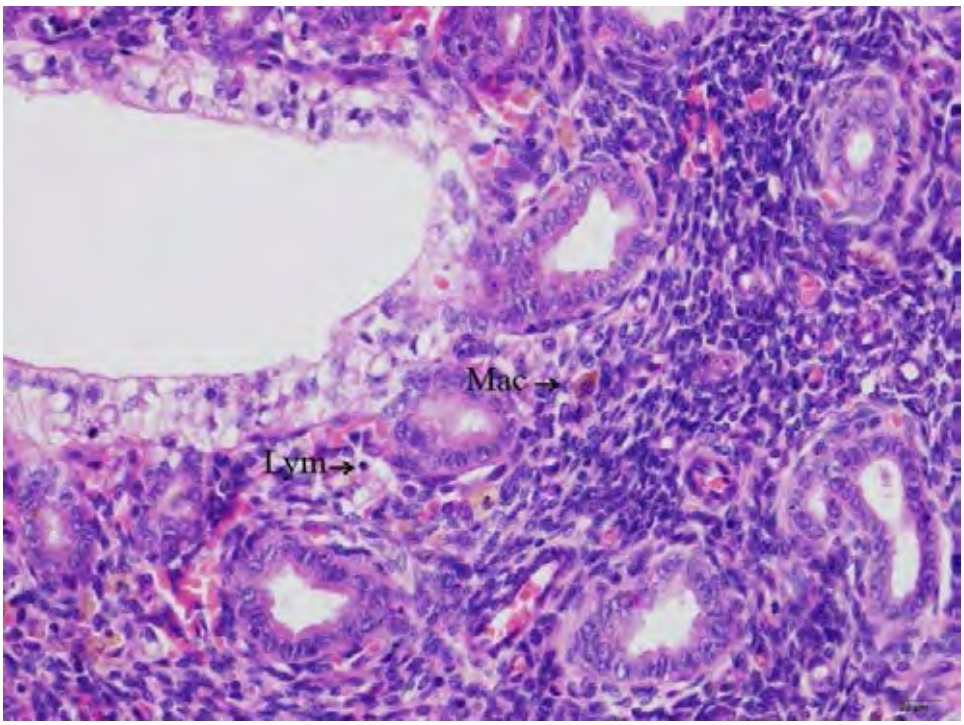
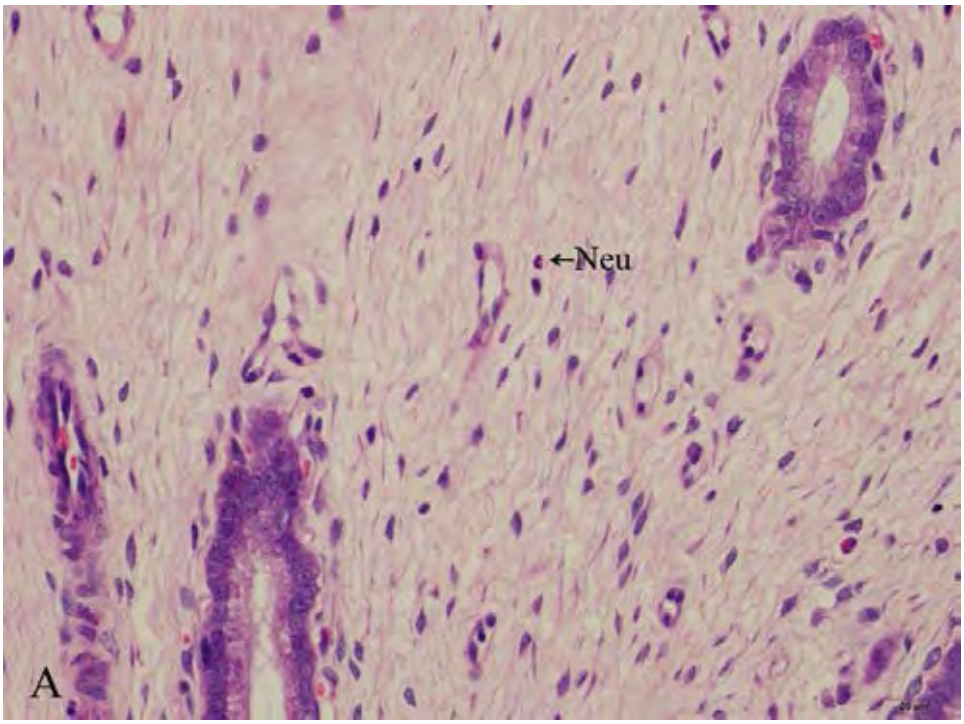


Figure 5 Hematoxylin and eosin staining of uterine body showing macrophage (Mac) and lymphocyte (Lym) in stroma at anestrus.



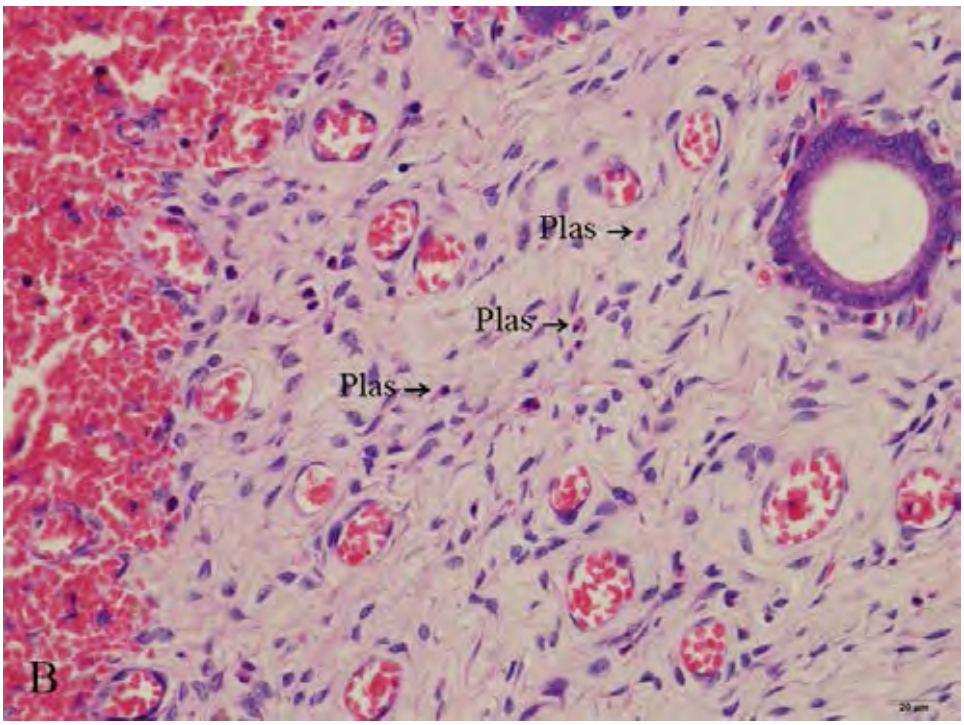
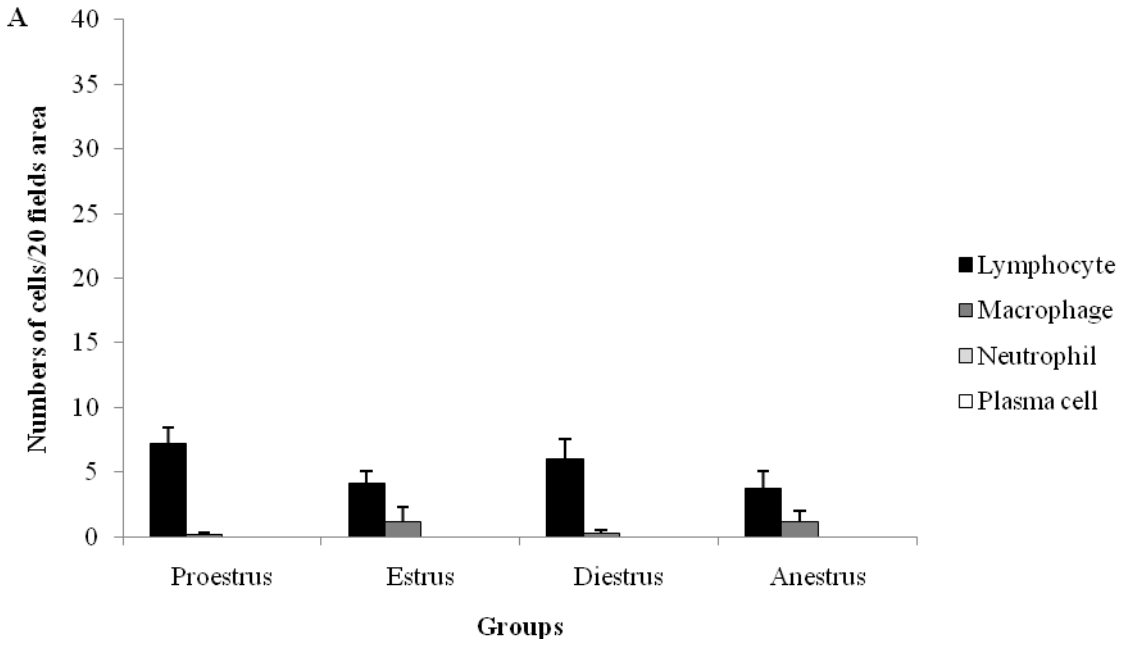


Figure 6 Hematoxylin and eosin staining of uterine body showing neutrophil (Neu) in stroma at proestrus (A) and plasma cells (Plas) in stroma at estrus (B).



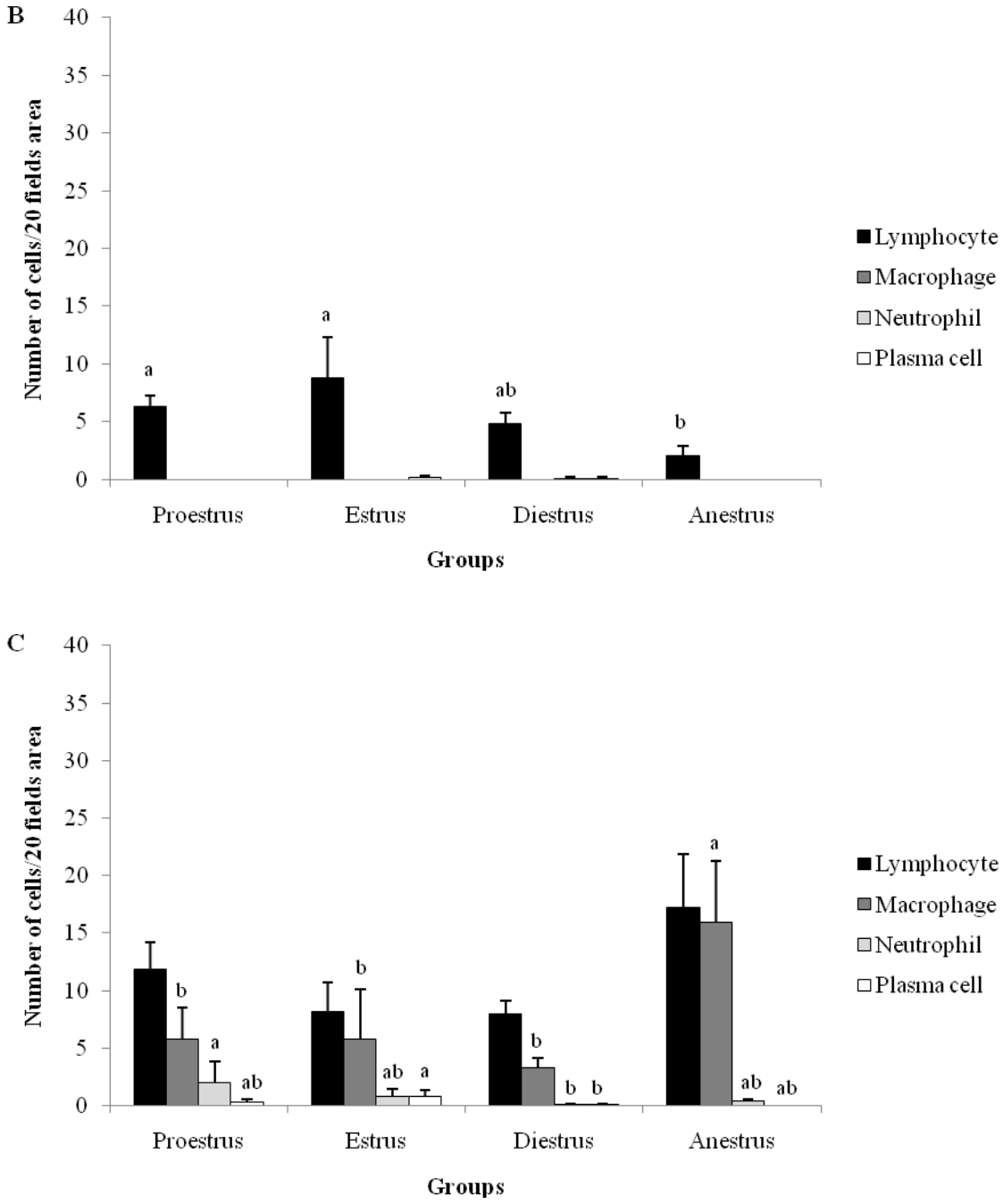


Figure 7 Number (Mean ± SEM) of different types of immune cells (lymphocyte, macrophage, neutrophil, plasma cell) in the surface epithelium (A), glandular epithelium (B) and stroma (C) of the endometrium (body part) during the estrous cycle. The letters "a" and "b" indicate differences in the number between groups within a similar type of immune cells. Different letters indicate a significant difference ($P < 0.05$).

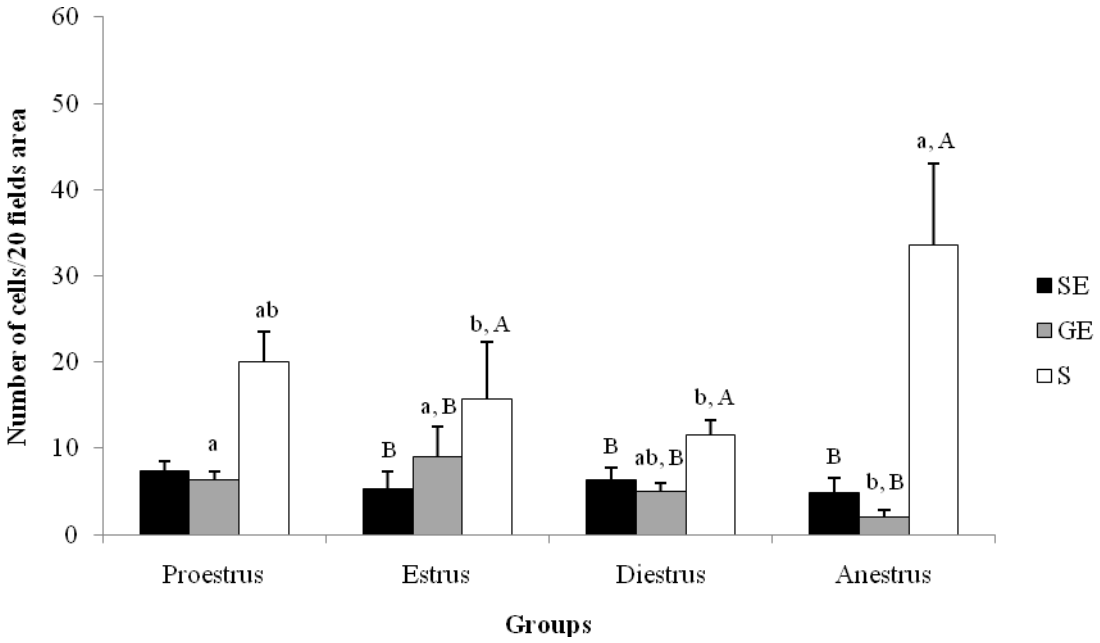


Figure 8 Number of total leukocytes (Mean ± SEM) in tissue layers (SE; surface epithelium, GE; glandular epithelium, S; stroma) of the endometrium (body part) during the estrous cycle. The letters "a" and "b" indicate differences in the number between groups within a similar tissue layer. The letters "A" and "B" indicate differences in the number between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

3.4.2 Leukocytes in the cervical tissues

The number of lymphocytes and total leukocytes in the surface epithelium of the cervix from this study was significantly decreased in anestrous stage compared to other stages ($P < 0.05$) (Figure 9). In diestrous dogs, the number of macrophages in the stroma of cervix was significantly higher than anestrous dogs ($P < 0.05$) from this study (Figure 10). The different types of immune cells and total leukocytes in all groups and layers were presented in Figure 11 and 12.

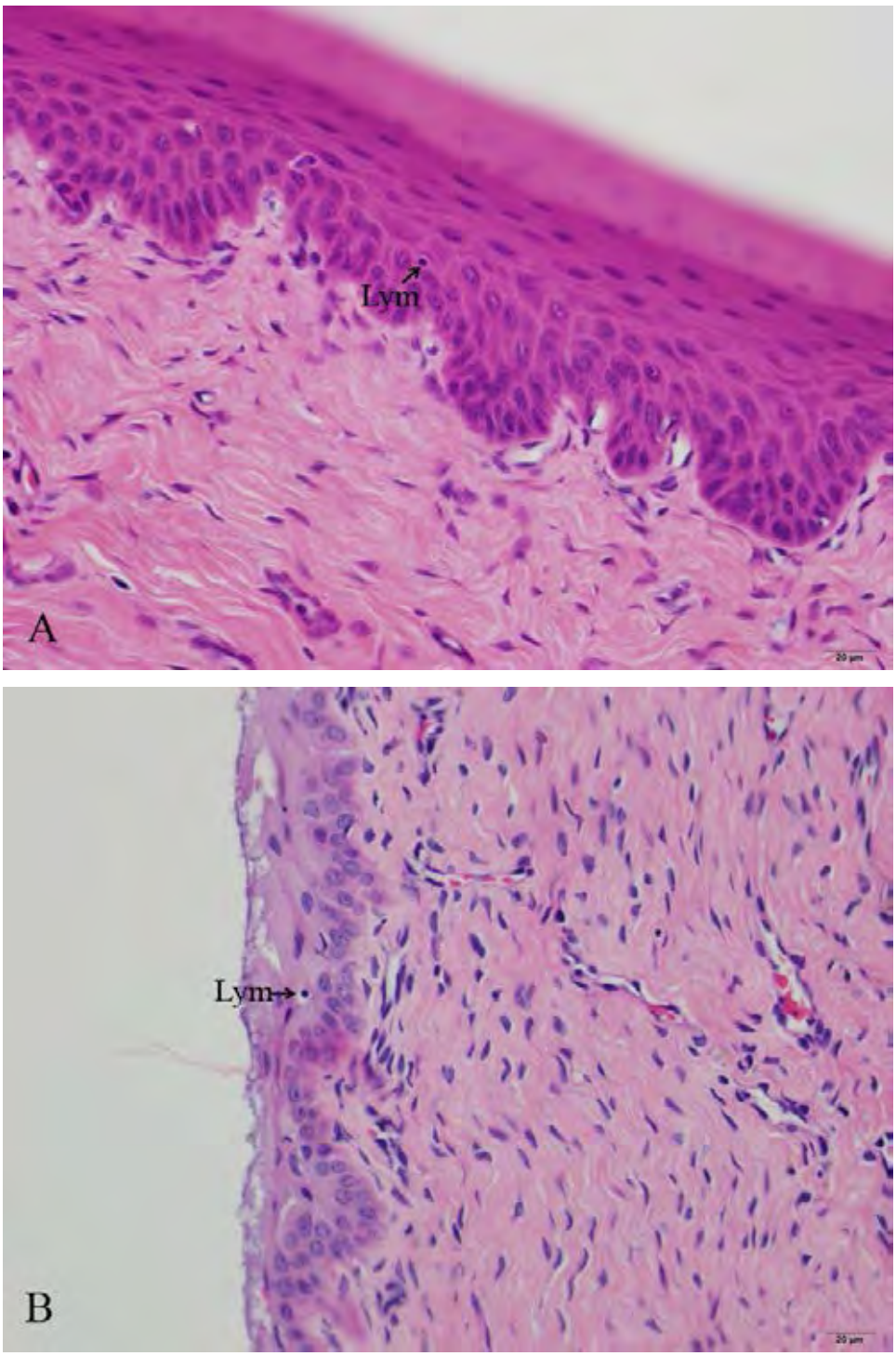


Figure 9 Hematoxylin and eosin staining of cervix showing lymphocyte (Lym) in surface epithelium at estrus (A) and diestrus (B).

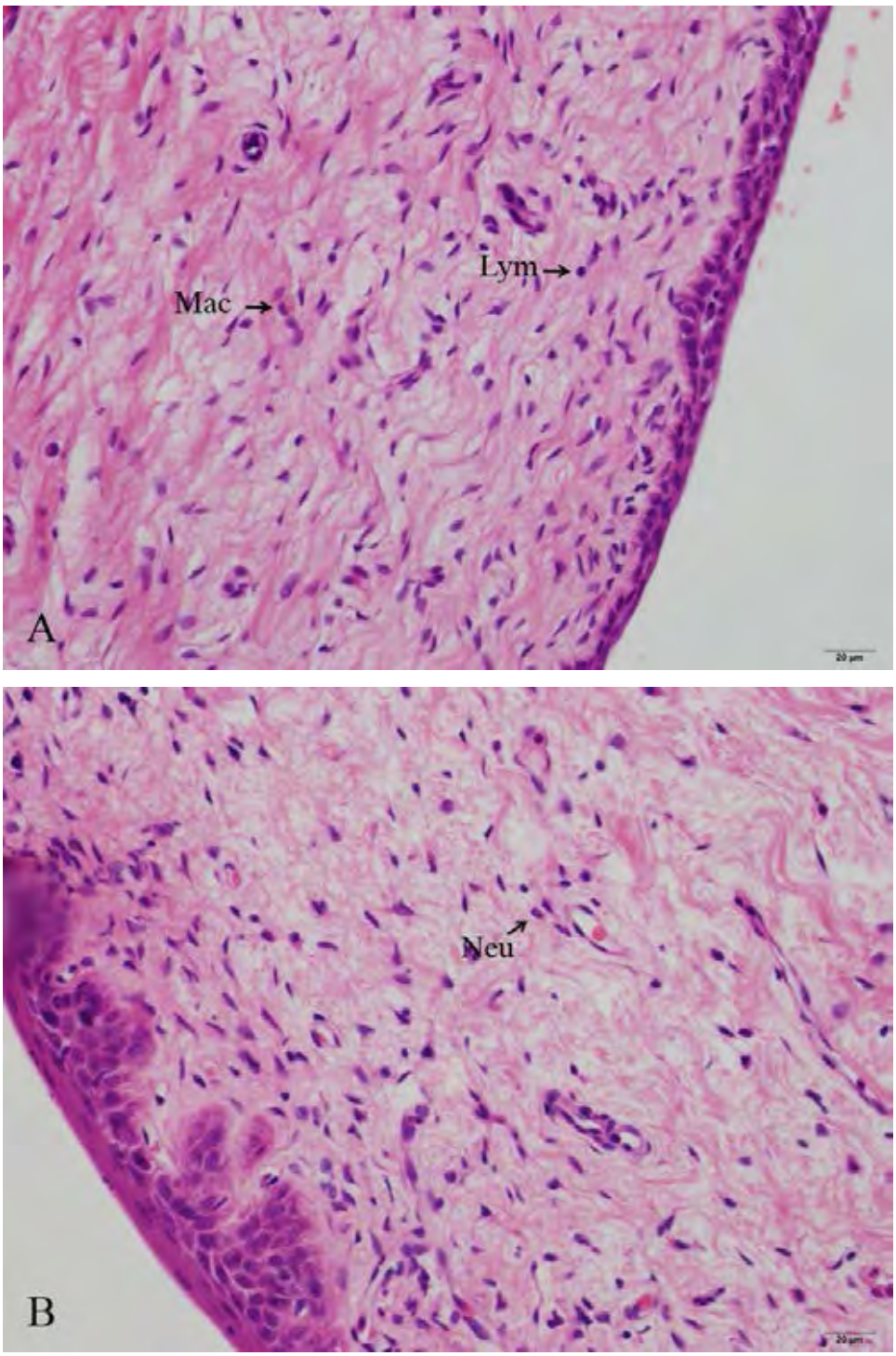


Figure 10 Hematoxylin and eosin staining of cervix showing macrophage (Mac) and lymphocyte (Lym) in stroma at diestrus (A) and neutrophils (Neu) in stroma at estrus (B).

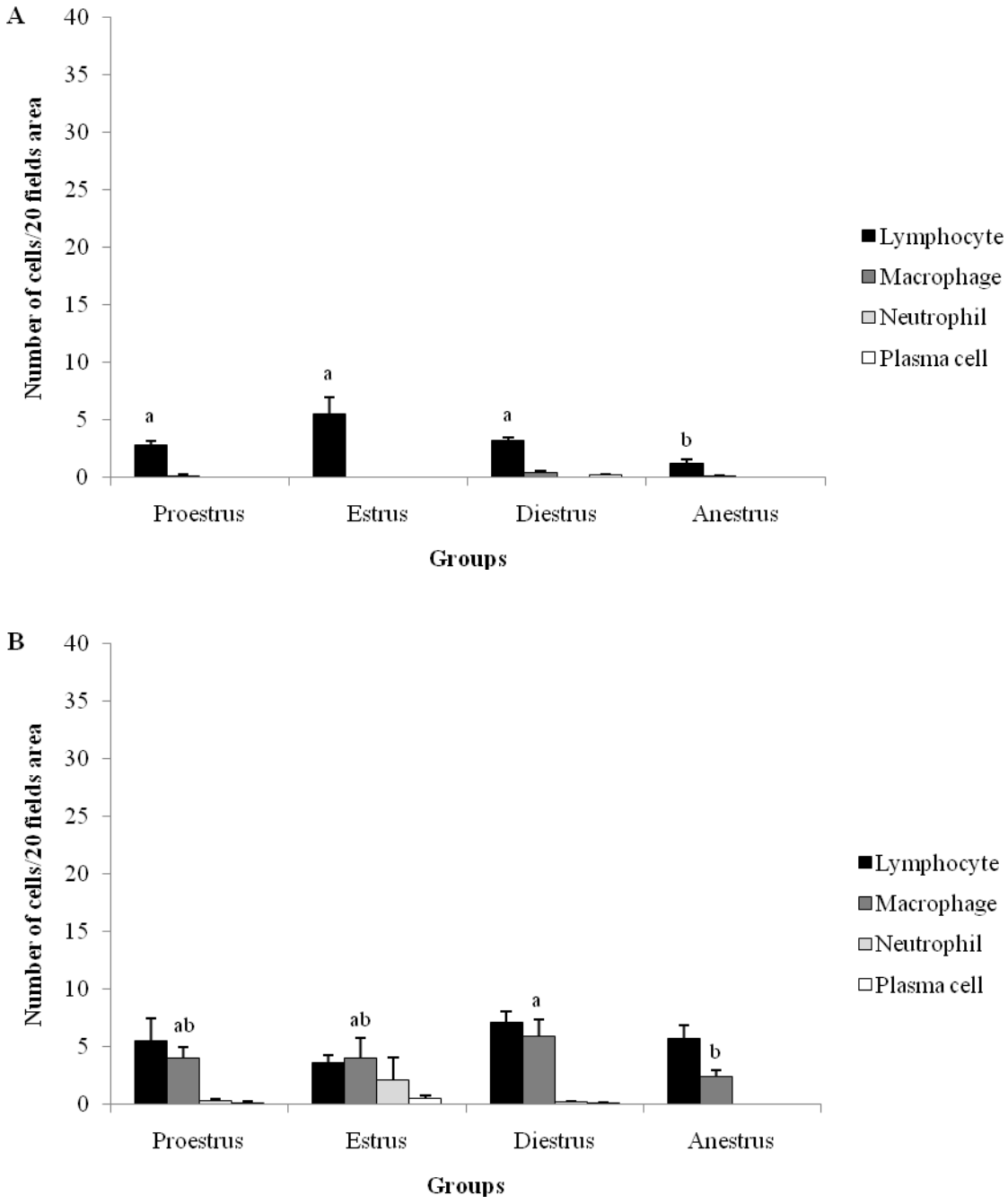


Figure 11 Number (Mean ± SEM) of different types of immune cells (lymphocyte, macrophage, neutrophil, plasma cell) in the surface epithelium (A) and stroma (C) of the cervix during the estrous cycle. The letters "a" and "b" indicate differences in the number between groups within a similar type of immune cells. Different letters indicate a significant difference ($P < 0.05$).

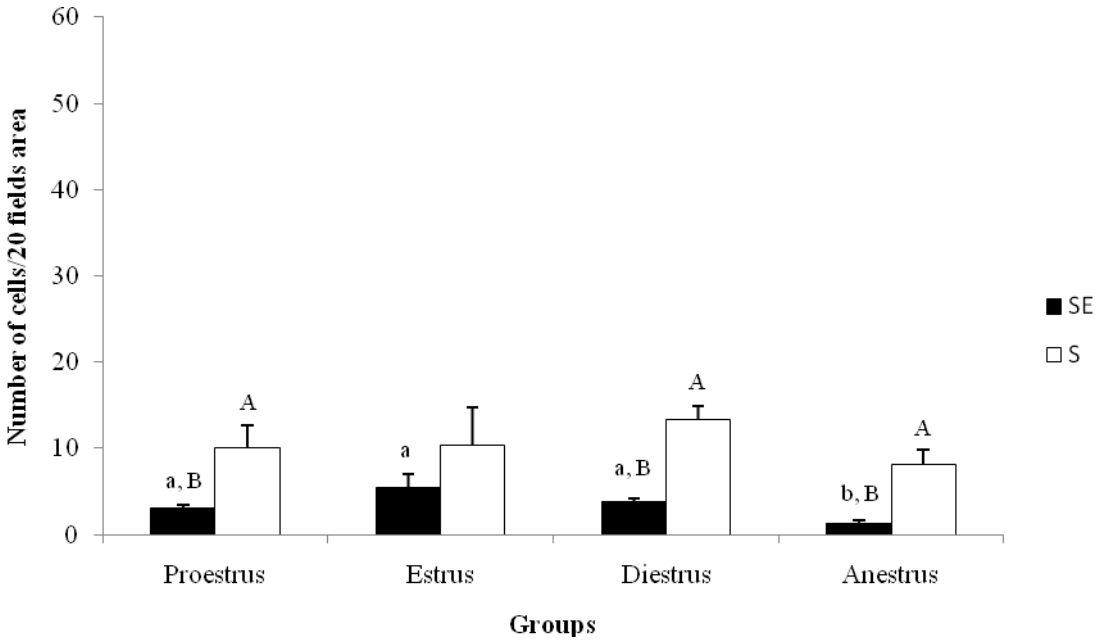


Figure 12 Number of total leukocytes (Mean ± SEM) in tissue layers (SE; surface epithelium, S; stroma) of the cervix during the estrous cycle. The letters "a" and "b" indicate differences in the number between groups within a similar tissue layer. The letters "A" and "B" indicate differences in the number between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

In the uterus of healthy dogs, when compared between tissue layers, the leukocytes were dominantly in the stroma. In the surface epithelium of this study, when compared between uterine sites, the number of lymphocytes and total leukocytes in the uterine horn was significantly higher than the cervix at proestrus ($P < 0.01$), diestrus ($P < 0.05$) and anestrus ($P < 0.01$) except only at estrus that the lymphocytes significantly dominant in the cervix ($P < 0.05$) compared to the uterine horn (Figure 13 and 14). In addition, the number of leukocytes was similar in the uterine horn and uterine body and the trend of the number of leukocytes was decreased in the lower part of the reproductive tract.

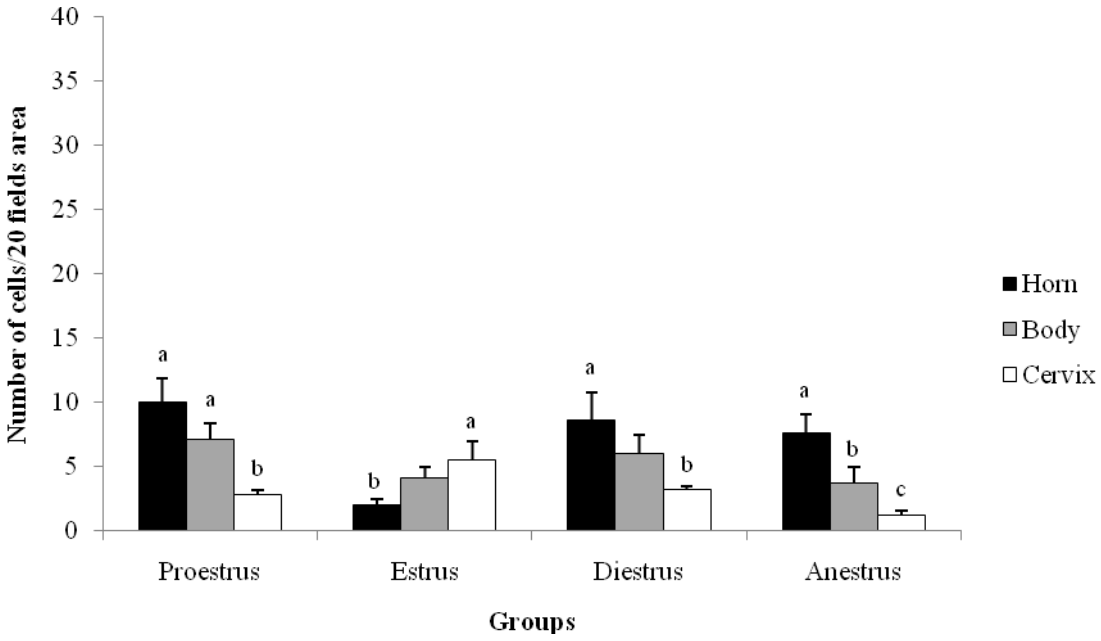


Figure 13 Number of the surface epithelial lymphocytes (Mean ± SEM) in different regions (Horn, Body, Cervix) of the uterus. The letters "a" and "b" and "c" indicate differences in the number between regions within a similar group. Different letters indicate a significant difference ($P < 0.05$).

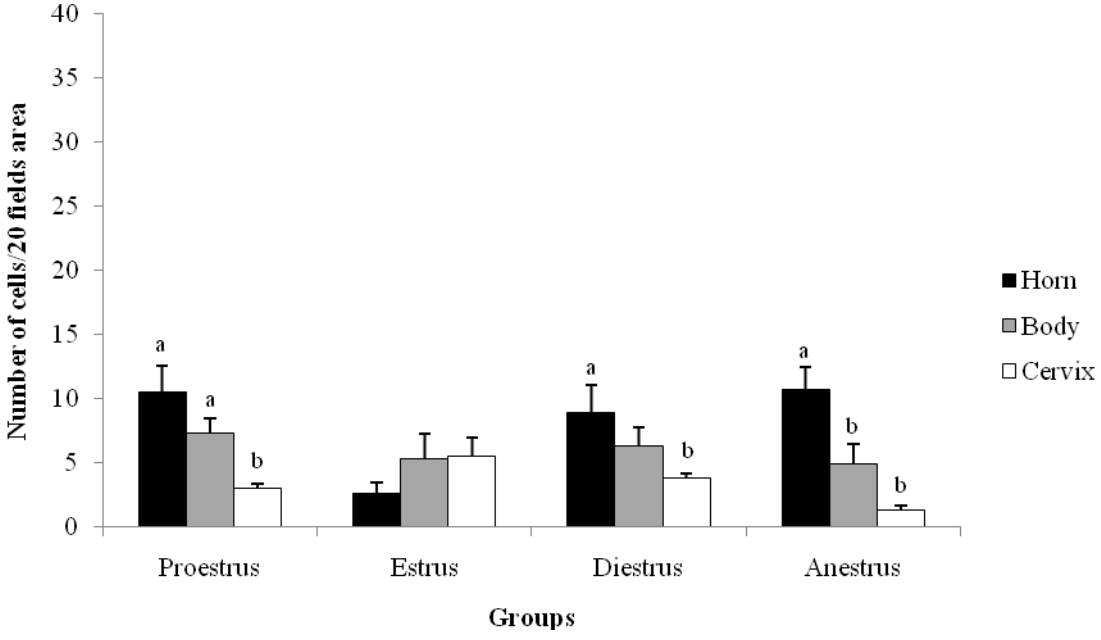


Figure 14 Number of the surface epithelial total leukocytes (Mean ± SEM) in different regions (Horn, Body, Cervix) of the uterus. The letters “a” and “b” and “c” indicate differences in the number between regions within a similar group. Different letters indicate a significant difference ($P < 0.05$).

3.5 Discussion

From this study, the distribution of leukocytes was found in the uterus of dogs during estrous cycle. The leukocytes that found from this study were composed of lymphocytes, macrophages, neutrophils and plasma cells. Lymphocytes were the most common immune cells found in the surface epithelium, glandular epithelium and stroma of the uterus. While, macrophages found dominantly in the stroma of the uterus from this study. Similarly, in pig uteri, T cells, macrophages and neutrophils appear to be the prominent leukocyte cell types which are responsible for the local cellular immune response (Bischof et al., 1994; Steffl et al., 2010). While, plasma cells are present in low number in porcine uterine tissue (Hussein et al., 1983; Steffl et al., 2010).

In the endometrial stroma, the total leukocytes and the number of macrophages were increased in anestrous dogs compared to other stages of the estrous cycle. And the number of lymphocytes in the uterine horn was also significantly higher in anestrus than diestrus. Moreover, the number of macrophages in the uterine horn was also significantly increased in the surface epithelium of anestrous dogs compared with dogs at other stages. In accordance with the previous experiment in chapter II that 10 out of 11 dogs, the bacteria could not be isolated from the uterus in anestrous stage. This finding may be indicated the role of the immune cells in the clearance of bacteria to maintain the sterile environment in canine uterus. In dogs, anestrus is important stage for the normal endometrial repair (Concannon, 2010). The number of canine endometrial leukocytes especially lymphocytes increased after degeneration of the surface epithelium at early anestrus. While, the number endometrial leukocytes decreased at diestrus (Chu et al., 2001, 2006) which may reflected the effect of progesterone in the suppression of the immune system (Hanson, 1998; Chu et al., 2006). In contrast, the withdrawal of progesterone is associated with

an influx of leukocytes in the endometrium (Loke and King, 1997; Critchley et al., 2001). In human, immediately prior to menstruation when estradiol and progesterone were maintain in a low levels (Maybin and Critchley, 2011), leukocytes in the uterine tissue contain up to 40% of the total cellular content (Salamonsen and Woolley, 1999; Kaitu'u-Lino et al., 2007) while the leukocyte was normally found in the human endometrium accounting for 10-20% of all endometrial cells (Givan et al., 1997; Yeaman et al., 2001).

In the tissue of human female reproductive tract, macrophages constitute nearly 10% of total number of leukocytes that are most abundant in the endometrial stroma of the uterus (Hunt et al., 1985; De and Wood, 1991; De et al., 1991; Starkey et al., 1991; De et al., 1993; Brandon, 1995; Salamonsen and Lathbury, 2000; Salamonsen et al., 2002; Wira et al., 2005; Howes, 2010; Yang et al., 2011). Macrophages are found through menstrual cycle but extremely increase in the late secretory phase (De and Wood, 1990; DeLoia et al., 2002; Jones et al., 2004; Keenihan and Robertson, 2004; Yang et al., 2011). The recruitment of uterine macrophages is regulated by the sex hormones throughout the menstrual cycle. Alterations of estradiol and progesterone levels are coincident with changes in the migration of macrophages to the endometrium (Jones et al., 1997; DeLoia et al., 2002; Wira et al., 2005). In the previous studies, macrophages selectively aggregate into premenstrual human endometrial stroma, concurrent with depression of estradiol and progesterone levels as a result of luteolysis (Kamat and Isaacson, 1987; Jones et al., 1997; Wira et al., 2005) in accordance with this study which found an increase number of macrophages in the endometrium of anestrous dogs. The possible mechanism of sex hormones is that progesterone withdrawal results in upregulation of monocyte chemotactic protein-1 (MCP-1) leading to chemotaxis and activation of monocytes (Critchley et al.,2001; Wira et al., 2005) while, estradiol significantly inhibits expression of MCP-1from human endometrial stromal cells, which correlated with suppression of macrophage migration (Arici et al., 1999; Wira et al., 2005). These may partially explain the correlation of macrophage accumulation with the fluctuation in estradiol and progesterone levels (De and Wood, 1990; DeLoia et al., 2002; Jones et al., 2004; Keenihan and Robertson, 2004; Yang et al., 2011). Nevertheless, increased number of endometrial macrophages when estradiol level is

high has been reported in the earlier studies. In pigs, macrophages were found in the surface and glandular epithelium at proestrus and the number of macrophages was also correlated with estradiol levels (Kaeoket et al., 2002a). In mouse, changes of macrophage population in the endometrium were influenced by both estradiol and progesterone (De and Wood, 1990; Kaeoket et al., 2002a). Accumulation of human endometrial macrophages also occurs during mid-secretory phase, when estradiol and progesterone levels are high (Arici et al., 1999; Wira et al., 2005) consistent with the report of macrophage chemokines that has been observed during these phase (Jones et al., 2004; Wira et al., 2005). The possibility is that regulation of MCP-1 is mediated by estradiol modulation of another factor such as IL-1 (Arici et al., 1999; Wira et al., 2005). Estradiol have been shown to stimulate IL-1, in turn, IL-1 has been shown to induce expression of MCP-1 (Akoum et al., 2000; Wira et al., 2005). Therefore, estradiol appears to have both positive and negative roles to regulate MCP-1 expression through different mechanisms. However, uterine macrophages are collectively regulated on their functions and localizations via dynamic interaction among cytokines, chemokines, hormones and other biological factors (Yang et al., 2011).

The total leukocytes and lymphocytes in the surface epithelium of the uterine horn significantly decreased at estrus compared with other stages of estrous cycle. This finding may corresponded to the previous experiment in the bacterial culture from the uterus of estrous dogs which found that 3 out of 5 dogs (60%) the bacteria could be isolated. Estrus in the dog occurs in response to the decline in estradiol concentration which normally begins shortly before LH surge while serum progesterone concentration rapidly increases (Concannon, 2010). Progesterone appears to inhibit the generation and activation of lymphocytes (Borel et al., 1999; Miyaura and Iwata, 2002; Sugiura et al., 2004) by directly inhibits the development of Th1 cells (Muyaura and Iwata, 2002; Sugiura et al., 2004) and may initiate synthesis of immunosuppressant proteins in the uterine lumen which then inhibit lymphocyte proliferation, rather than acting itself as an immunosuppressant (Chacin et al., 1990; Dhaliwal et al., 2001). The increased susceptibility of the uterus to infections under progesterone dominance might be influenced by the immunosuppressant proteins present in the uterine lumen, which inhibit lymphocyte proliferation or by the

inflammatory mediators, (Dhaliwal et al., 2001). However, in diestrous stage which the progesterone dominance, the number of leukocytes was not significantly lower than other stages. This may be indicated that factors other than sex hormones may be involved in the influx of leukocytes in the endometrial surface epithelium.

Uterine epithelial cells are involved in both innate and adaptive immunity of the female reproductive tract. These cells produce a variety of substances that links for the innate and adaptive immune system (Fahey et al., 2005; Wira et al., 2005). The innate immune system has evolved to recognized foreign structures that are not normally found in the host. The pattern recognition receptors (PRRs) are expressed on the female reproductive tract epithelial cells to detect potential microbial pathogen and also involved through the secretion of cytokine and chemokine which may lead to the recruitment of the leukocytes in the uterus (Wira et al., 2005). Furthermore, the female reproductive tract epithelial cells secrete soluble factors such as defensins and secretory leukocyte protease inhibitor (SLPI) that inhibit the growth of microorganisms. In addition to their antimicrobial role, human β-defensins (HBDs) have been shown to have other properties including chemotactic activity suggesting a link between the innate and adaptive immune systems (Yang et al., 1999; King et al., 2007). And SLPI is also required for the production of pro-inflammatory chemokines and cytokines in response to microbial antigen (Zhang et al., 2002; Wira et al., 2005).

In bovine endometrium, the numbers of CD4+ and CD8+ T cells have been reported to increase in the follicular phase (Cobb and Watson, 1995). Furthermore, the predominance intraepithelial CD8+ lymphocytes have been reported in the endometrium in non-pregnant women and pigs and most of the CD8+ cells were associated with the glandular epithelium (Pace et al., 1991; Bischof et al., 1994; Cobb and Watson, 1995; Kaeoket et al., 2002b). In accordance with the present study that found an increased number of total leukocytes and lymphocytes in the endometrial glandular epithelium at proestrus and estrus. Earlier studies, the administration of estrodiol to rats induced a significant increase in the number of CD4+ T cells. Thus, CD4+ cells may be responses to estradiol (Zheng et al., 1989; Cobb and Watson, 1995). In addition, estradiol appears to directly activate immune cells including T cells. Estradiol enhances Th1 cell responsiveness via estradiol receptor-α and -β

(Erlandsson et al., 2001; Rider et al., 2001; Maret et al., 2003; Sugiura et al., 2004) and B cells (Erlandsson et al., 2003; Grimaldi et al., 2002; Sugiura et al., 2004).

Neutrophils are prototypical innate immune leukocytes that form the first line of defense against infection and also initially response to bacteria that enter the uterus. The ability of neutrophils to respond to intrauterine bacteria may be the important component of the uterine immune defense mechanism (Hussain, 1989; Saad et al., 1989; Hussain and Daniel, 1991; Lewis, 2004). From the current study, Neutrophils were found in the stroma of the uterine horn which significantly higher at proestrus and estrus. And neutrophils were also significantly higher in the stroma of the uterine body at proestrus. Neutrophils contain 40-75% of circulating white blood cells and they transiently interact with the wall of small capillaries (Downey et al., 1990; Wira et al., 2005). During the estradiol dominance, there is increased blood flow to the uterus, increased mucus production and intensified PMN activity (Hawk et al., 1960, 1964; Killingbeck and Lamming, 1963; Dhaliwal et al., 2001). Estradiol may affect chemotaxis (Hoedemaker et al., 1992; Lamote et al., 2004; Singh et al., 2008) and phagocytosis by enhanced the production of IFN-γ and promotes recruitment of neutrophils and macrophages into uterus as a pro-inflammatory activity (Tibbetts et al., 1999; Sugiura et al., 2004). In pigs during proestrous and estrous stages, the number of neutrophils was highly in the stroma (Kaeoket et al., 2002a). The possibility is that the high level of estradiol increase the permeability of blood capillaries (Key and King, 1988; Kaeoket et al., 2002a) leading to the high infiltration of neutrophils in the endometrium (Kaeoket et al., 2002a). Another possibility is explained by the influence of cytokines and chemokines on inflammatory cell migration (Dunon et al., 1996; Kaeoket et al., 2002a). In the previous study, epithelial cells in the human female reproductive tract secreted chemokines to attract neutrophils to the epithelium. Moreover, they produce a higher amount of chemoattractant that may induce neutrophils to cross the epithelium and enter the lumen which they would contribute to innate protection of the endometrium and proactively remove microorganisms (Wira et al., 2005). In addition, degranulation of neutrophils would release antimicrobial peptides such as defensins, SLPI, lactoferrin that are present in blood neutrophils (Spitznagel et al., 1974; Cowland et al., 1995; Faurschou et al., 2002; Wira et al., 2005). And earlier studies on endometrial

neutrophils also described the expression of α-defensins (Lea and Sandra, 2007) and elafin, a neutrophil protease inhibitor and microbicide related to SLPI (King et al., 2003; Wira et al., 2005).

In mice, numerous plasma cells were also found in the uterus and oviduct at proestrus and estrus. In addition, the uterus contained more plasma cells than the cervix and vagina (Gu et al., 2005) which in accordance with this study in dogs. The number of plasma cells in the uterine horn at proestrus was significantly higher than anestrus and the number of plasma cells in the uterine body at estrus was significantly increased compared to diestrus in this study. The presence of plasma cells in the uterus indicated that immunoglobulins should be secreted in the reproductive tract (Perez-Martinez et al., 2002). However, small amount of plasma cells was found in canine uterus from this study. This may suggested that antibodies in the canine reproductive tract may be derived mainly from other sources such as serum rather than local production (Gu et al., 2005). The low number of plasma cells was found also in normal premenopausal human endometrium (Sen and Fox, 1967; Perez-Martinez et al., 2002) and in female goat reproductive tract (Perez-Martinez et al., 2002). In addition, function of plasma cells seemed to be distinct in different species, for example, plasma cells increased during estrus in sow whereas they did not changes in the mare (Hussein et al., 1983; Watson and Thomson, 1996; Perez-Martinez et al., 2002).

In diestrous dogs, the number of macrophages in the stroma of cervix was significantly higher than anestrous dogs. While, the number of total leukocytes and lymphocytes in the surface epithelium of the cervix from this study was significantly increased in proestrus, estrus and diestrus. In the increased number of lymphocytes and total leukocytes in proestrous and estrous dogs may be explained by the influence of estradiol in the activation and influx of leukocytes (Sugiura et al., 2004). Whereas, in the secretory phase when progesterone dominance, the number of lymphocytes significantly increased in the goat cervix which related to the hormonal levels. The presence of immune cells the cervix in this stage when progesterone dominance indicated a reinforcement of the mucosal barrier in the anatomical region that exposed to pathogens in a critical period while embryo implantation and development may occur (Perez-Martinez et al., 2002). However, in human cervix, no differences in the

epithelial lymphocyte cell densities were noted when comparing between the proliferative and secretory phase (Poppe et al., 1998). Thus, these findings may confirm that the sex hormones are not only one factor that involve in the immune system of the uterus, some other factors may play a role by influencing the lymphocytes traffic in the canine reproductive tract.

The uterine horn was in the sterile upper female reproductive tract (Horne et al., 2008). In the surface epithelium of the uterus from this study, when compared between uterine regions, the number of lymphocytes in uterine horn was significantly higher than cervix at proestrus, diestrus and anestrus except only at estrus that the surface epithelial lymphocytes were significantly dominant in the cervix compared to the uterine horn. This increased number of lymphocytes in the endometrial surface epithelium indicated the major role of lymphocytes in the immune surveillance to maintain the sterile environment in the upper part of canine reproductive tract. Meanwhile, bacterial contamination of the uterus usually occurs prior to diestrus when the cervix is open (Rietschel et al., 1982; McAnulty, 1983; Pretzer, 2008). To protect the uterus from the pathogens invasion in this stage the leukocytes should be accumulate in the cervix preparing for combat to the infection. This may indicated an increased number of lymphocytes in the cervix at estrous stage in this study which indicated the protective mechanism of the immune system in canine reproductive tract.

In the uterus of healthy dogs, when compared between tissue layers, the leukocytes were dominantly in the stroma. The possibility is that the endometrium may be considered as a tertiary lymphoid site characterized by a remarkable population of lymphocytes in a microenvironment of stromal cells (Kämmerer et al., 2004). The lymphoid aggregates which presented in the endometrial stroma beneath the epithelium commonly process antibodies in human and other species (Segerson et al., 1991; Watson and Dixon, 1993; Yeaman et al., 1997; Perez-Martinez et al., 2002). Besides, endometrial cytokines are produced by tissues and leukocytes. Stromal cells produced GM-CSF which has many effects, one of them to prolong life of endometrial neutrophils (Chegini et al., 1999; Wira et al, 2005) which appear to be the most predominant stromal cell type producing IFN-γ. This cytokine is a potent

activator of macrophages (Appelberg, 1994; Wira et al., 2005) and therefore may help to promote endometrial innate immunity (Wira et al., 2005).

The number of leukocytes seem varied in a different tissue layers and different regions between the upper and lower part of the female reproductive tract at different stages of the estrous cycle, indicated the different role in the uterine immune surveillance protecting the host from the pathogen invasion. The sex hormone may be involved in the infiltration of leukocyte. However, the uterine leukocytes may not only depend on the sex hormones, other factors such as the cytokines, physiological structure of the uterus and different arms of innate immunity may also regulate the mechanisms of leukocytes infiltration.

CHAPTER IV

THE EXPRESSION OF TLR2 AND TLR4 IN THE UTERUS OF HEALTHY BITCHES AT DIFFERENT STAGES OF THE ESTROUS CYCLE AND BITYCHES WITH PYOMETRA

4.1 Abstract

This study provides the first report into immunohistochemical localization of Toll-like receptor (TLR) in the canine reproductive tract. TLR2 and TLR4 were investigated in the uterus during the estrous cycle and in pyometra. Pyometra is the most important pathological condition of the uterus due to bacterial infection in dogs. To protect against invading pathogens, the female reproductive tract has evolved immune mechanisms. TLRs are the cellular components of the afferent arm of the innate immune system. The expression of TLR4 in the endometrial surface epithelium was higher in dogs with pyometra compared with all other groups ($P < 0.01$). While, the expression of TLR2 in the surface epithelium of the uterine body was significantly decreased in pyometra dogs ($P < 0.05$). Interestingly, TLR2 was expressed in endometrial epithelium but absent in the endometrial stroma of healthy dogs at all stages. In dogs suffering from pyometra, when compared between the uterine regions, the expression of TLR4 was significantly more intensely in the surface epithelium and stroma of the uterine horn compared to the uterine body and the cervix ($P < 0.01$). Conversely, the expression of TLR2 in the surface epithelium of the cervix was significantly higher than the uterine horn and body ($P < 0.01$). Furthermore, the different levels of TLR2 and TLR4 expression seems related to physiological changes in distinct cell types of the uterus, leukocytes populations and sex hormones.

4.2 Introduction

Many cases of inflammatory disease in the reproductive tract of dogs and cats are considered to be caused by infectious agents (Schultheiss et al., 1999). In the dog, pyometra (chronic uterine inflammation with an accumulation of pus in the uterus) is the most important pathological condition of the uterus as a result of uterine infection (Ishiguro et al., 2007). Pyometra seems to be a result from the interplay of pathogenic bacteria and the progesterone primed uterus, however in a sequence that still need to be validated (Verstegen et al., 2008). To protect against invading pathogens, the female reproductive tract has developed immune mechanisms (Wira et al., 2005). The immune system has been classified into innate and adaptive immune system. However, innate immune system is the most universal, the most rapidly acting and may be the most important type of immunity (Beutler, 2004). Mucosal surfaces are the first line of defense against pathogens. And, mucosal epithelial cells have developed innate immune system which can inhibit pathogen growth immediately (Hecht, 1999; Fahey et al., 2005). Furthermore, epithelium is in constant contact with the commensal microorganisms of the female reproductive tract and must differentiate between commensal microorganisms and pathogens in order to activate protective immune mechanisms. Therefore, the control of commensal microorganisms and detection of pathogens by cells of the innate immunity rapidly is important to survival of the host (Soboll et al., 2006). The key mediators of the innate immune system are natural antimicrobial peptides (NAPs) and pattern recognition receptors (PRRs) (Wira et al., 2005; Horne et al., 2008). These receptors that express in the epithelial cells of the female reproductive tract recognize conserved pathogen-associated molecular patterns (PAMPs) which present on microorganisms (Lea and Sandra, 2007) such as bacterial, viral and fungal microorganisms (Medzhitov and Janeway, 2000; Janeway and Medzhitov, 2002; Aflatoonial et al., 2007). Toll-like receptors (TLRs) are the main family of PRRs (Akira et al., 2001; Takeda and Akira, 2005; Aflatoonian and Fazeli, 2008), and they are expressed by cells of host that related to the first line of defense such as neutrophils, macrophages, dendritic cells, dermal endothelial cells and mucosal epithelial cells (Aflatoonian et al., 2007). To date, 13 murine TLRs and 10 human TLRs have been identified. TLR2 and TLR4 are the best characterized of

innate responses to bacteria (Pioli et al., 2004). TLR4 is identified as the first mammalian TLR and it is also the best described of the family (Rock et al., 1998; Takeda et al., 2003; Linde et al., 2007). TLR4 is the receptor which recognizes lipopolysaccharide (LPS) (the endotoxic component of Gram-negative bacteria) (Poltorak et al., 1998; Hoshino et al., 1999; Yu et al., 2010). Meanwhile, TLR2 is reported to detect lipoteichoic acid from Gram-positive bacteria (Silva et al., 2010). TLRs are the cellular components of the afferent arm of the innate immune system (Beutler, 2004). Thereby, various types of the TLR family which are expressed on various cells seem to mediate signaling transduction to a range of antigenic stimuli by binding to unique ligands in order to produce different proinflammatory cytokines, chemokines and effecter components upon the type of cell that is stimulated (Aflatoonian et al., 2007). Following results, such as influx of neutrophils and stimulation of macrophages, lead to the destroying of pathogens directly (Takeda et al., 2003; Pasare and Medzhitiv, 2004). Recognition of pathogens by TLRs also triggers the stimulation of adaptive immune system which making these receptors as a critical link between the innate and adaptive immune system (Takeda et al., 2003; Werling and Jungi, 2003; Linde et al., 2007).

The innate immunity is essential to control uterine infection and to maintain the uterine homeostasis for normal physiological functions. Also, the bacterial species frequently isolated from canine uterus in the previous study in chapter II is not only the Gram-negative *Escherichia coli* but also the Gram-positive bacteria. And, gene transcriptions of TLR2 and TLR4 by real-time PCR have been recently reported in endometrium of diestrous dogs and dogs with pyometra (Silva et al., 2010). However, the hypothesis is that TLR2 and TLR4 may be involved in the pathogenesis of uterine bacterial infection or pyometra and TLR2 and TLR4 may be expressed differentially in distinct tissue layers which the real-time PCR (Silva et al., 2010) could not be differentiated TLR2 and TLR4 expression between the layers. Accordingly, the protein expression of TLR2 and TLR4 which is the best characterized with respect to innate responses to Gram-positive and Gram-negative bacteria respectively were investigated in healthy and infected canine endometrium in different tissue layers by immunohistochemistry.

4.3 Materials and methods

4.3.1 Animals

In total, 67 nulliparous bitches, ranging in age from 1-13 years, were submitted for ovariohysterectomy at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. All bitches were at puberty and none of bitches had received hormonal treatment. The bitches were allocated into five groups. Group 1-4 were healthy bitches which were classified depending on the stage of the estrous cycle; group1, bitches in proestrus (n = 7); group 2, bitches in estrus (n = 10); group 3, bitches in diestrus (n = 16); group 4, bitches in anestrus (n = 11). Group 5 were bitches with pyometra (n = 23). All healthy bitches were examined for vulva swelling, estrous behaviour and blood collection. Stages of the estrous cycle were confirmed by vaginal cytology, gross aspect of the ovaries and serum progesterone levels, none of bitches presented abnormal clinical signs and the blood profile was normal (Lumsden et al., 1979). In bitches with pyometra, the diagnosis of pathology is based on clinical signs, blood collection and usually confirmed by radiography and/or ultrasonography (Sandholm et al., 1975).

4.3.2 Hormonal analysis

A blood sample was collected from the cephalic vein of bitches before ovariohysterectomy. For serum production, blood samples were centrifuged for 5 min at 2500 × *g*. Peripheral blood serum progesterone concentrations were measured using chemiluminescent assay (Chapwanya et al., 2008).

4.3.3 Tissue collection

Uterine tissues were collected from each group of bitches undergoing ovariohysterectomy. Each tissue sample was divided in to horn, body and cervix of the uterus. Tissue collected from the uterine horn in all samples was collected from the left horn and the middle portion. Full thickness segments of the uterus,

approximately 1 cm in length were removed. Tissue samples were fixed in 4% paraformaldehyde for immunohistochemical examination (Wassef et al., 2004).

4.3.4 Estrous cycle stage determination

The stage of the estrous cycle of healthy bitches was confirmed by vaginal cytology (proestrus: mixed types of epithelial cells, red blood cells and white blood cells may be present in early to midproestrus; estrus: > 90% cornified cells with fewer red blood cells than proestrus, few to no white blood cells; diestrus: > 50% parabasal and intermediate cells on first day of diestrus, white blood cells may be present with fewer red blood cells than proestrus; anestrus: > 90% parabasal and intermediate cells with few white blood cells, fewer bacteria), by gross aspect of the ovaries (proestrus and estrus: presence of follicles; diestrus: large corpora lutea; anestrus: regressed corpora lutea), and by serum progesterone concentration (anestrus: ≤ 0.5 ng/ml; proestrus: < 1 ng/ml; estrus: 1-15 ng/ml; diestrus: > 1 ng/ml) (Van Cruchten et al., 2004; Kida et al., 2010).

4.3.5 Immunohistochemical staining

Samples fixed in 4% paraformaldehyde were embedded in paraffin and cut in to 4 μm sections. Sections were mounted on positive charged slides and dried overnight at 37 °C. After deparaffinization in xylene and rehydration in a graded series of ethanol, slides were boiled in citrate buffer (10 mM, pH 6.0) at 95 °C for 40 minutes for antigen retrieval and then cooled down at room temperature for 20 minutes. Sections were immersed in 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. Non-specific binding was blocked in 3% bovine serum albumin (BSA) for 30 minutes. Slides were incubated in a humidified chamber overnight at 4°C with the primary antibody, mouse anti-human TLR2 (TL2.1; eBioscience, San Diego, CA, USA) in a dilution of 1:100 and goat anti-mouse TLR4 (sc-12511; Santa Curz Biotechnology, Inc., Santa Cruz, CA, USA) in a dilution of 1:100. The sections were then incubated with horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulin (DakoCytomation, Denmark) for

TLR2 and horseradish peroxidase (HRP) conjugated anti-goat immunoglobulin (DakoCytomation, Denmark) for TLR4 for 30 minutes with three washes in PBS in-between. Bound antibody conjugates were visualized using NovaRED peroxidase substrate as a chromogen and sections were counterstained with hematoxylin and mounted with glycerine gelatine. In each run, negative controls (the substitution of the primary antibody with PBS) were run along with the samples.

4.3.6 Quantification of immunohistochemical staining

All the immunostaining evaluation was carried out under light microscope. The expression of TLR2 and TLR4 was analyzed blind by one experienced scorer. The expression was assessed semi-quantitatively incorporating both the proportion of cells stained positively and the intensity of specific staining. Scoring of the horn and body of uterus in three different layers; surface epithelium, stroma and glandular epithelium was evaluated separately. And scoring of the cervix in two different layers; surface epithelium and stroma was evaluated separately. The proportionate area in each tissue later showing a positive staining was taken into account to the nearest 5% and defined as the percentage expression of the cell layer. The intensity of staining was classified on a scale of 1 to 3 (1 = weak, 2 = moderate, 3 = strong). These scores were then used to generate an expression index by multiplying the percentage expression with the average intensity score. The expression index was derived by multiplying percentage expression (PE) with an average intensity score (AIS) and this index was subsequently used for the statistical analyses (Ponglowhapan et al., 2010).

4.3.7 Statistical analysis

The mean expression index at each tissue layer of uterus for each group of dog was calculated. Multiple analysis of variance using SAS was used to compare the differences in protein expression level between groups (proestrus, estrus, diestrus, anestrus, pyometra) and tissue layers (surface epithelium, stroma, glandular epithelium). Differences with $P < 0.05$ were regarded as statistically significant, $P < 0.01$ as highly statistically significant. Results are shown as mean ± S.E.M.

4.3.8 Experimental design

The expression of TLR2 and TLR4 in horn, body and cervix of the uterus of healthy dogs during estrous cycle and dogs with pyometra were investigated. The tissue samples of healthy dogs used in this study were the same as the previous experiment in chapter III. While, in pyometra dogs, the tissue were collected as the same method as in healthy dogs. The immunohistochemical staining of TLR2 and TLR4 were performed in the tissue sections from the same sample. In each run, the substitution of the primary antibody with PBS was run as the negative controls along with the samples. The evaluation was carried out under light microscope. The section of horn and body of the uterus were evaluated and scoring in three different layers (surface epithelium, glandular epithelium and stroma). And the section of the cervix was evaluated and scoring in two different layers (surface epithelium and stroma). The immunostaining was presented in the term of expression index.

4.4 Results

4.4.1 TLR2 and TLR4 expression in endometrial tissue

4.4.1.1 The part of horn

In this study, no pathological changes of uterine samples were found from all healthy dogs. And, the mean of progesterone concentration in each group were 1.07 ng/ml (proestrus), 8.04 ng/ml (estrus), 25.93 ng/ml (diestrus), 0.48 ng/ml (anestrus) and 11.17 (pyometra). From current study, TLR4 protein was expressed mainly in the surface epithelium, glandular epithelium and stroma of the endometrium in pyometra dogs. At different stages in healthy dogs, the differential expression of TLR4 was observed in different layers of endometrium. The expression of TLR4 on immune cells such as macrophages was found at different stages of estrous cycle (Figure 15).

In proestrus, the expression of TLR4 was higher in the endometrial stroma compared to the endometrial surface epithelium, glandular epithelium ($P < 0.01$). And, endometrial stroma expressed TLR4 dominantly when compared to the group of estrus and anestrus ($P < 0.05$) (Figure 16).

The glandular epithelium and stroma at the diestrous stage significantly expressed TLR4 more intensely than the surface epithelium ($P < 0.01$). Furthermore, when compared to other healthy groups, the glandular epithelium of diestrus also expressed TLR4 more intensely than bitches in proestrus, estrus and anestrus ($P < 0.01$) (Figure 17). Whereas, the expression levels of TLR4 in the endometrial stroma in this diestrus were dominant when compared with anestrus ($P < 0.05$).

The surface epithelium of the endometrium expressed a wide range of TLR4 in both healthy and pyometra dogs, except for a group of estrous dogs for which the expression of TLR4 in surface epithelium was not observed. In infected uteri, the expression of TLR4 in the surface epithelium was higher in dogs with pyometra compared with all other groups of healthy dogs ($P < 0.01$) (Figure 18). Moreover, when compared with the endometrial layers, the surface epithelium of dogs suffering from pyometra also expressed TLR4 more intensely than the glandular epithelium ($P < 0.05$). Meanwhile, the expression level of TLR4 in the glandular epithelium was higher in dogs with pyometra compared with healthy dogs at proestrus, estrus and anestrus ($P < 0.01$) except for one group of diestrous dogs which showed no significant difference. In addition, the expression of TLR4 in endometrial stroma was higher in pyometra dogs compared to the group of estrous ($P < 0.05$) and anestrous dogs ($P < 0.01$). The immunostaining of TLR4 in all groups and layers were differential expressed as presented in Figure 19.

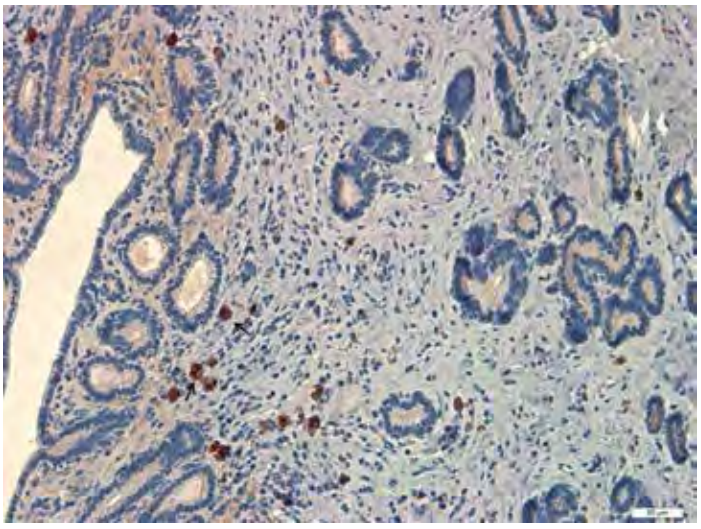
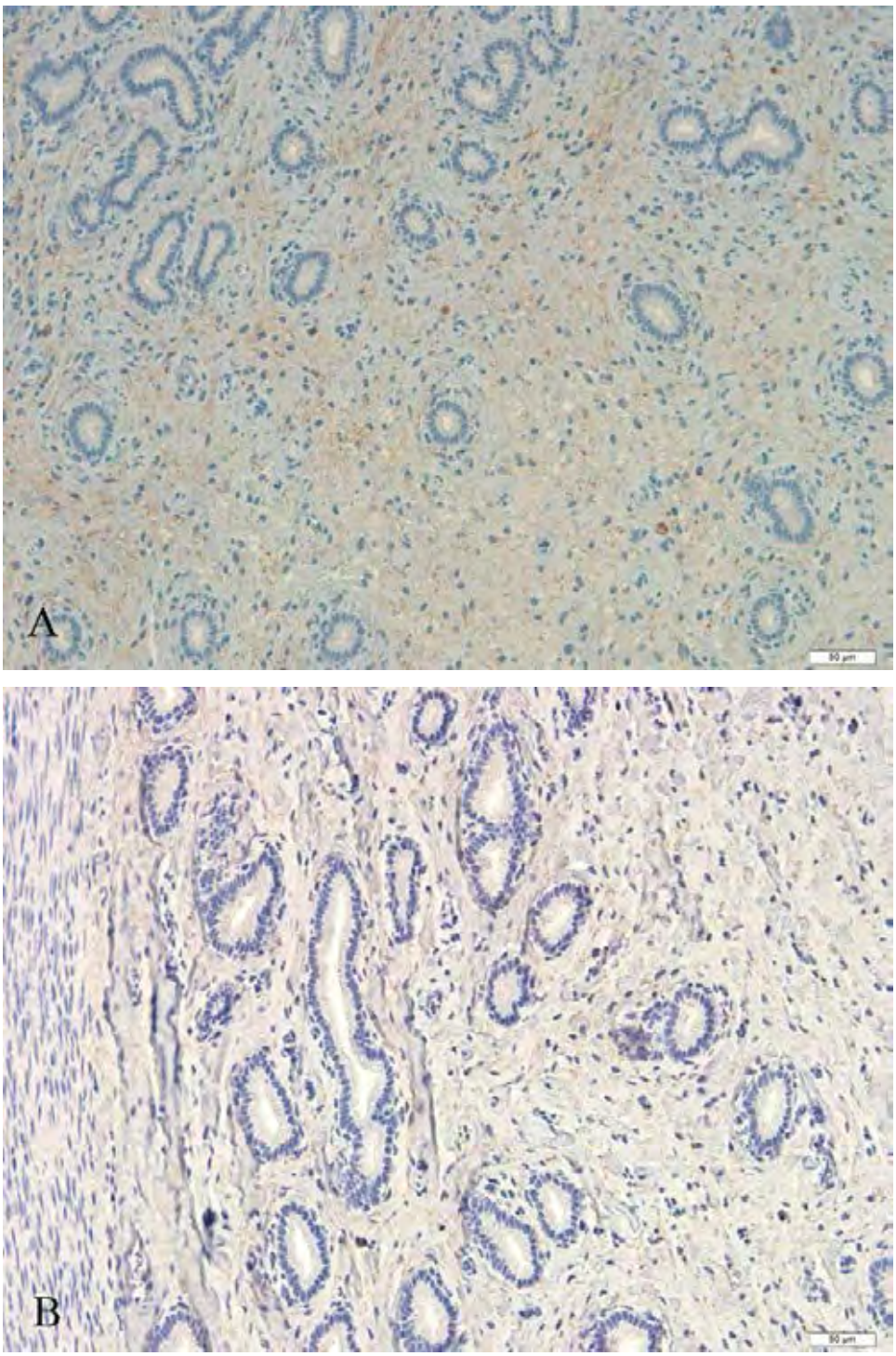


Figure 15 Immunohistochemistry showing the expression of TLR4 in immune cells of uterine horn at diestrus (black arrow).


A
B

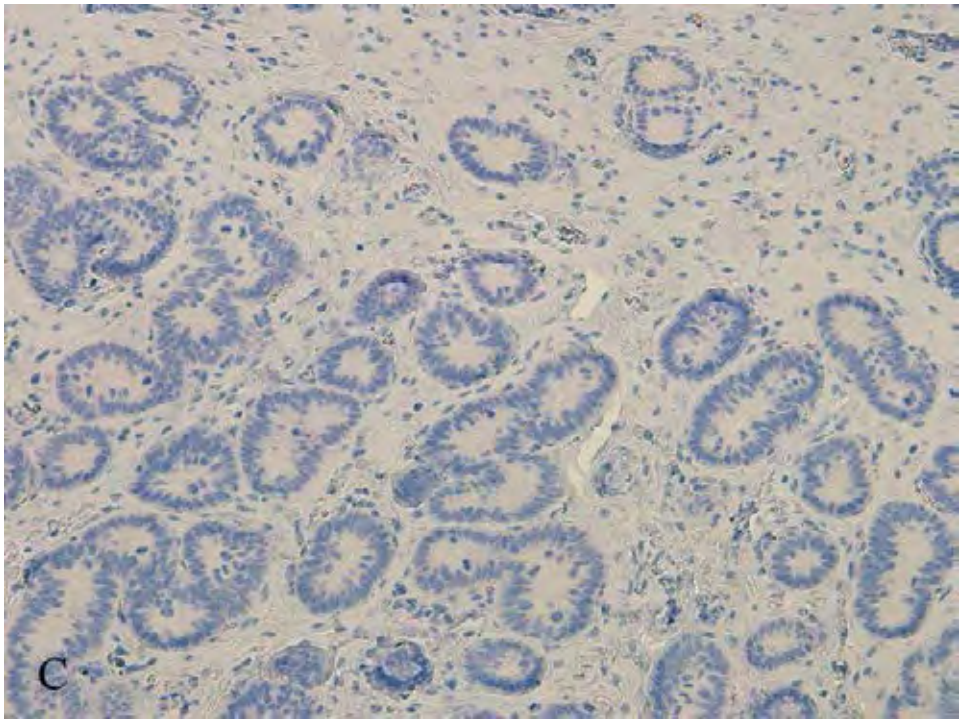
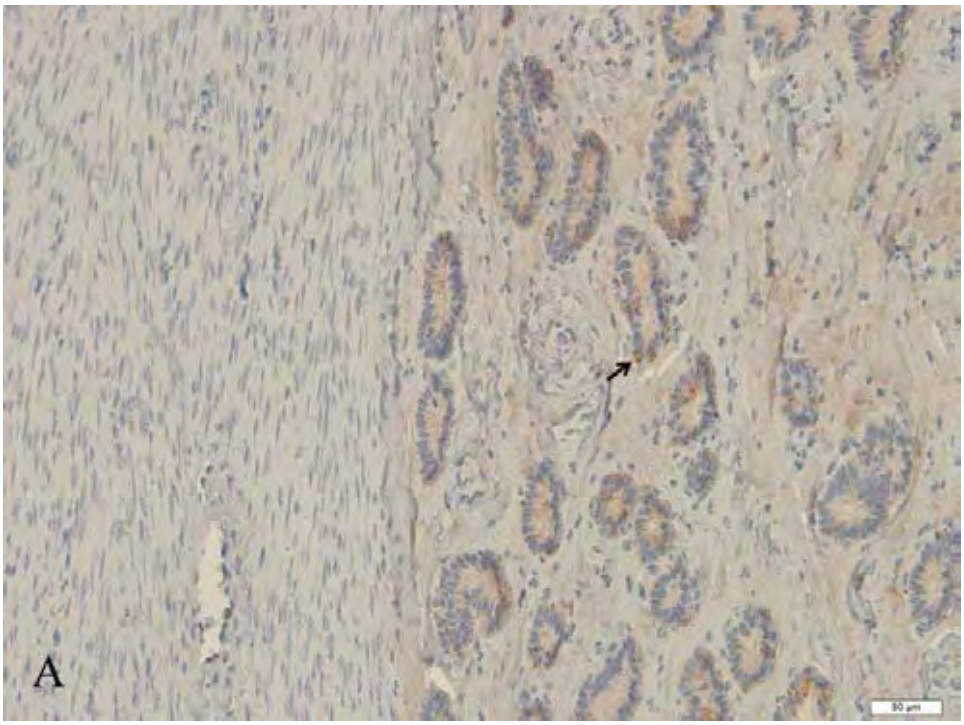


Figure 16 Immunohistochemistry showing the intense staining of TLR4 in stroma of uterine horn at proestrus (A) and weak staining at estrus (B) can be noted. Negative control is shown in (C).



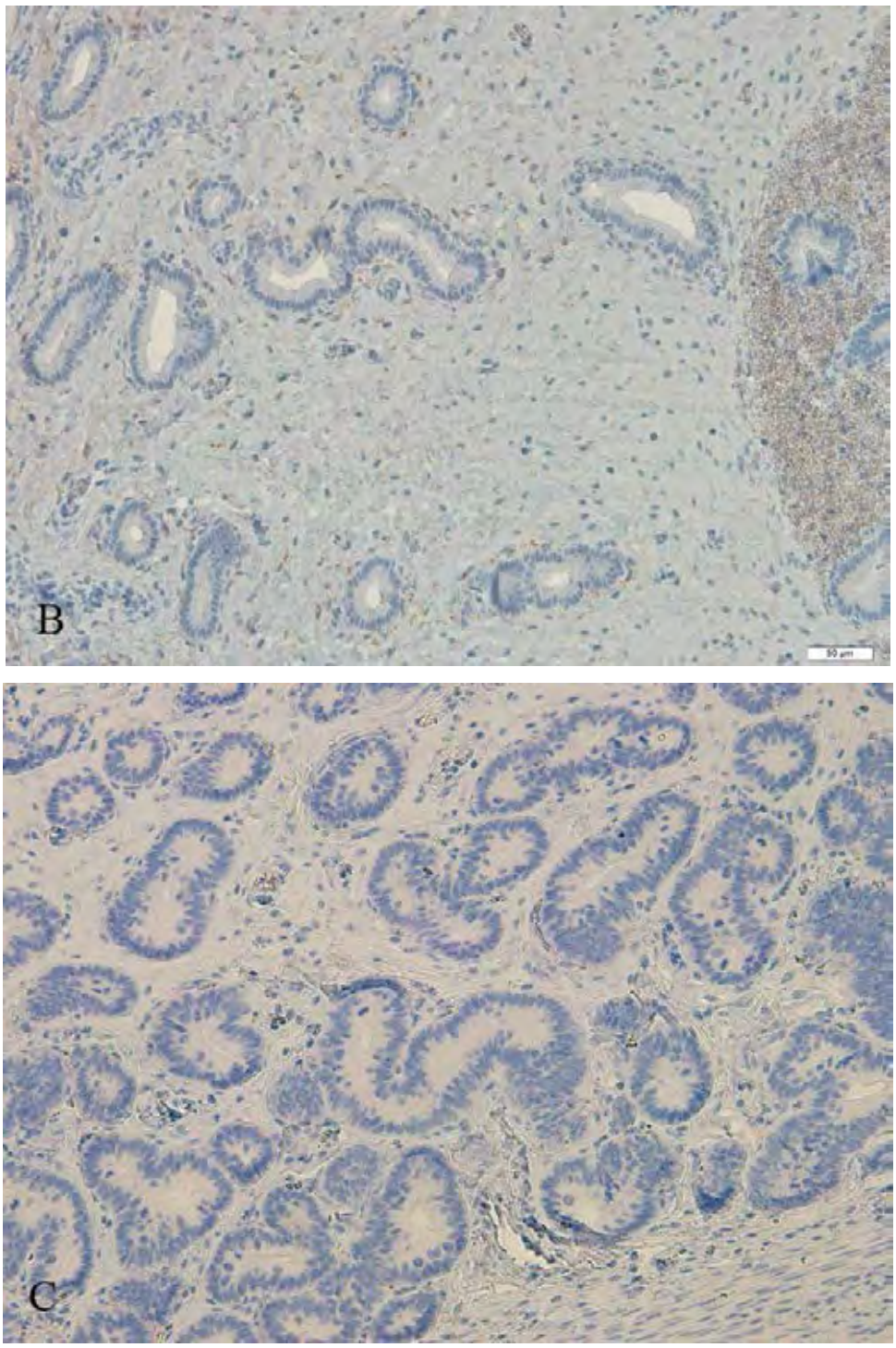


Figure 17 Immunohistochemistry showing the intense staining of TLR4 in glandular epithelium of uterine horn at diestrus (A, black arrow) and weak staining at proestrus (B) can be noted. Negative control is shown in (C).

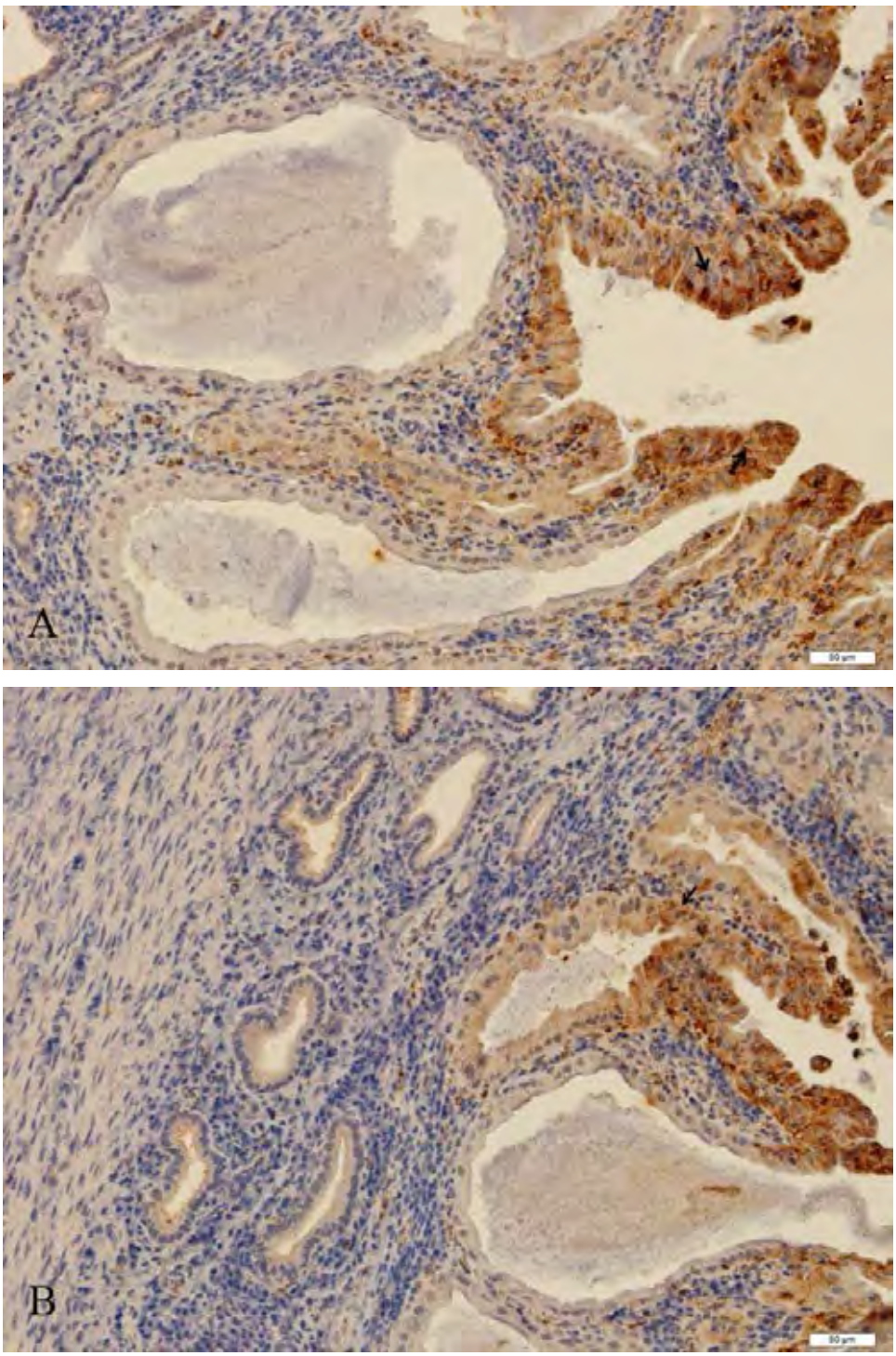


Figure 18 Immunohistochemistry showing the intense staining of TLR4 in surface epithelium of uterine horn in pyometra dog (A, B, black arrow).

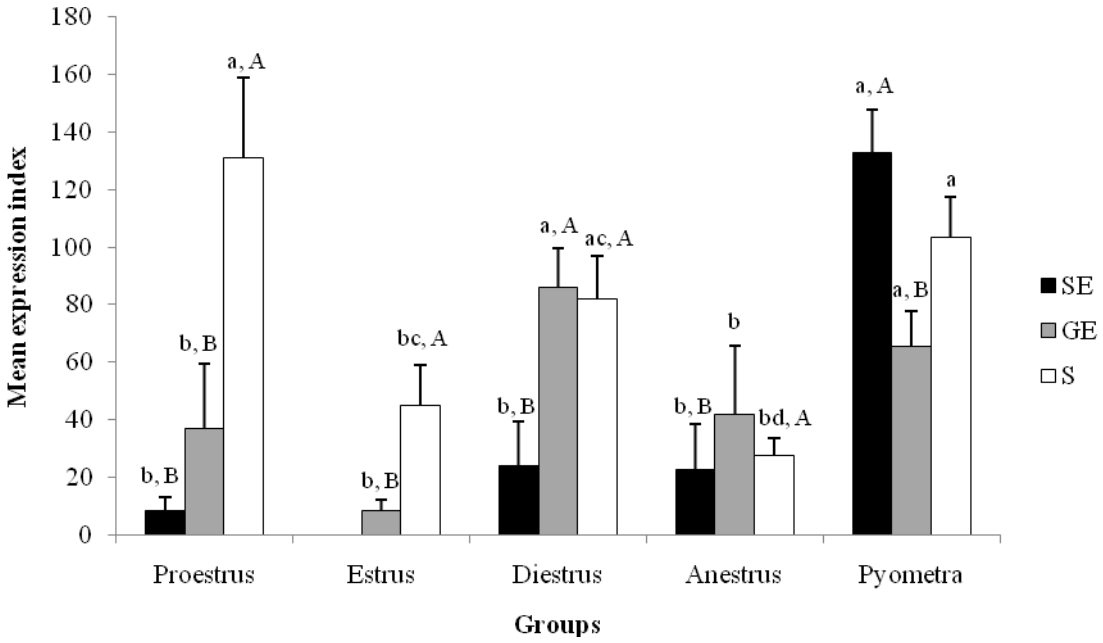
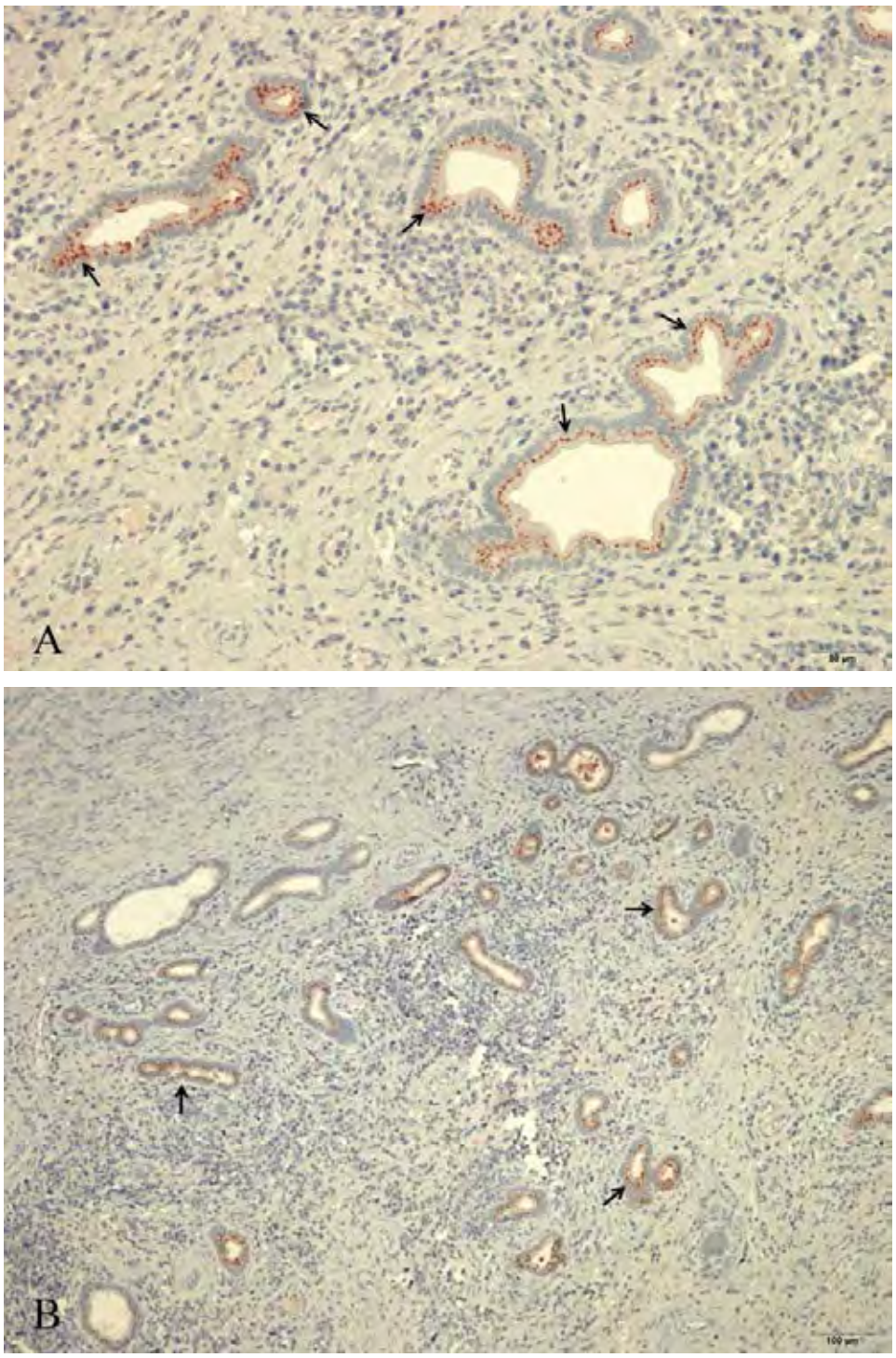


Figure 19 The mean expression index (± S.E.M.) for TLR4 in tissue layers (SE; surface epithelium, GE; glandular epithelium, S; stroma) of endomerium (horn part) in proestrus, estrus, diestrus, anestrus and pyometra. The letters "a" and "b" and "c" and "d" indicate differences in the expression between groups within a similar tissue layer. The letters "A" and "B" indicate differences in the expression between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

In pyometra, the glandular epithelium expressed TLR2 more intensely than the surface epithelium ($P < 0.05$). The expression of TLR2 in the glandular epithelium was significantly higher in healthy dogs at estrus, diestrus and dogs with pyometra compared with anestrous dogs ($P < 0.01$) (Figure 20 and 21). While, the expression of TLR2 in the stroma was not observed in the group of healthy dogs at all stages. The immunostaining of TLR2 in all groups and layers were differential expressed as shown in Figure 22.


A
B

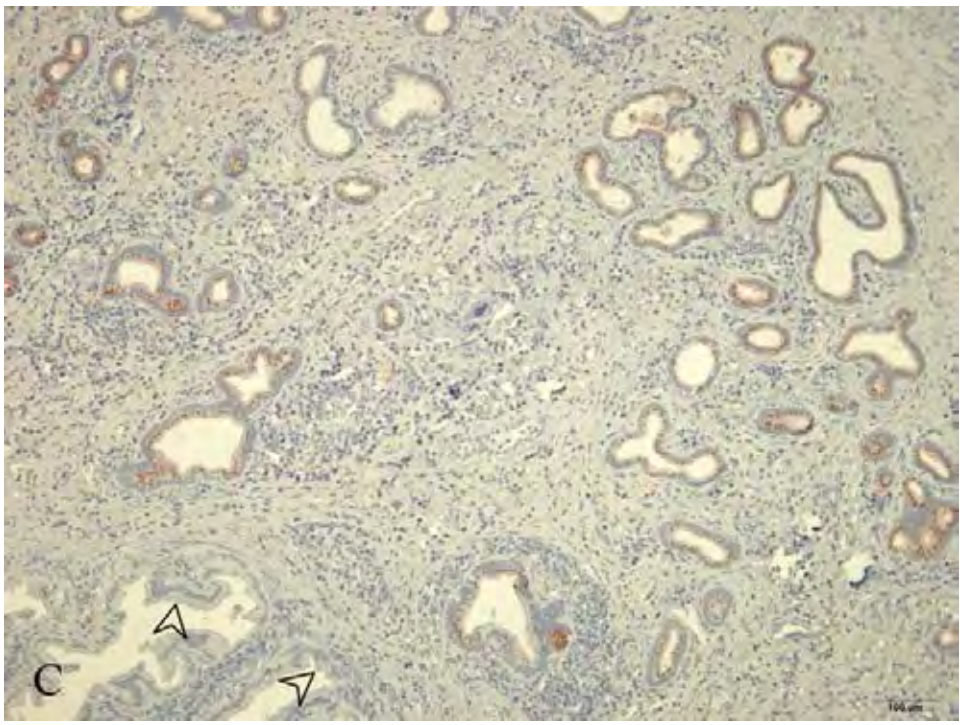
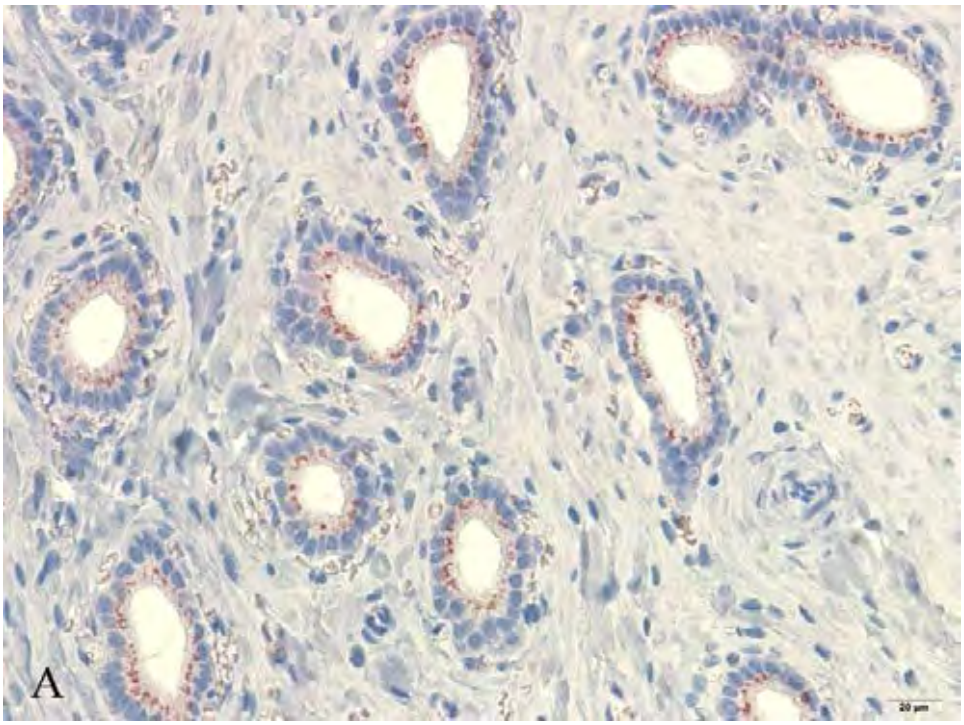
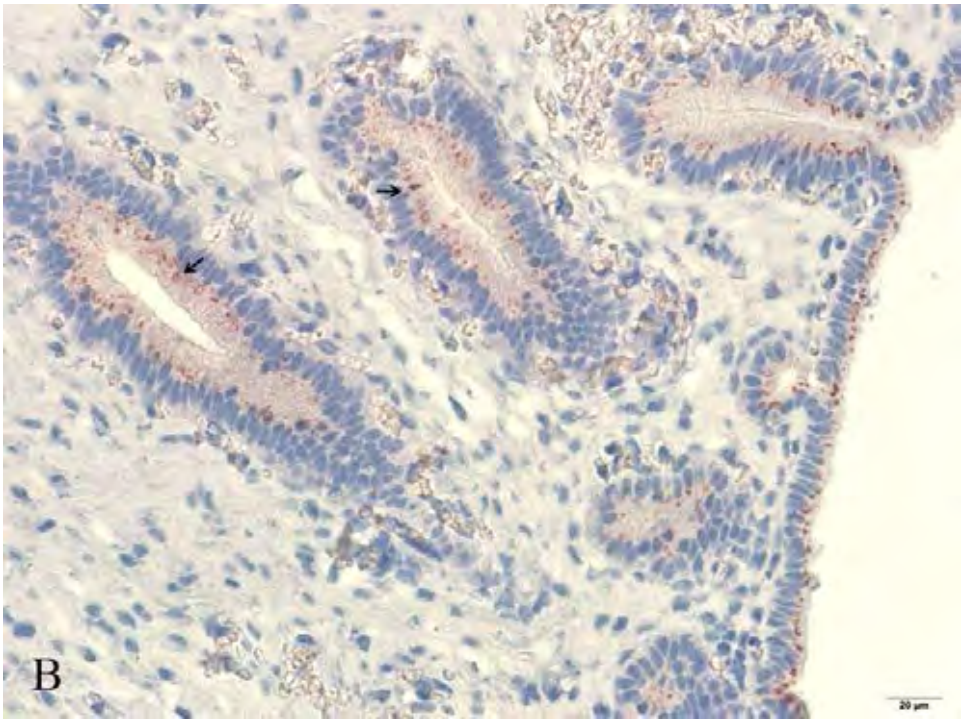
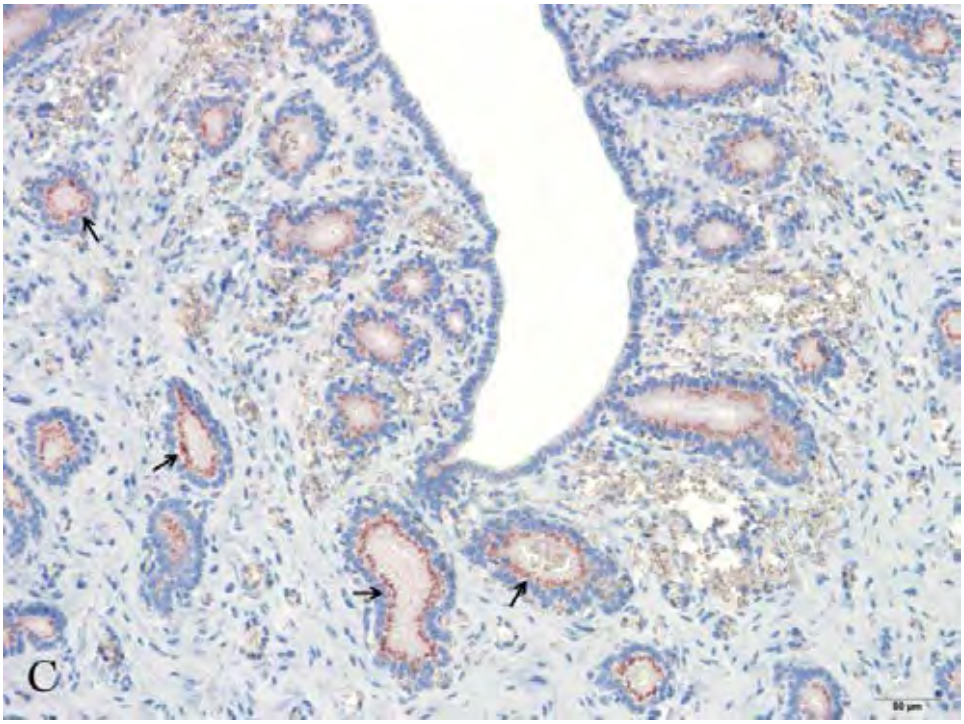
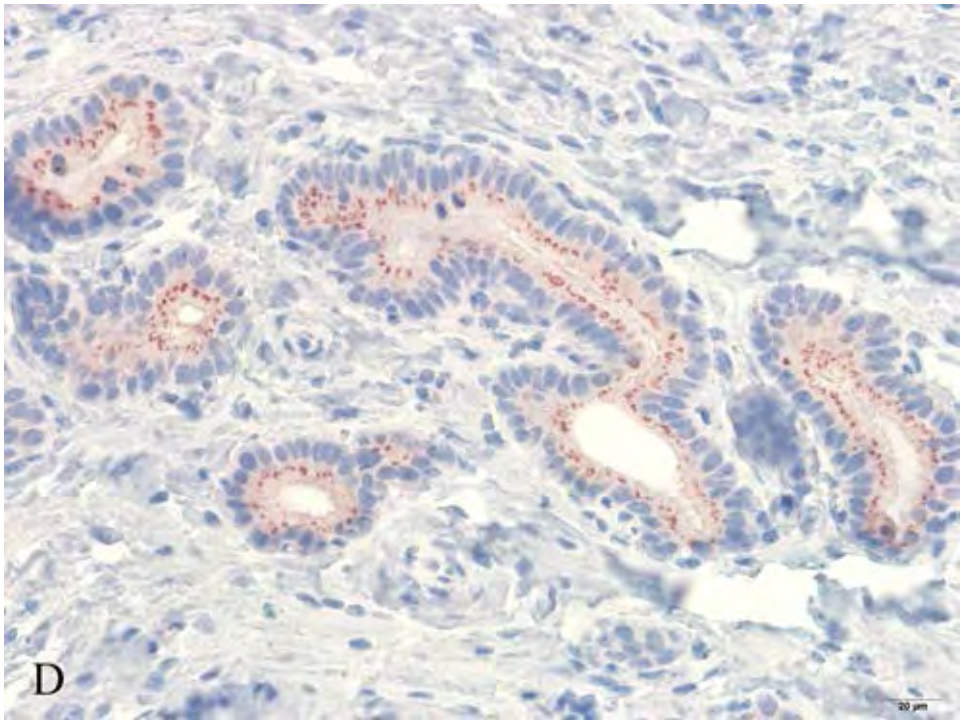


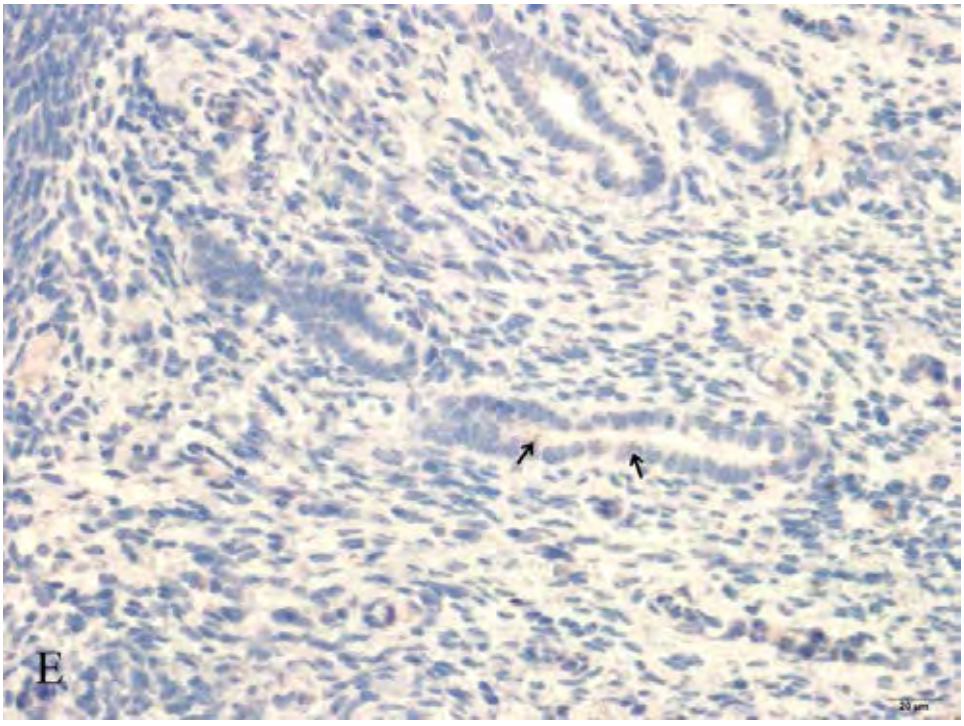
Figure 20 Immunohistochemistry showing the intense staining of TLR2 in glandular epithelium (A, B, black arrow) and weak staining in surface epithelium of uterine horn in pyometra dog (C, open arrow head).




B


C


D


E

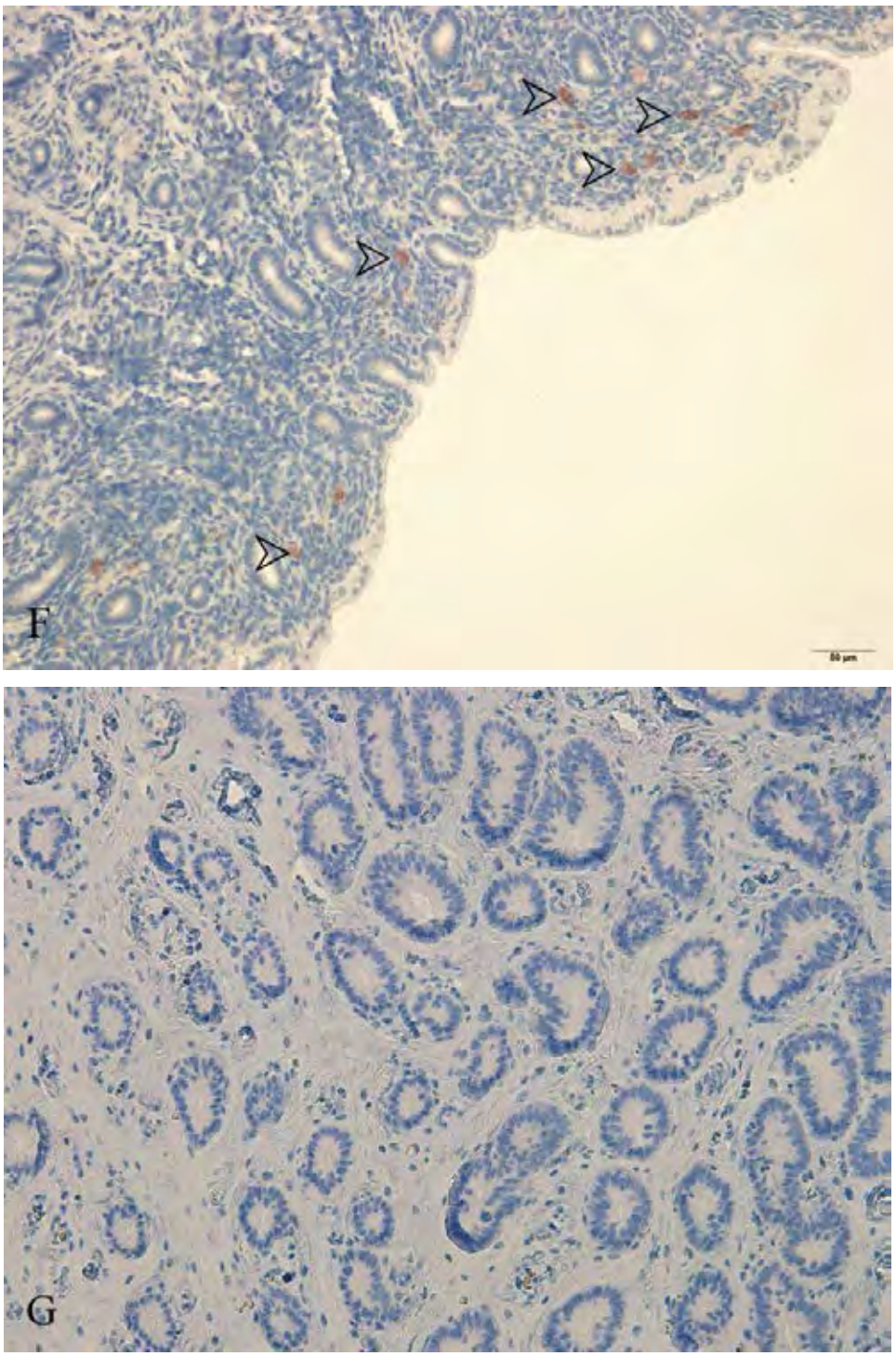


Figure 21 Immunohistochemistry showing the intense staining of TLR2 in glandular epithelium of uterine horn at estrus (A, B, black arrow), diestrus (C, D, black arrow) and weak staining at anestrus (E, F, black arrow). The immunostaining of TLR2 in immune cells at anestrus (F, open arrow head) can be noted. Negative control is shown in (G).

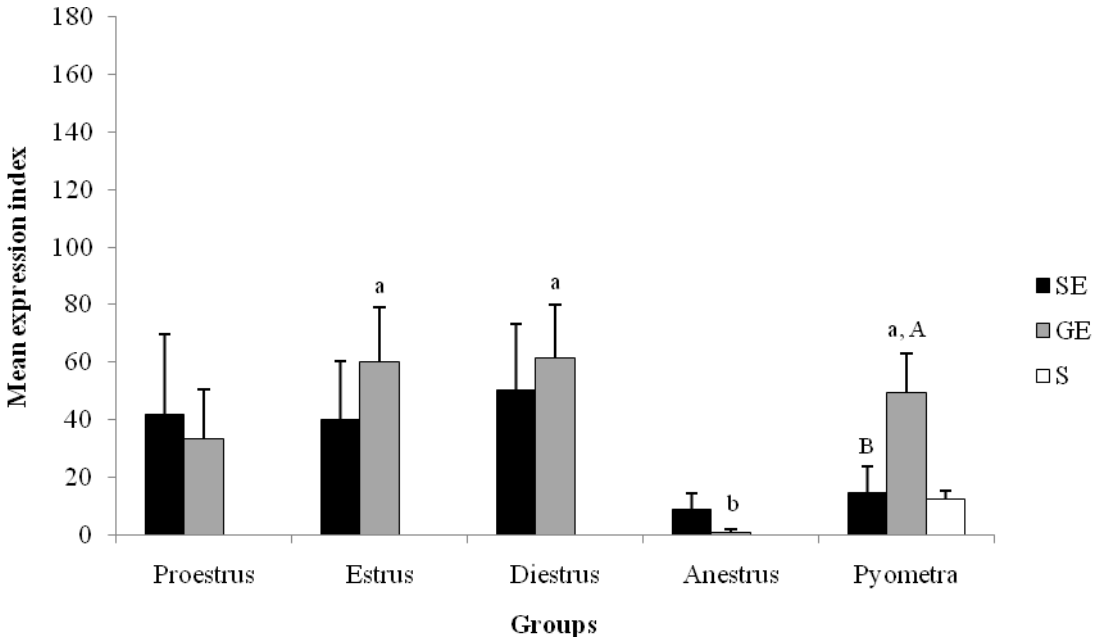
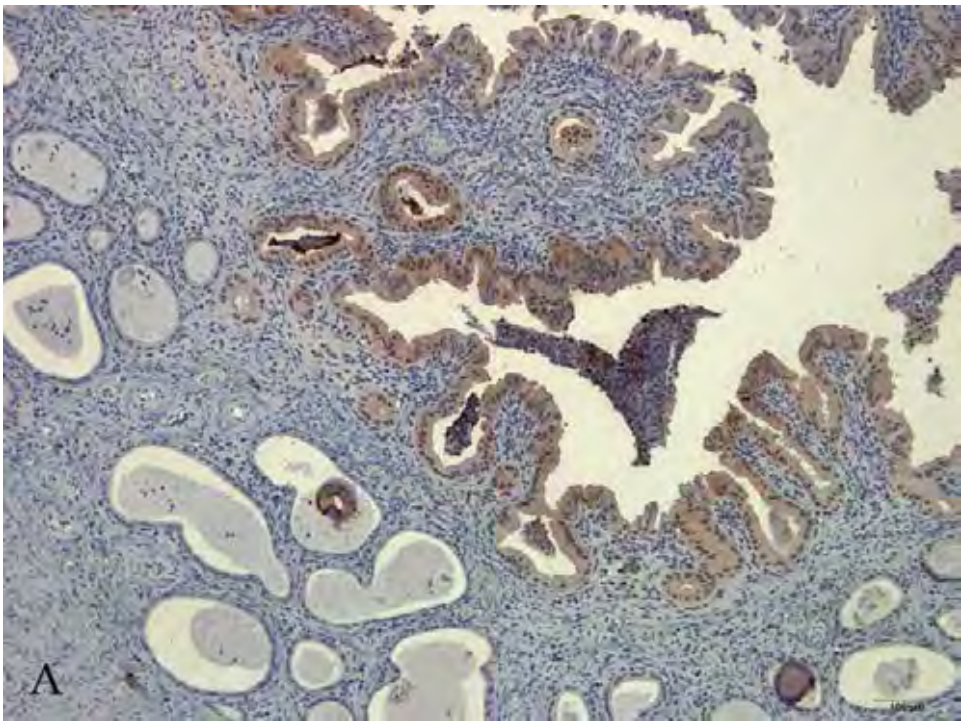


Figure 22 The mean expression index (± S.E.M.) for TLR2 in tissue layers (SE; surface epithelium, GE; glandular epithelium, S; stroma) of endomerium (horn part) in proestrus, estrus, diestrus, anestrus and pyometra. The letters "a" and "b" indicate differences in the expression between groups within a similar tissue layer. The letters "A" and "B" indicate differences in the expression between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

4.4.1.2 The part of body

The expression of TLR4 in proestrus and estrus was significantly higher in the stroma compared to the surface and glandular epithelium ($P < 0.05$) and the glandular epithelium of estrous dogs also expressed TLR4 significantly more intensely than the surface epithelium ($P < 0.01$). In diestrus, the expression of TLR4 was significantly higher in the glandular epithelium and stroma compared to the surface epithelium ($P < 0.01$). However, when compare between groups, the expression of TLR4 in the surface epithelium was significantly higher in dogs suffering from pyometra compared with all other groups of healthy dogs ($P < 0.01$) (Figure 23). In the glandular epithelium, the expression of TLR4 was significantly higher in proestrus ($P < 0.05$), diestrus ($P < 0.01$) and dogs with pyometra ($P < 0.01$) compared to

anestrous dogs (Figure 24). And, the expression of TLR4 was significantly more intensely in glandular epithelium of diestrus compared to estrus ($P < 0.05$). In the stroma, the expression of TLR4 was significantly higher in proestrus compared to diestrus ($P < 0.01$), anestrus ($P < 0.01$) and pyometra dogs ($P < 0.05$) (Figure 25). And the expression of TLR4 in the stroma was higher in estrus ($P < 0.01$), diestrus ($P < 0.05$) and dogs with pyometra ($P < 0.01$) compared to anestrous dogs. The immunostaining of TLR4 in all groups and layers were differential expressed as presented in Figure 26.



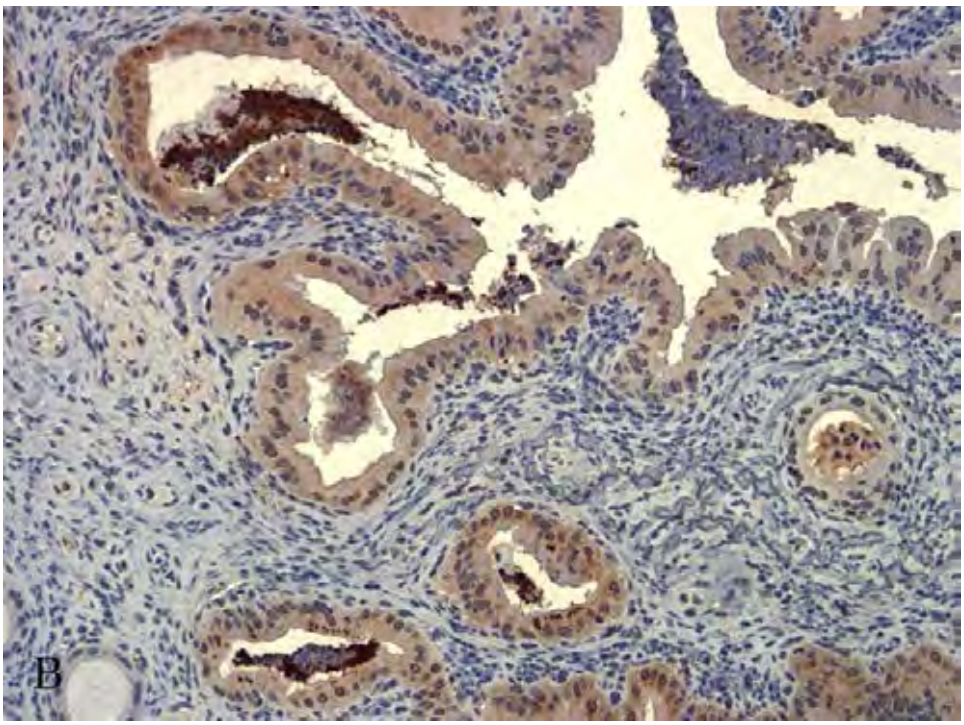
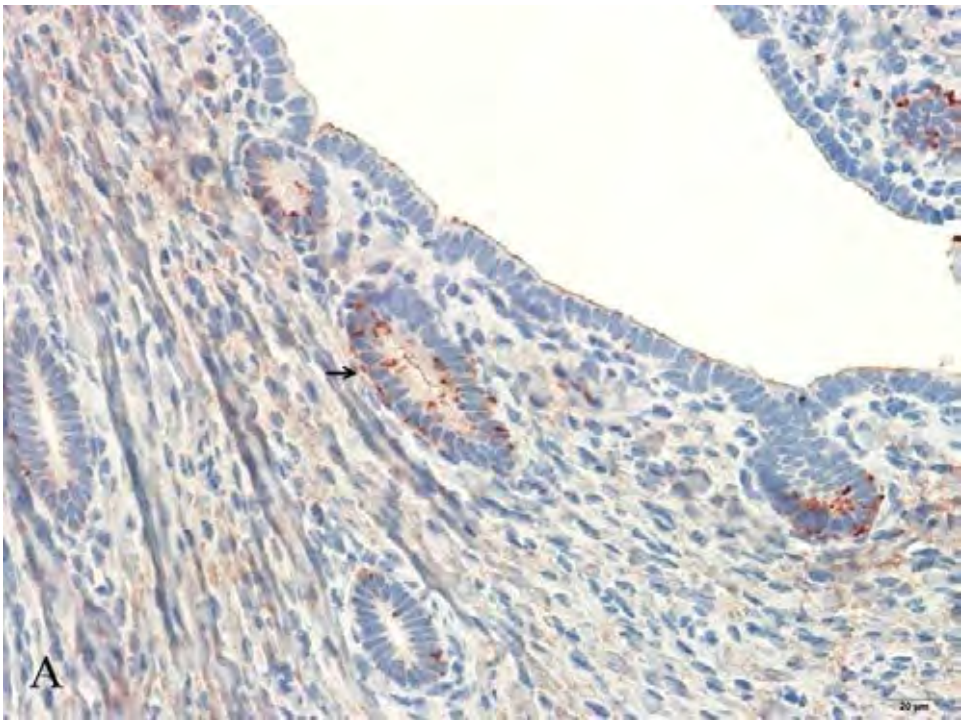


Figure 23 Immunohistochemistry showing the intense staining of TLR4 in surface epithelium and glandular epithelium of uterine body in pyometra dog (A, B).



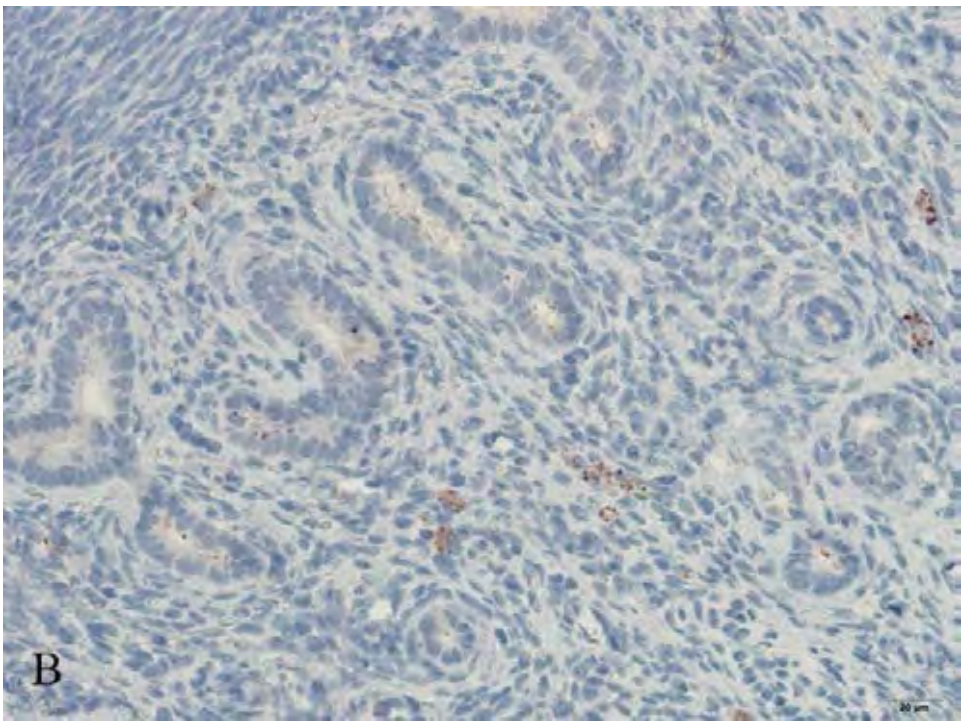
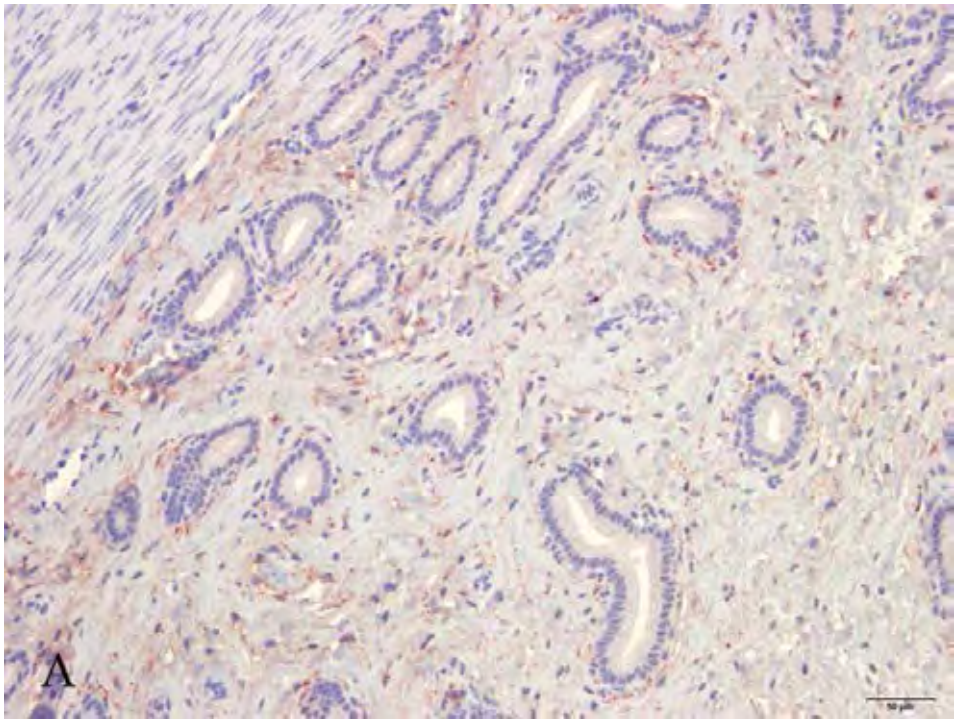


Figure 24 Immunohistochemistry showing the intense staining of TLR4 in glandular epithelium of uterine body at diestrus (A, black arrow) and weak staining at anestrus (B) can be noted.



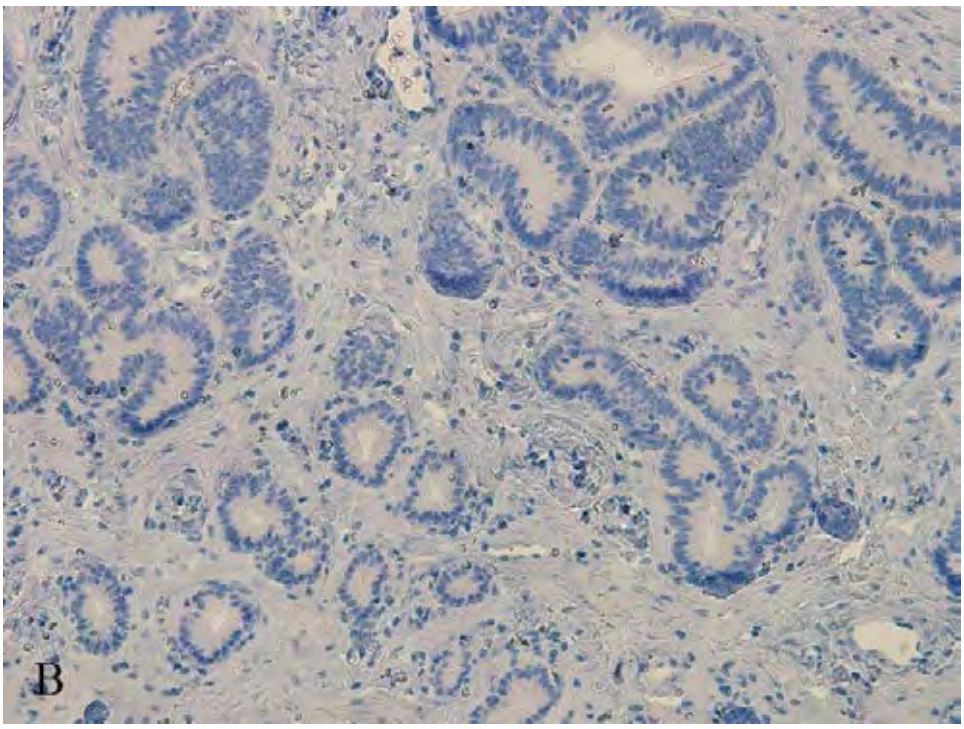


Figure 25 Immunohistochemistry showing the intense staining of TLR4 in stroma of uterine body at proestrus (A) can be noted. Negative control is shown in (B).

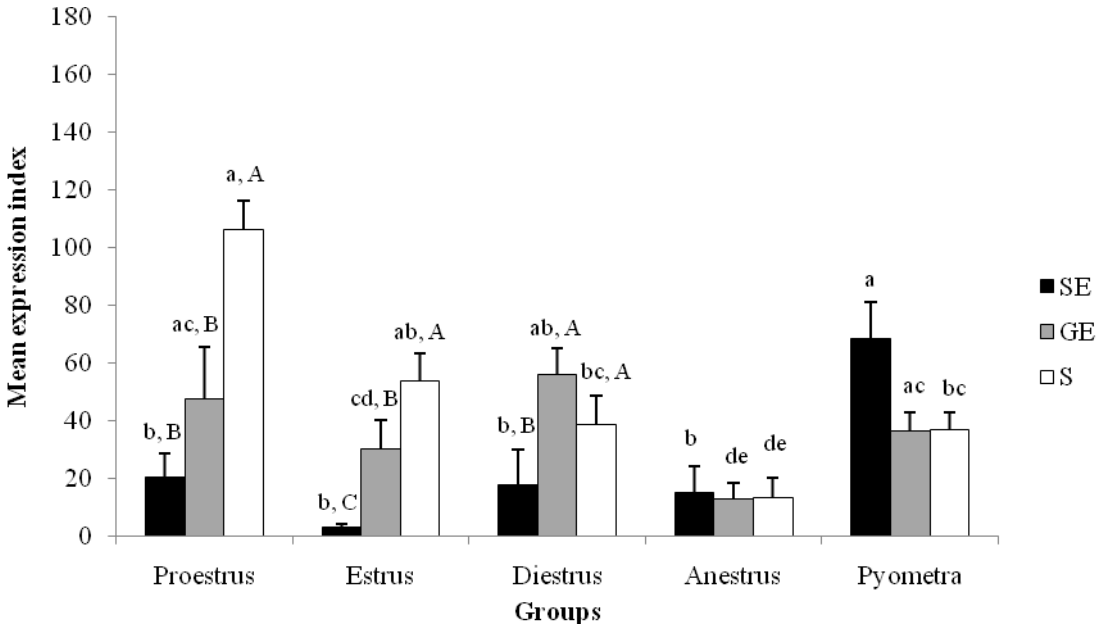
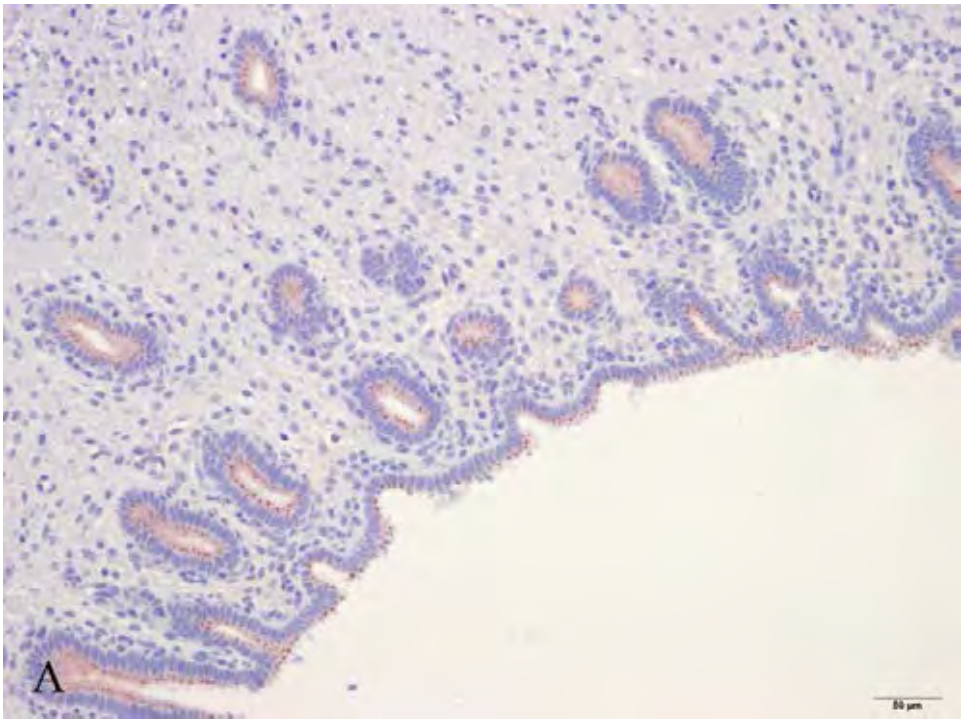
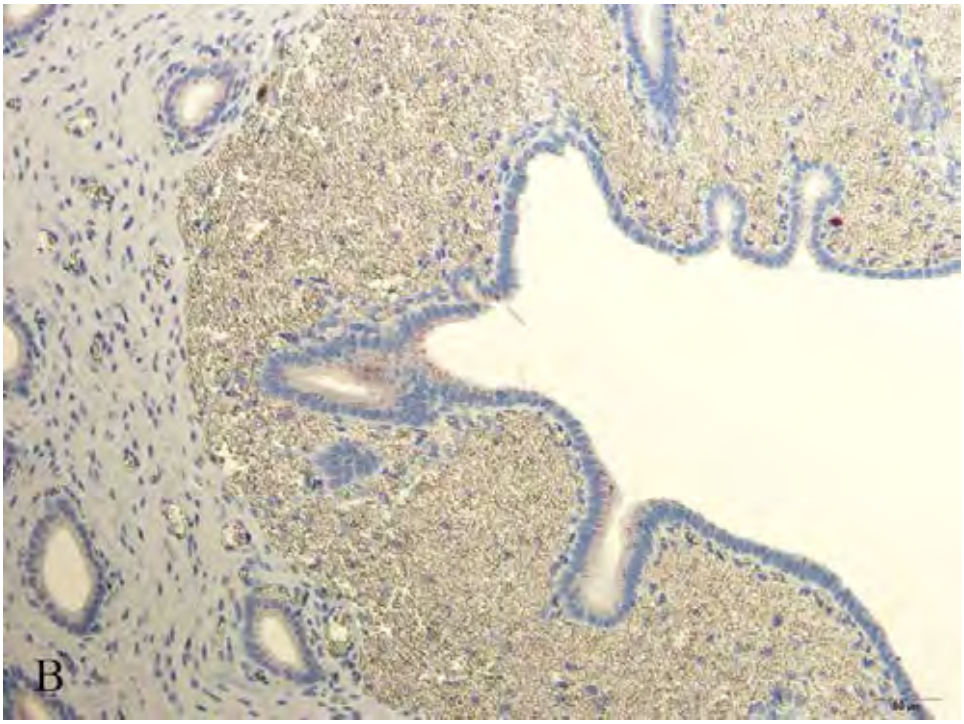
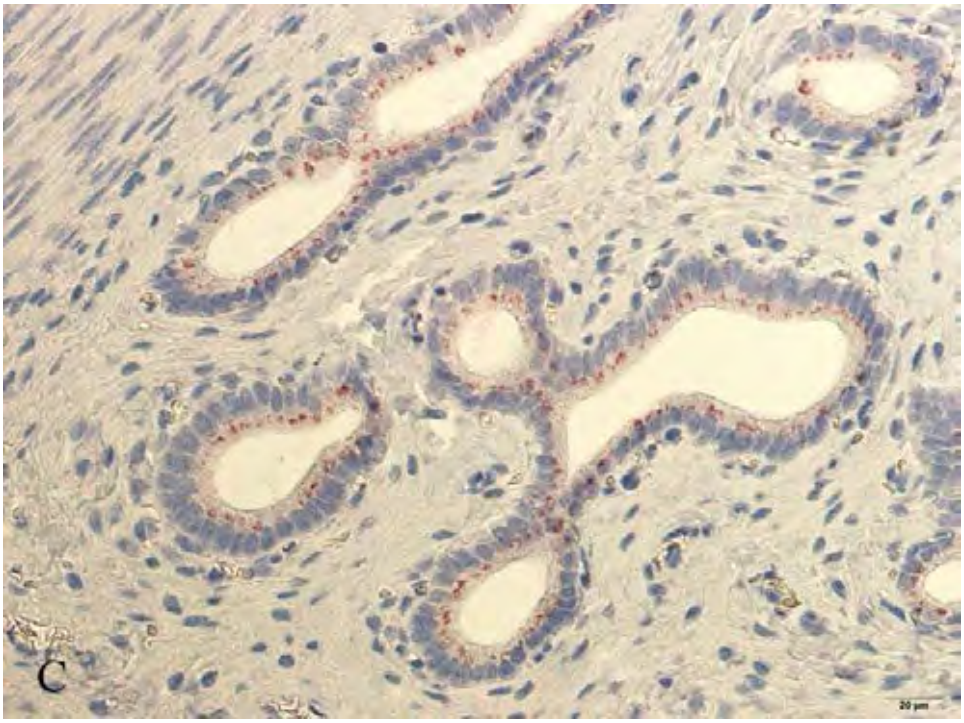


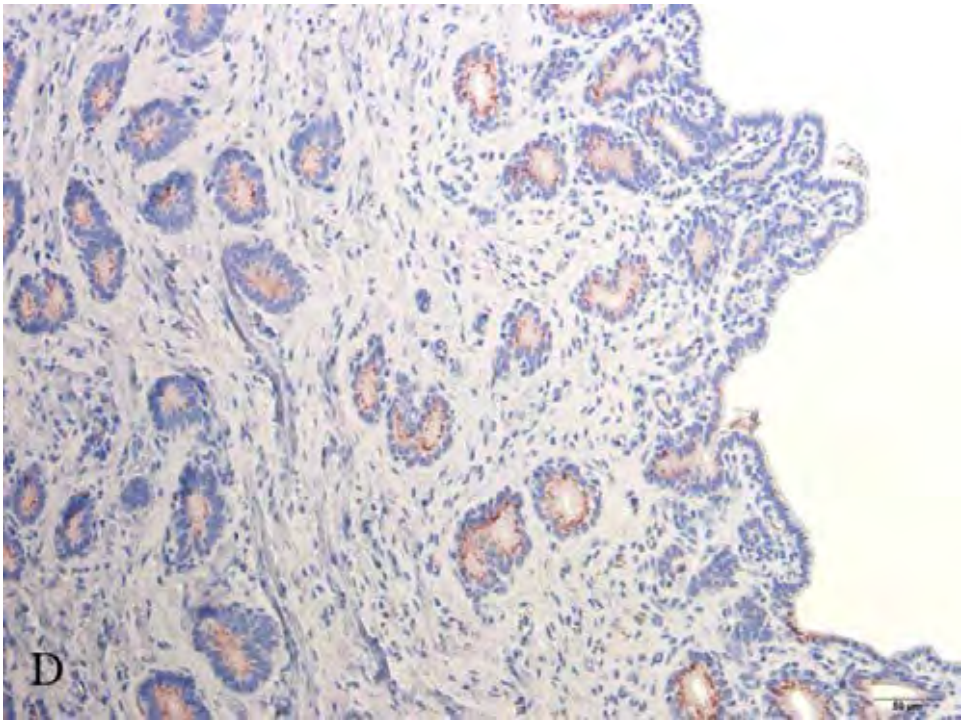
Figure 26 The mean expression index (± S.E.M.) for TLR4 in tissue layers (SE; surface epithelium, GE; glandular epithelium, S; stroma) of endomerium (body part) in proestrus, estrus, diestrus, anestrus and pyometra. The letters “a” and “b” and “c” and “d” and “e” indicate differences in the expression between groups within a similar tissue layer. The letters “A” and “B” indicate differences in the expression between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

The expression of TLR2 in diestrous dogs was significantly higher in the surface epithelium and glandular epithelium compared to the stroma ($P < 0.01$). And TLR2 was expressed in the glandular epithelium significantly more intensely than the surface epithelium of dogs with pyometra ($P < 0.05$). In the surface epithelium, the expression of TLR2 was significantly higher in proestrus ($P < 0.01$), estrus ($P < 0.05$) and diestrus ($P < 0.05$) compared to dogs with pyometra (Figure 27). The expression of TLR2 was significantly higher in the glandular epithelium of proestrus, estrus, diestrus, and dogs with pyometra compared to anestrous dogs ($P < 0.01$). The stroma of dogs with pyometra expressed TLR2 more intensely than diestrous ($P < 0.01$) and anestrous dogs ($P < 0.05$). The immunostaining of TLR2 in all groups and layers were differential expressed as shown in Figure 28.


A


B


C


D

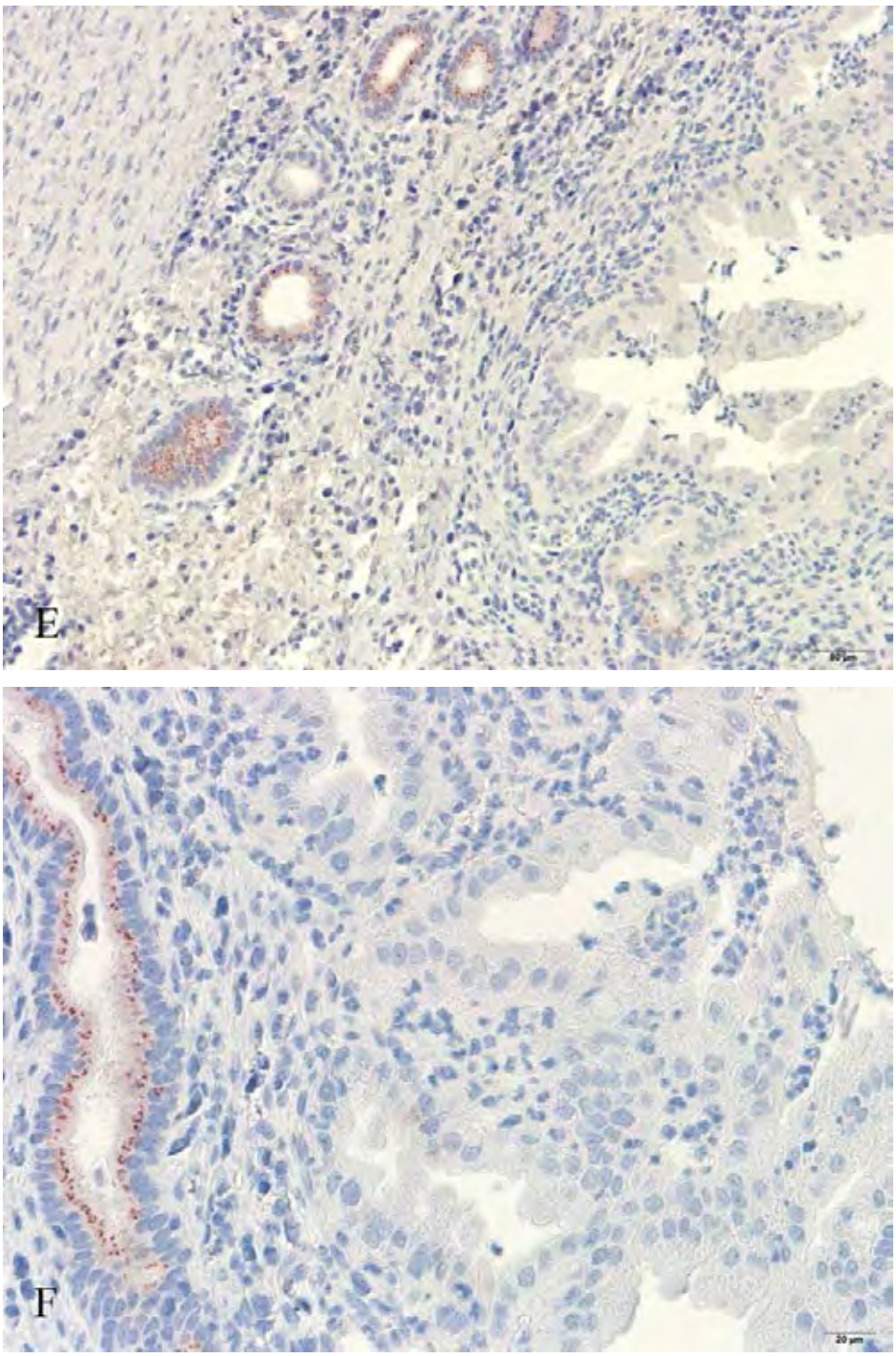


Figure 27 Immunohistochemistry showing the intense staining of TLR2 in surface epithelium and glandular epithelium of uterine body at proestrus (A), estrus (B, C), diestrus (D) and weak staining in surface epithelium in pyometra dog (E, F) can be noted.

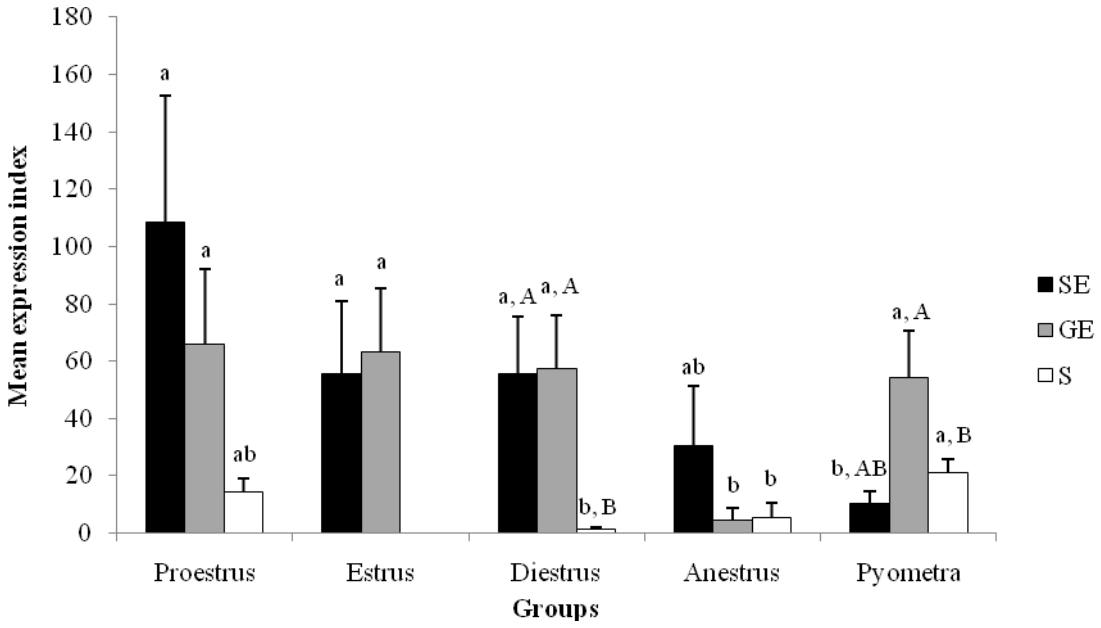


Figure 28 The mean expression index (± S.E.M.) for TLR2 in tissue layers (SE; surface epithelium, GE; glandular epithelium, S; stroma) of endomerium (body part) in proestrus, estrus, diestrus, anestrus and pyometra. The letters "a" and "b" indicate differences in the expression between groups within a similar tissue layer. The letters "A" and "B" indicate differences in the expression between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

4.4.2 TLR2 and TLR4 expression in cervical tissue

The expression of TLR4 was significantly higher in the stroma at proestrus and estrus compared to the surface epithelium ($P < 0.01$) and other groups ($P < 0.01$) (Figure 29). Conversely, the expression of TLR4 in diestrus was significantly more intensely in the surface epithelium compared to the stroma ($P < 0.01$) and other groups ($P < 0.05$) (Figure 30). In addition, the expression of TLR4 in the surface epithelium was significantly higher in dogs with pyometra compared to estrous dogs ($P < 0.05$) (Figure 31). The immunostaining of TLR4 in all groups and layers were differential expressed as presented in Figure 32.

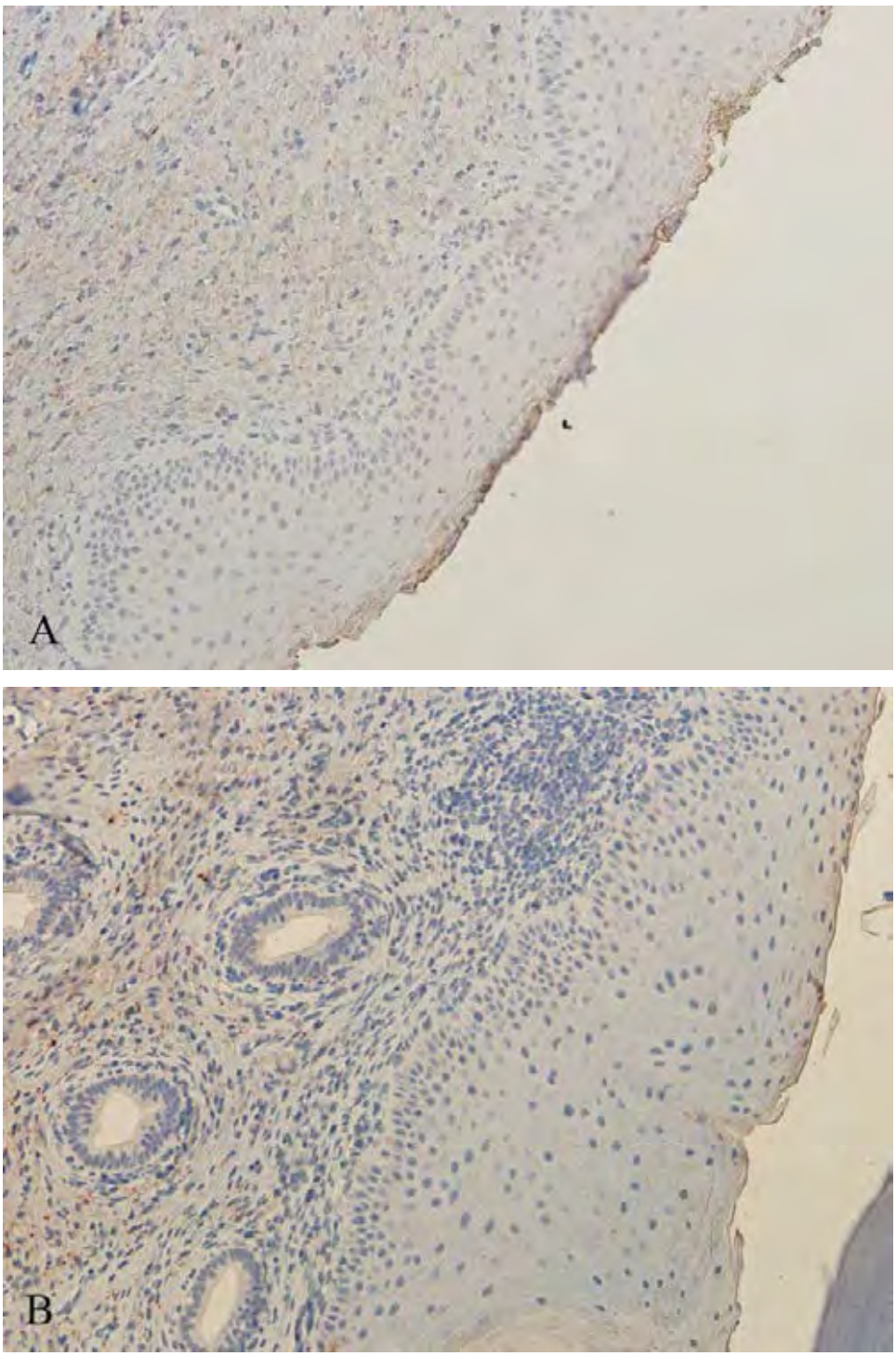
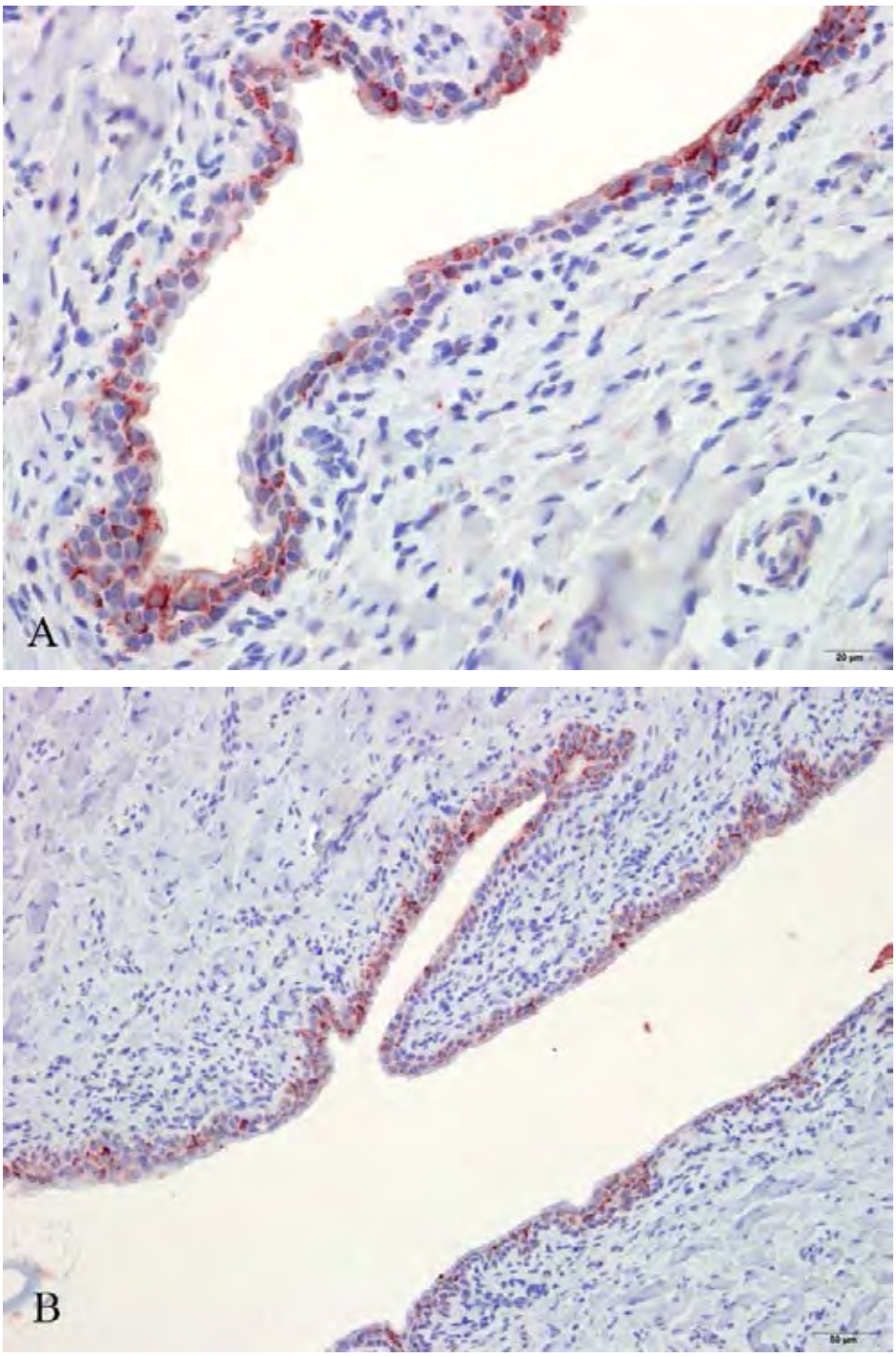


Figure 29 Immunohistochemistry showing the intense staining of TLR4 in stroma of cervix at proestrus (A) and estrus (B) can be noted (Magnification ×200).


A
B

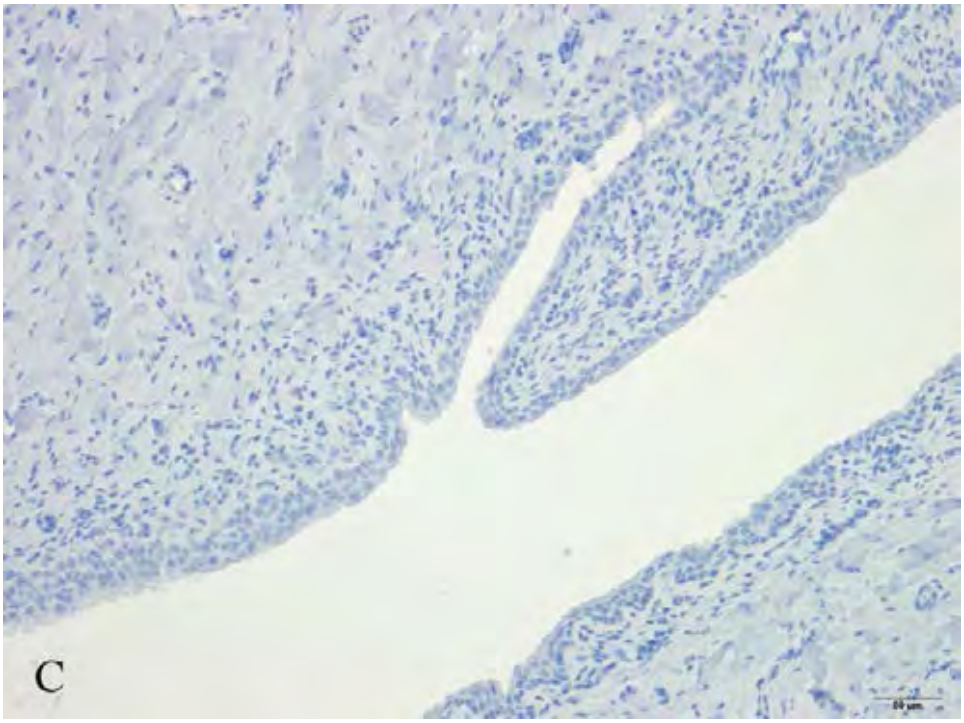
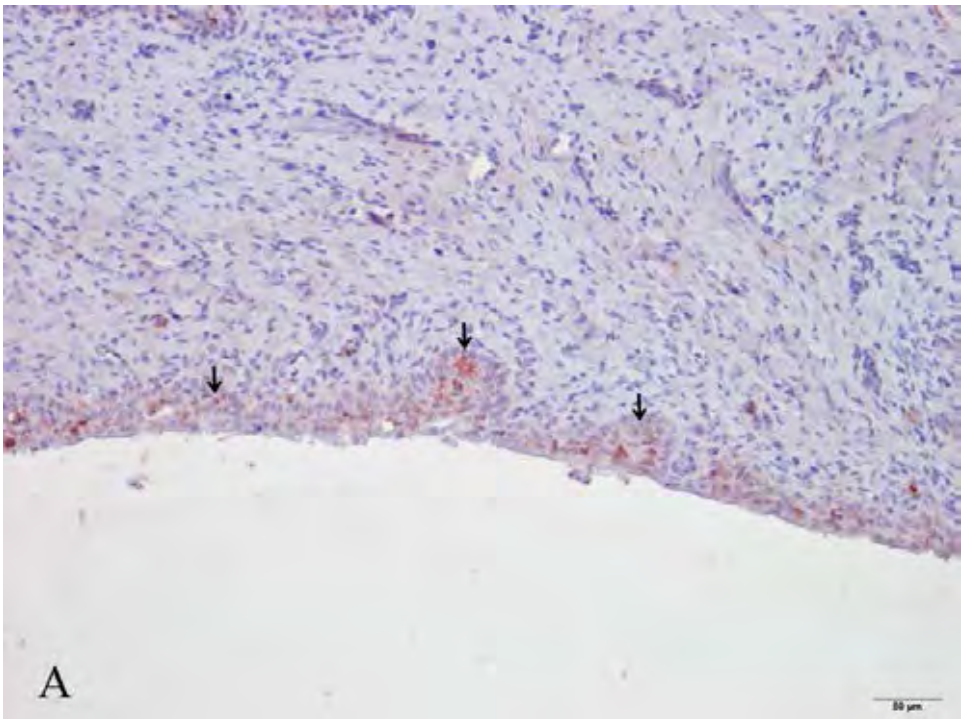


Figure 30 Immunohistochemistry showing the intense staining of TLR4 in surface epithelium of cervix at diestrus (A, B) can be noted. Negative control is shown in (C).



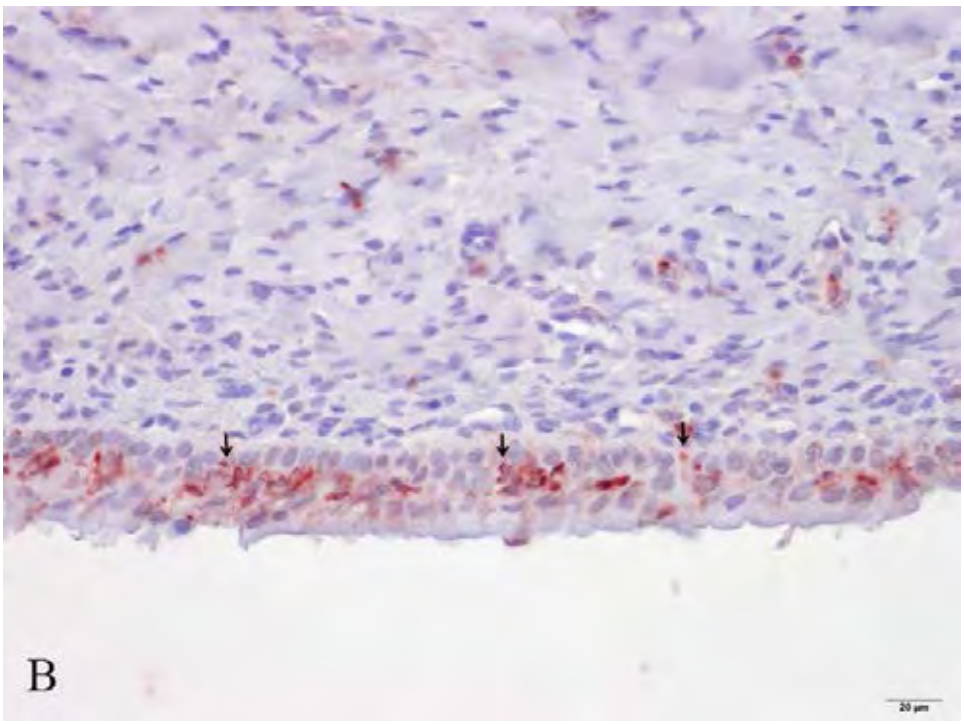


Figure 31 Immunohistochemistry showing the staining of TLR4 in surface epithelium of cervix in pyometra dog (A, B, black arrow).

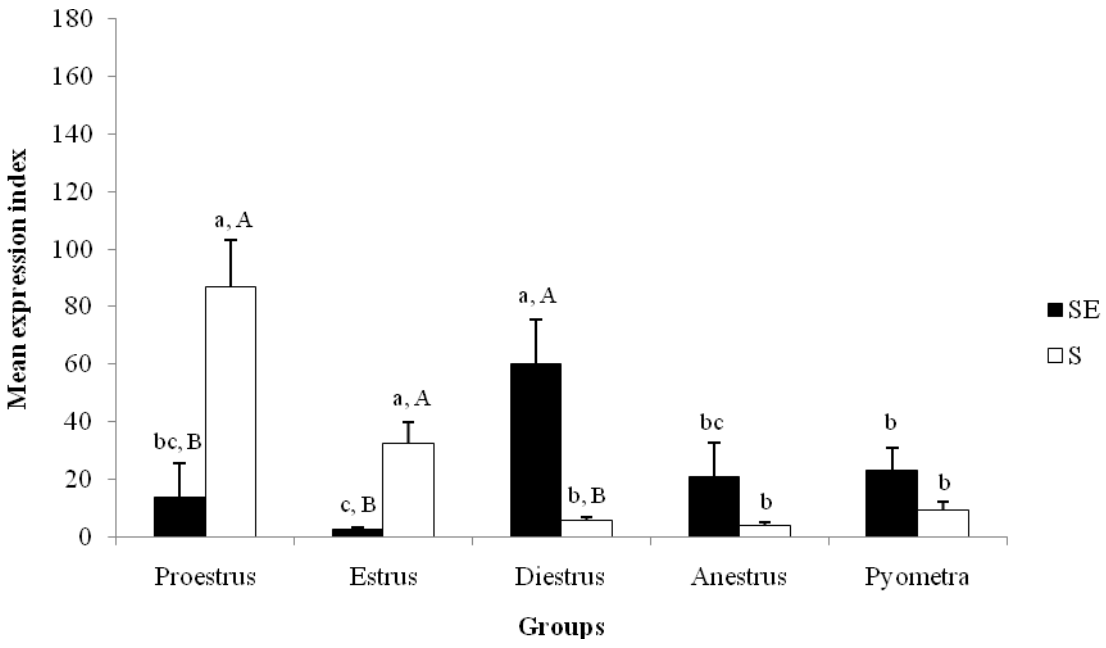


Figure 32 The mean expression index (± S.E.M.) for TLR4 in tissue layers (SE; surface epithelium, S; stroma) of cervix in proestrus, estrus, diestrus, anestrus and pyometra. The letters “a” and “b” and “c” indicate differences in the expression between groups within a similar tissue layer. The letters “A” and “B” indicate differences in the expression between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

The surface epithelium in dogs with pyometra expressed TLR2 significantly more intensely than the stoma ($P < 0.01$) (Figure 33). While, the expression of TLR2 at estrous and diestrous stage were absent in the stroma. The immunostaining of TLR2 in all groups and layers were differential expressed as shown in Figure 34 and 35.

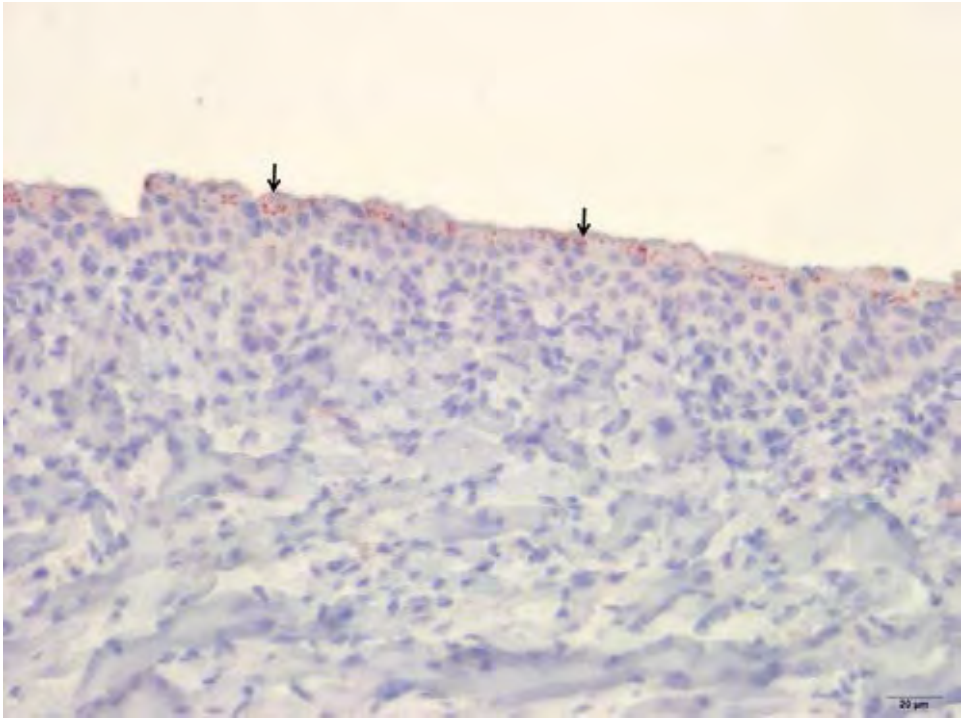
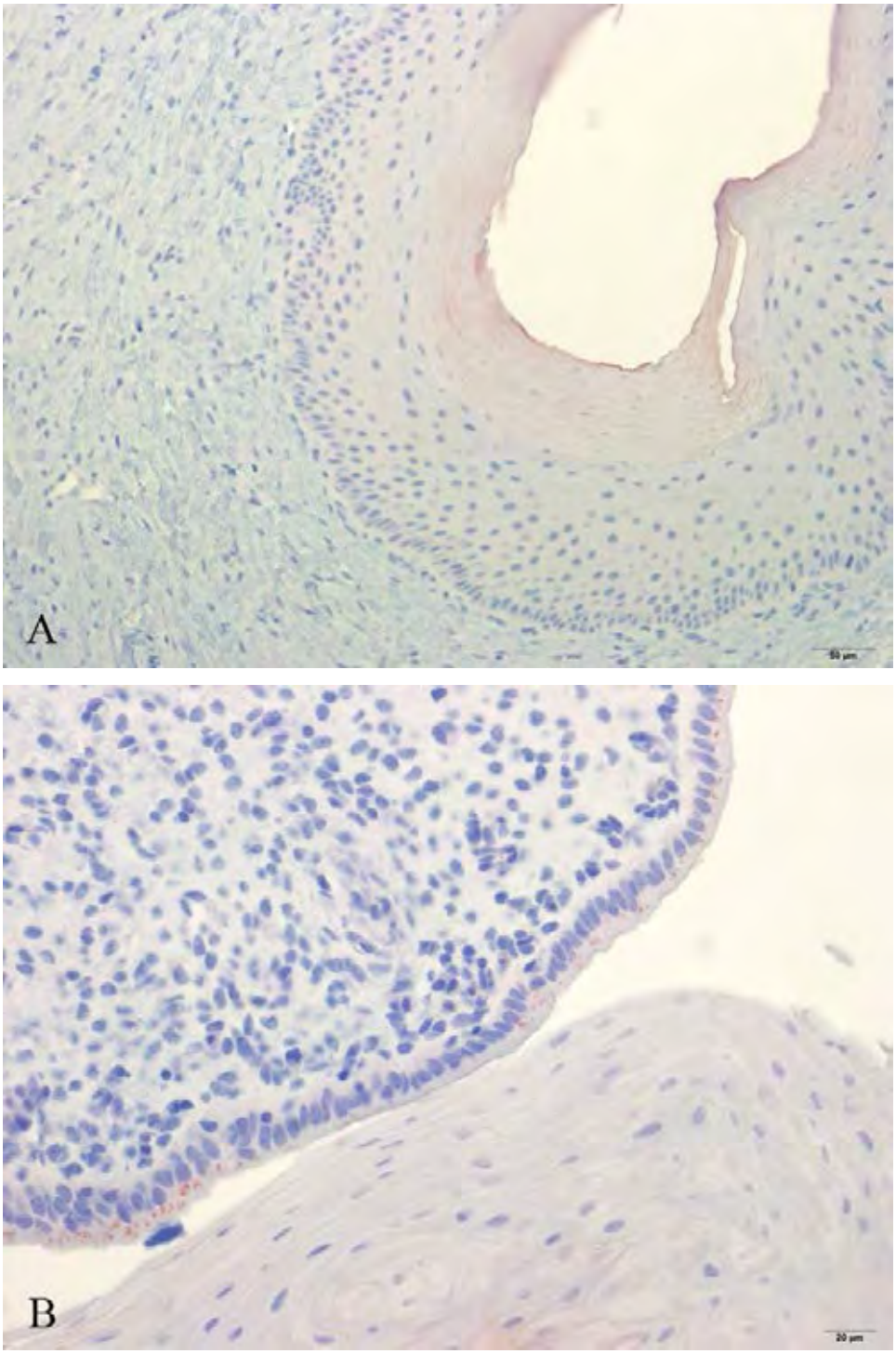
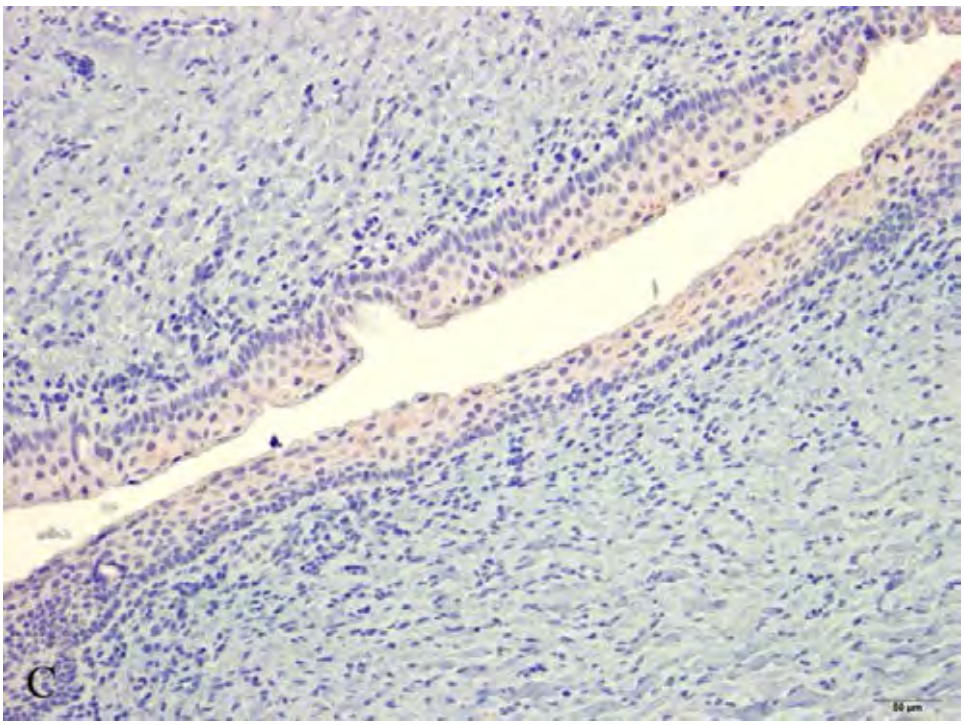
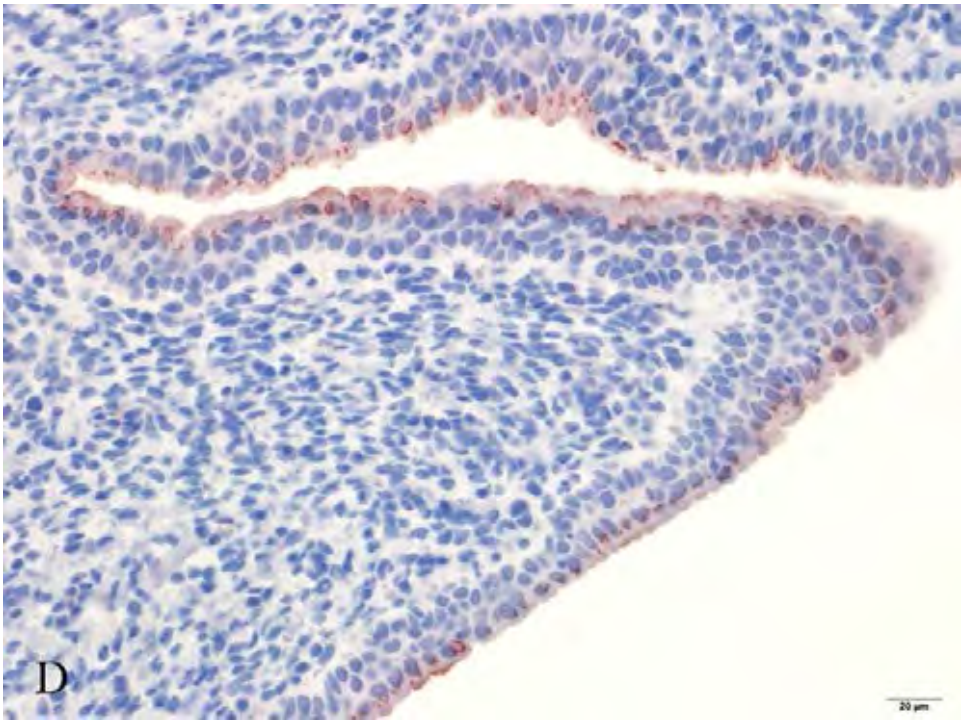


Figure 33 Immunohistochemistry showing the intense staining of TLR2 in surface epithelium of cervix in pyometra dog (black arrow).


A
B


C


D

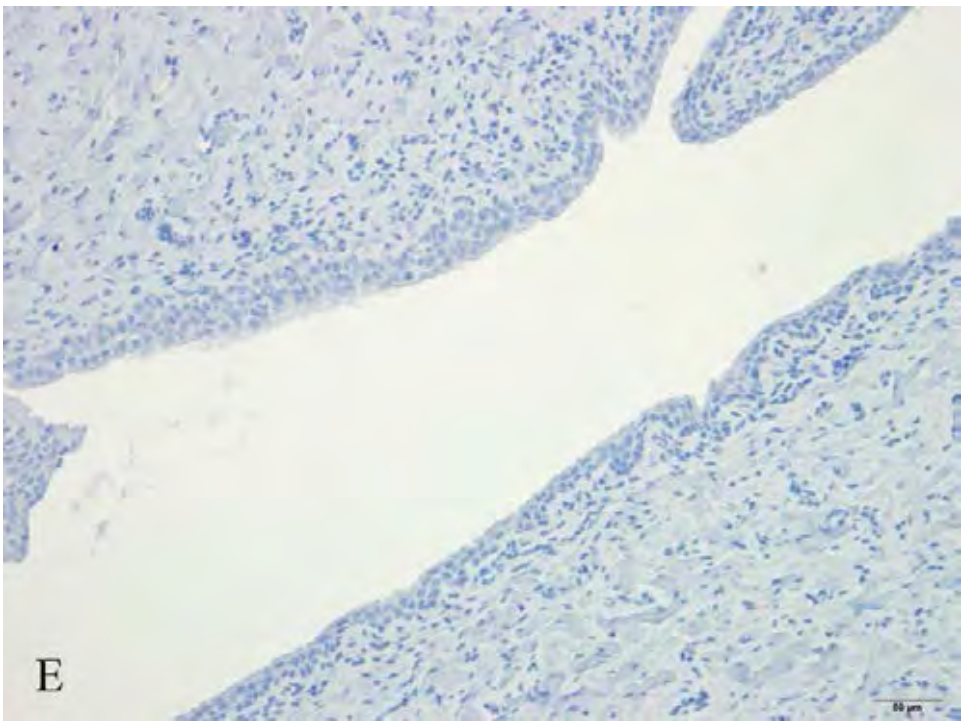


Figure 34 Immunohistochemistry showing the staining of TLR2 in surface epithelium of cervix at proestrus (A), estrus (B), diestrus (C) and anestrus (D) can be noted. Negative control is shown in (E).

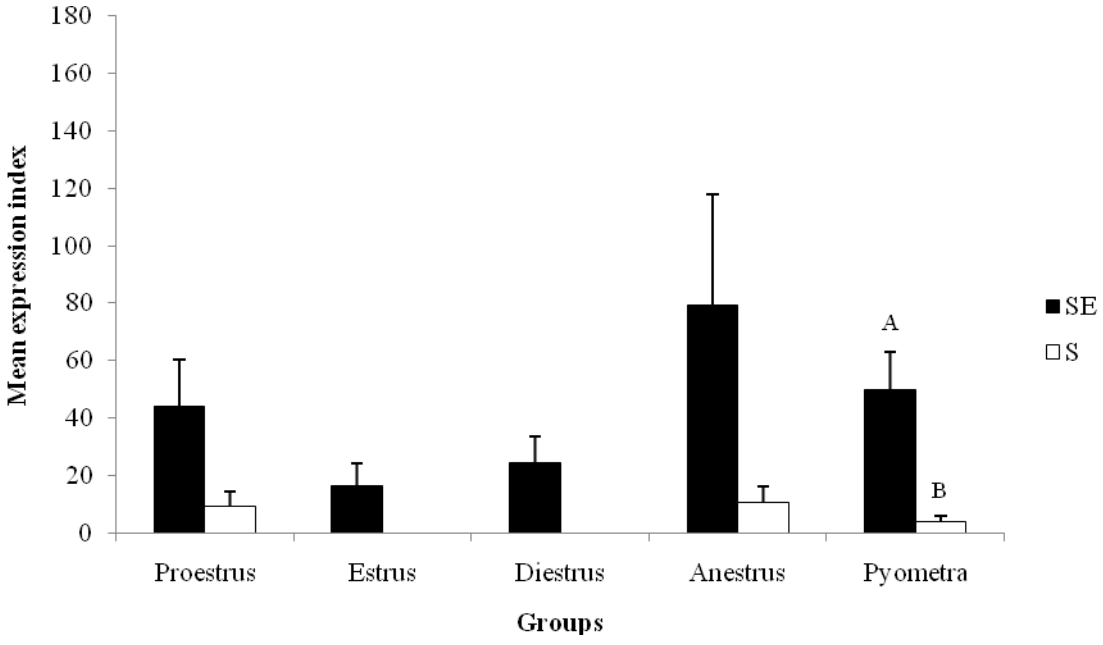


Figure 35 The mean expression index (± S.E.M.) for TLR2 in tissue layers (SE; surface epithelium, S; stroma) of cervix in proestrus, estrus, diestrus, anestrus and pyometra. The letters "A" and "B" indicate differences in the expression between tissue layers within a similar group. Different letters indicate a significant difference ($P < 0.05$).

The expression of TLR4 in the surface epithelium of dogs with pyometra and in the stroma of diestrous and pyometra dogs were significantly more intensely in the uterine horn compared to the uterine body and the cervix ($P < 0.01$) (Figure 36 and 37). Conversely, the expression of TLR2 in the surface epithelium of the cervix was significantly higher than the uterine horn and body ($P < 0.01$) (Figure 38). However, the expression of TLR4 in the surface epithelium of diestrous dogs was significantly increased in the cervix compared with the uterine horn and body ($P < 0.01$).

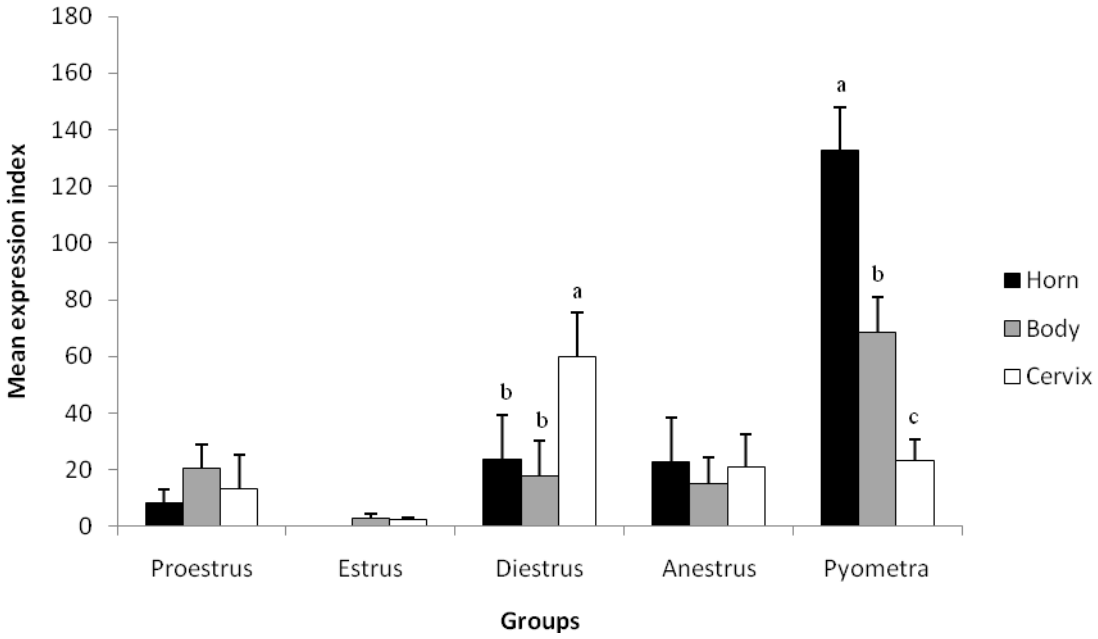


Figure 36 The mean expression index (± S.E.M.) for TLR4 in surface epithelium at different regions of uterus (Horn, Body, Cervix) in proestrus, estrus, diestrus, anestrus and pyometra. The letters "a" and "b" and "c" indicate differences in the expression between groups within a similar tissue layer. Different letters indicate a significant difference ($P < 0.05$).

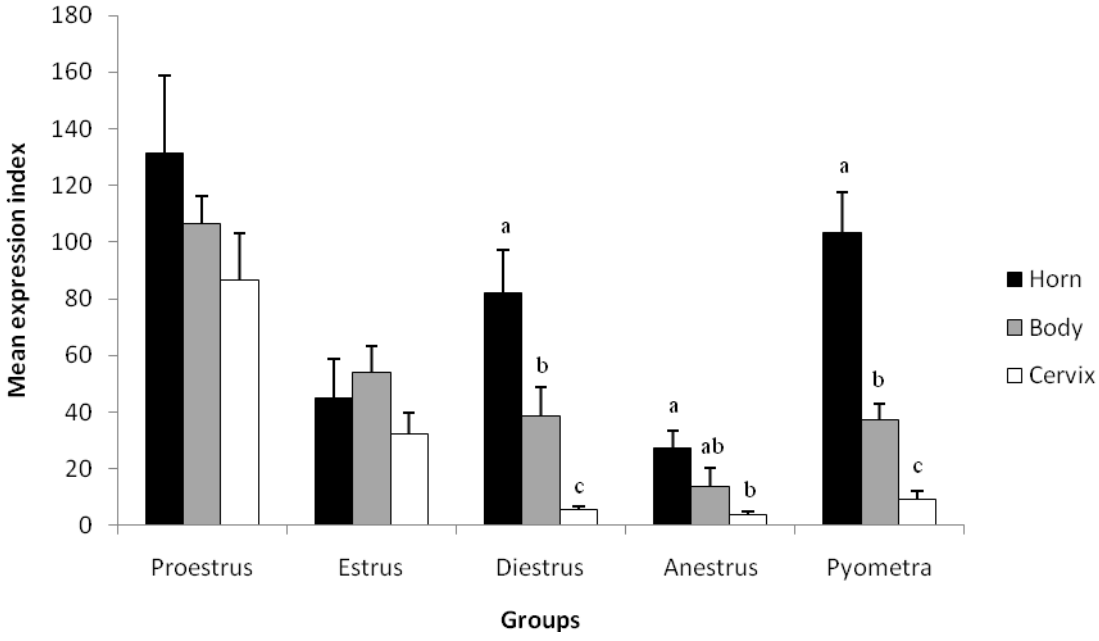


Figure 37 The mean expression index (± S.E.M.) for TLR4 in stroma at different regions of uterus (Horn, Body, Cervix) in proestrus, estrus, diestrus, anestrus and pyometra. The letters "a" and "b" and "c" indicate differences in the expression between groups within a similar tissue layer. Different letters indicate a significant difference ($P < 0.05$).

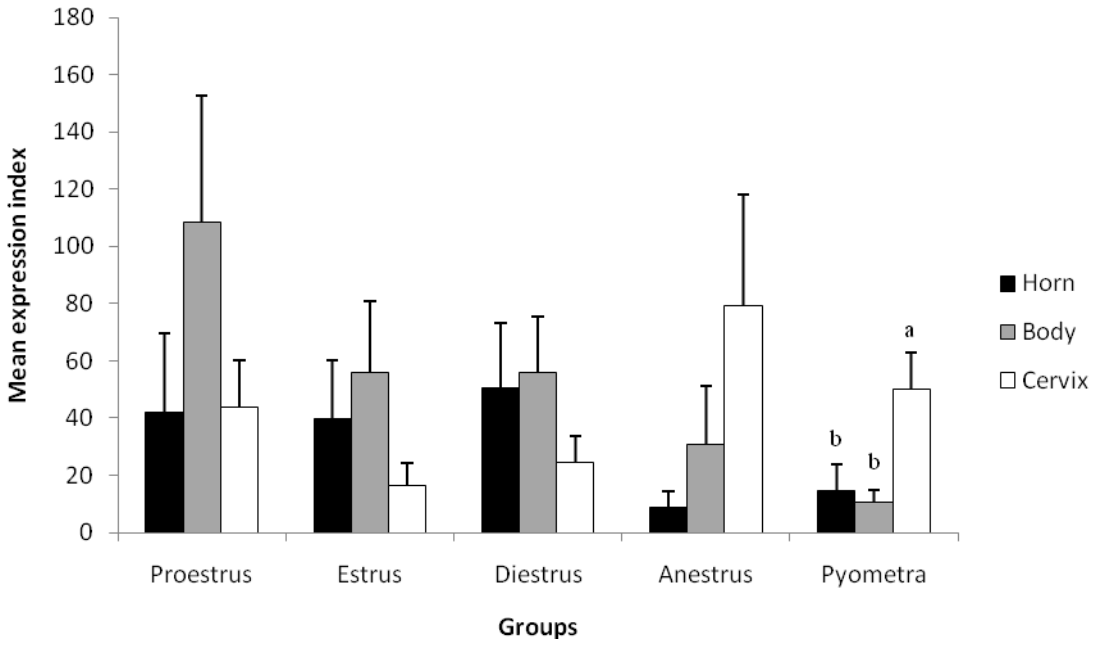


Figure 38 The mean expression index (± S.E.M.) for TLR2 in surface epithelium at different regions of uterus (Horn, Body, Cervix) in proestrus, estrus, diestrus, anestrus and pyometra. The letters "a" and "b" indicate differences in the expression between groups within a similar tissue layer. Different letters indicate a significant difference ($P < 0.05$).

4.5 Discussion

This study provides the first report in immunohistochemical localization of TLR in the canine reproductive tract. For most reproductive cycles in animals and humans, the uterus is thought to be sterile or at least clear of pathogenic bacteria, but it is readily contaminated with bacteria during sexual intercourse and around the time of parturition. Furthermore, the microorganisms in the lower reproductive tract may be able to spread to the upper reproductive tract, resulting in the development of uterine infection such as endometritis and pyometra (Khan et al., 2009).

In healthy uteri during reproductive cycle, TLR4 is also activated due to sterile inflammation (Takeda and Akira, 2005; Jiang et al., 2006; Allhorn et al., 2008). Therefore, the expression of TLR4 in the endometrium may reflect the occurrence of sterile inflammation during the reproductive cycle (Allhorn et al., 2008). In the

current study, the endometrial stroma of proestrus, estrus, diestrus and anestrus expressed TLR4 more intensely compared with the endometrial epithelium. In humans, TLR4 was also expressed more intensely in endometrial stormal cells than in endometrial epithelial cells (Hirata et al., 2007). TLR4 recognizes lipopolysaccharide (LPS) through a complex mechanism involving several accessory molecules. For example, CD14, a high-affinity LPS receptor. CD14 is secreted into serum as a soluble CD14 (sCD14). Moreover, MD2 is also required for LPS recognition. In the previous studies, CD14 was found to be expressed on the cell surface of the endometrial stromal cells but not the endometrial epithelial cells. TLR4 activation with the aid of sCD14 is also known in intestinal epithelial cells and bladder epithelial cells, in which the tolerant status for LPS in a basal condition is supposed to suppress any unnecessary inflammation induced by commensal bacteria (Cario et al., 2000; Backhed et al., 2002; Hirata et al., 2005). TLR4 expression in the epithelial cells was unresponsive to LPS, unless soluble CD14 was supplied (Hirata et al., 2007; Allhorn et al., 2008). Conversely, stromal cells, which have membranous CD14, were responsible for LPS. The lack of membranous CD14 in the epithelial cells may prevent harmful hyper-responsiveness in response to microorganisms, while stromal cells may react promptly once the epithelial barrier has been breached (Hirata et al., 2007). Thus, the notion that the TLR4 system might be inactive in endometrial epithelial cells relative to endometrial stromal cells in basal status also appears to be consistent with the finding that expression levels of TLR4, CD14 and MD2 mRNA are lower in endometrial epithelial cells, compared with endometrial stromal cells (Hirata et al., 2005). However, in the bovine endometrium, epithelial and stromal cells expressed CD14 protein, in addition to expressing CD14 at the mRNA level. These results may reflect a species difference (Herath et al., 2006a). On the other hand, other factors such as sex hormones and leukocyte populations may be involved in the expression of TLR4 in canine endometrium.

White blood cells are the main source of interferon gamma (IFN-γ) in the endometrium (Herath et al., 2006a; Hirata et al., 2007) and IFN-γ upregulates expression of TLR4 in endometrial stromal cells (Hirata et al., 2005, 2007). Invading microorganisms will be recognized by TLRs and activate NK cell cytokine production such as IFN-γ resulting in further activation of the innate immunity. Earlier studies in

canine endometrial leukocytes discussed only the total number of endometrial leukocyte at diestrus and anestrus. The data of the average number of endometrial leukocytes is shown to be high in stroma at late diestrous and early anestrous stages (Chu et al., 2006). However, these findings were supported by the in investigation of uterine leukocytes infiltration of healthy dogs at different stages of the estrous cycle in the previous experiment that the total leukocytes also significantly increased in anestrous stage compared to other stages of the estrous cycle. In addition, in the previous experiment which study in different stages of canine estrous cycle found that the number of total leukocytes in the endometrial stroma was higher than the surface epithelium in all stages. While, in estrous stage, the total leukocytes in the endometrial surface epithelium was significantly lower than other stages which may related to the absent of TLR4 expression in the endometrial surface epithelium of estrous dogs. These results support the previous studies which conclude that leukocytes and TLRs may coordinately protect the endometrium against microbial invasion (Hirata et al., 2007). From the present study, the expression of TLR4 on leukocytes such as macrophages and lymphocytes was also found varies during estrous cycle. Thus, the differential expression levels of TLR4 in different layers (epithelium, stroma) of the canine endometrium are shown to be influenced by the number of endometrial leukocytes.

The most important role of TLRs in host defense is the regulation of innate and adaptive immune responses. Nevertheless, another important role of TLR besides this is maintaining tissue homeostasis by regulating tissue repair and regeneration. Recent reports have suggested that activation of TLR can also induce apoptosis (Xu et al., 2004; Kawai and Akira, 2007; Ding et al., 2010). TLR signaling has been shown to regulate apoptosis with the expression of antiapoptotic protein or inhibitors of apoptosis (Ioannou and Voulgarelis, 2010). In the present study, the expression of TLR4 at the diestrous stage was significantly dominant in the glandular epithelium compared with the surface epithelium and other groups of healthy dogs. In the normal canine estrous cycle, the endometrium undergoes two phases of growth and differentiation. The first growth phase begins during the late stage of anestrus. The second growth period begins around the middle of estrus, peaks near the end of estrus and rapidly subsides during the first week of metestrus. Late metestrus and early

anestrus are characterized by an involution of the endometrium (Barrau et al., 1975; Galabova et al., 2003). The regression of the glandular epithelium was commenced by the end of the first week of diestrus (Barrau et al., 1975; Chu et al, 2006). This regression involved apoptosis in dogs both in natural and in hormone-induced stimulated diestrus. The apoptosis in the basal glandular epithelium preceded the degeneration of the cells of luminal epithelium (Chu et al., 2002, 2006). The higher incidence of apoptosis in the basal glandular epithelial cells at early diestrus confirmed the findings in earlier studies (Chu et al., 2002, 2006; Van Cruchten et al., 2003). The lack of apoptosis in the luminal epithelial cells was also reported in the previous studies (Van Cruchten et al., 2003; Chu et al., 2006). In TLR4-wild-type mice with acute pancreatitis, the apoptotic index and the expression of cytochrome C and Fas-associated protein with the death domain (FADD) were significantly higher in the pancreas at two hours after injection compared with TLR4-deficient mice. These results suggest that TLR4 mediates apoptosis during the early stage of acute pancreatitis, via activation of intrinsic and extrinsic apoptotic signaling pathways (Ding et al., 2010). Accordingly, the physiological changes in the glandular epithelium by apoptosis at diestrus may be related to the higher expression of TLR4 in the glandular epithelium of this stage from the present study. Nevertheless, the role and mechanism of TLR4 in apoptosis of dogs should be further investigated.

Interestingly, at estrus, the expression of TLR4 was absent in the surface epithelium of the endometrium. Estrus in dogs is a stage of receptivity to mounting by males (Concannon, 2010). Low expression of TLR4 might prevent an unfavorable inflammatory response of the endometrium evoked by microbial contaminants with upcoming sperms (Friberg et al., 1987; Svenstrup et al., 2003; Hirata et al., 2007). Incidentally, it was noticeable that TLR4 was expressed at low levels in the surface epithelium in groups of healthy dogs. This is in accordance with the previous studies into lungs where TLR4 was also characterized as an intracellular localization in pulmonary epithelium, and its special localization is expected to play an important role in the prevention of the development of chronic inflammatory disease (Guillot et al., 2004; Yu et al., 2009). Moreover, the TLR pathway can also stimulate the production of prostaglandins by immune cells (Uematsu et al., 2002; Herath et al., 2006a). These chemical mediators, produced by uncontrolled inflammatory responses,

can disturb uterine peristalsis for normal fecundability (IJland et al., 1997; Hirata et al., 2007). However, the low responsiveness or unresponsiveness to PAMPs in most of the epithelial cells may be related to the inability of the cells to discriminate pathogens from commensal bacteria by the mere recognization of PAMPs. Additional factors such as cellular invasion or other signs of pathogenicity are required to induce inflammatory responses which in turn prevent tissue destruction due to excessive inflammatory innate immune responses to bacterial stimuli (Uehara et al., 2007). Hence, it is essential that the upper reproductive tract epithelium has the capacity to recognize and respond to ascending pathogens while simultaneously avoiding a state of unnecessary inflammation that might disrupt the epithelial barrier (Nasu and Narahara, 2010).

In the earlier report, TLR3 and TLR4 proteins in human endometrial hyperplasia and endometrial carcinoma were mostly localized to the luminal and glandular epithelium (Allhorn et al., 2008). In this study, the extremely high levels of TLR4 expression was found in the surface epithelium, glandular epithelium and stroma of pyometra dogs compared with other groups of healthy dogs. This indicated the effect of the innate immune defense mechanism against bacterial infection in pyometra dogs resulting in the elevation of TLR4 expression in the endometrium. TLR4 was found to be localized mainly in the upper part of the female reproductive tract (Aflatoonian and Fazeli, 2008) and mediates the response to bacterial endotoxins. So, ascending bacterial infection could have contributed to the TLR4 dominance in the endometrium (Allhorn et al., 2008). Furthermore, TLR4 was found to be expressed more intensely in the surface epithelium than the glandular epithelium of the endometrium in pyometra dogs. This may be due to the fact that the surface epithelial cells are the first line of defense and are directly in contact with the invading pathogens especially the LPS of Gram-negative bacteria. Thus, TLR4 is supposed to be expressed abundantly in the surface epithelium of the endometrium to produce a differential of inflammatory cytokines, chemokines leading to the direct killing of the invading bacteria (Aboussahoud et al., 2010).

The endometrial environment is under the control of sex hormones during the reproductive cycle. In addition, sex hormones are also involved in the influx and localization of immune cells in the endometrium (Spornitz et al., 1992; Yeaman et al.,

1997; Aflatoonian and Fazeli, 2008). Estradiol has proinflammatory effects in the uterus and has been linked to an influx of leukocytes at estrous in the mouse (De and Wood, 1990; Lea and Sandra, 2007). Conversely, progesterone has been associated with anti-inflammatory activity (Lea and Sandra, 2007) by suppresses the immune response to make the uterus more susceptible to spontaneous bacterial infection (Olson et al., 1984; Lewis, 2003; Herath et al., 2006b). In humans, rodents and cattle, progesterone suppresses uterine immune function by decreasing the proliferative capacity of lymphocytes (Beagley and Gockel, 2003; Herath et al., 2006a; Khan et al., 2009).

From the current study, at diestrus, the glandular epithelium of endometrium expressed TLR4 more intensely than at other stages of estrous cycle. Meanwhile, endometrial storma expressed TLR4 dominantly at diestrus and also in proestrus. Similarly, gene expression of TLRs in human endometrium through menstrual cycle reveals that their highest levels are observed in the late secretory and early proliferative phases (Girling and Hedger, 2007; Yang et al., 2011). The high levels of TLR4 expression in the glandular epithelium and stroma at the diestrous stage may be related to the high levels of progesterone at this stage. Furthermore, most dogs with pyometra from this study that expressed high levels of TLR4 in endometrium were found to be at the stage of diestrus (21/23) when progesterone is dominant. These findings may support the influence of progesterone on the innate immunity by enhancing the expression of TLR4 in the canine endometrium. This is in agreement with the previous report into human endometrial tissue during the menstrual cycle, that demonstrated that TLR2, 3, 4, 5, 6, 9 and 10 genes showed a significantly higher expression in the secretory phase when progesterone levels were high compared with the proliferative phase when estrogen levels are high. This result may indicate a supporting effect of progesterone and/or suppressing effect of estrogen on the expression of TLRs in the endometrium (Aflatoonian et al., 2007; Aflatoonian and Fazeli, 2008). However, the expression of TLR4 in endometrial stroma was not significantly different between diestrus and proestrus. But, the relative expression of TLR4 in the stroma seemed dominant at both diestrus and proestrus. In human endometrial stromal cells, progesterone treatment enhanced expression of TLR4 mRNA and estradiol treatment suppressed expression of TLR4 mRNA. Whereas,

hormonal treatment did not affect the expression of TLR4 in the endometrial epithelial cells (Hirata et al., 2007). These findings may give an initial explanation of the role of sex hormones in the regulation of innate immunity especially in different cell types of the canine endometrium. In contrast to the previous study, exogenous replacement of estradiol but not progesterone has been shown to elevate LPS-binding protein levels and cell surface expression of TLR4 and CD14 on macrophages. Moreover, the replacement of both estradiol and progesterone demonstrated the higher LPS binding capacity than received estradiol alone without significant difference in TLR4 or CD14 expression in macrophages (Rettew et al., 2009). In male reproductive tract, estrogens also upregulated TLR9 in prostate cancer cell line which implicating estrogen's role in prostatitis (Ilvesaro et al., 2009; Kannaki et al., 2011). Another possibility is that the sex hormones might have an indirect effect in the modulation of TLR function. For instance, treatment of endometrial epithelial cell lines with estradiol did not affect TLR3 mRNA or protein expression but, suppressed cytokine and chemokine production resulting from TLR3 stimulation with poly I:C (Lesmeister et al., 2005). Nevertheless, to increase the understanding of the role of sex hormones, the molecular mechanisms of sex hormones and the manner of progesterone in concert with high level of estradiol influencing the expression of TLR4 in canine endometrium should be further investigated. On the other hand, factors other than sex hormones might modulate expression of TLR4 in the canine endometrium. This is in accordance with the earlier studies which demonstrated that estrogen did not influence the expression of TLR4 in the retina (Paimela et al., 2007; Allhorn et al., 2008) and in macrophages (Vegeto et al., 2004; Allhorn et al., 2008). So, additional factors are required to decrease TLR-expression in endometrium (Allhorn et al., 2008).

In the present study, TLR2 was expressed in endometrial epithelium but absent in the endometrial stroma of healthy dogs at all stages. These specific expressions of TLR in a different tissue layers have been reported in the earlier studied. For example, TLR3 expression was presented in epithelial cells to combat the viral infection. Meanwhile, TLR4 was highly expressed in the stromal cells in order to prevent hyperresponsiveness to microorganisms and commensal bacteria (Hirata et al., 2007). These findings may indicate the type-dependent differential expression of

TLR in a distinct cell types. However, the expression levels of TLR2 in human endometrial stromal cells were comparable to those of endometrial epithelial cells (Compton et al., 2003; Aflatoonian et al., 2007; Lin et al., 2009; Nasu and Narahara, 2010). So, on the other hand, the lack of TLR2 in the stroma of healthy uteri of dogs may promote the infection from the invading pathogens once the epithelial cells have been destroyed by the pathogens especially Gram-positive bacteria. Nevertheless, TLR2 would be inducible post-infection by TNF-α. The engagement of TLR4 resulting in the production of TNF-α which leading to increased expression of TLR2 (Pioli et al., 2004). These may explain why TLR2 expression was found increased in the endometrial stroma of dogs with pyometra in this study.

Recently, the study of TLR2 and TLR4 mRNA in pyometra dogs found that these genes were significantly up-regulated in pyometra dogs infected with *E.coli* when compared to diestrous stage (Silva et al., 2010). In agreement with the protein expression of TLR4 in the endometrium of dogs with pyometra in this study, but the protein expression of TLR2 in this study was different from the previous study in pyometra dogs. In infected endometrium of dogs suffering from pyometra, TLR2 was not highly expressed in the endometrial surface epithelium as found in TLR4 expression. And, the expression of TLR2 in the surface epithelium of the uterine body was also significantly decreased in pyometra dogs compared to dogs at proestrus, estrus and diestrus. The possibility is that the endometrium has increased expression of other receptors such as TLR4 which also increased production of cytokines in response to infection (Darville et al., 2003). In accordance with the finding in TLR4 in this study which found the extremely high level of TLR4 in infected endometrium. This result may indicate a protective role for TLR2 in canine uterine infection which can decrease the uterine pathology due to unfavorable inflammation and improved balance between protective and pathological inflammatory responses to maintain the uterine homeostasis (Si-Tahar et al., 2009).

In addition, the expression of TLR2 was more intensely in the endometrial glandular epithelium of estrus, diestrus and dogs with pyometra compared to anestrus. In the previous study, significantly higher levels of expression of TLR2 in the human endometrium have been observed during the secretory phase than other phases of the menstrual cycle (Compton et al., 2003; Aflatoonian et al., 2007; Lin et al., 2009; Nasu

and Narahara, 2010). However, TLR2 was also expressed dominantly in the uterine body at proestrus compared to anestrus. This finding may support the influence of not only progesterone but also estrogen in the expression of TLR2 and TLR4 in canine endometrium.

In dogs with pyometra, the expression of TLR2 was significantly more intensely in the surface epithelium of the cervix. The surface epithelium of the cervix maintains a first line of defense for the lower female reproductive tract (Pioli et al., 2004). To protect the host from the infection, the recognition of the invading bacteria in dogs suffering from pyometra shown to be mediated by TLR2 in the cervix this may resulting in an increased expression of TLR2 in the surface epithelium of these tissues. Interestingly, the expression of TLR2 were absent in the stroma of the cervix at estrus and diestrus. In dog, the cervix usually opens about 2 days before the LH peak which close to the time of mating in estrous stage (Silva et al., 1995). In this period, bacteria could enter to the uterus easily. Consequently, from the result of this study, if the epithelial barrier has been destroyed at this time, these pathogens may invade into the reproductive tract and cause the uterine infection in dogs.

In murine vaginal epithelium, TLR2 and TLR4 significantly increased in diestrus or following Depo-treatment and the expression is significantly lower at estrus (Yao et al., 2007). In accordance with this study in the cervix that the expression of TLR4 was significantly more intensely in the surface epithelium of diestrous dogs compared to other groups and the stroma. And the surface epithelium of dogs with pyometra also expressed TLR4 higher than estrus. Conversely, this study found the lowest expression of TLR2 and TLR4 in the surface epithelium of estrous dogs. Furthermore, the expression of TLR4 was also significantly increased in the stroma of the cervix at proestrus and estrus compared to the surface epithelium. The possibility is that female dogs and mice only mate at estrus, thus it may be important to have the low level of TLR expression at this time preventing the inappropriate immune responses against upcoming sperm (Yao et al., 2007). Interestingly, even though the expression of TLR in the surface epithelium of the cervix was lowest at estrus, the number of resident leukocytes in the surface epithelium of the cervix from the previous experiment in chapter III was highest at this stage. These findings indicated the interplay of different arms of canine uterine immune system to facilitate

the fertilization along with the elimination of pathogens that may transmitted in period of mating.

In human primary endocervical epithelial cells, LPS leads increased expression of TLR4 and more release of antimicrobial peptides human defensin5 and cytokines such as IL-6 and TNF-α which indicated that the signaling pathways of TLR4 are involved in the innate response against LPS by these cells (Ma and Yang, 2010). Moreover, in the earlier study found the higher tissue expression of TLR4 protein in the squamous epithelium of the cervix in the group with bacterial infection due to the translocation of TLR4 as a protective mechanism (Adams et al., 2007; Dubicke et al., 2010). However, the expression of TLR4 in the surface epithelium of the cervix in dogs with pyometra was significantly lower than healthy dogs at diestrus in this study. It was possible that TLR4 expression in human endocervical epithelial cells showed a quick response in 24 hours after LPS stimulation and decreased sharply after 48 hours (Ma and Yang et al., 2010). In agreement with this study that most of dogs that was diagnosed as pyometra and treated by the ovariohysterectomy, the period was usually more than 24 hours. Thus, these may explained why the expression of TLR4 from this study was not significantly high level in pyometra dogs. The downregulation of TLR4 could be benefiting the epithelial cells from cytokine overproduction. Since, the overproduction of cytokine may serious inflammatory reaction and long-terms complications (Ma and Yang et al., 2010).

In dogs suffering from pyometra, when compared between the uterine regions, the expression of TLR4 was significantly more intensely in the surface epithelium and stroma of the uterine horn compared to the uterine body and the cervix which found the lowest expression in dogs with pyometra and also in the stroma of diestrous dogs. Conversely, the expression of TLR2 in the surface epithelium of the cervix was significantly higher than the uterine horn and body. In accordance with the previous study in the human female reproductive tract that the expression of TLR4 has been shown decline progressively along the female reproductive tract, with the highest levels of expression in the endometrium followed by the cervix. Meanwhile, the highest levels of TLR2 mRNA expression have been observed in the cervical tissues followed by the endometrium. The possibility is that under condition of low TLR4 expression in the cervical tissue, TLR2 is activated either because of redundant or

alternative pathogen recognition (Pioli et al., 2004). Whereas, the low levels of TLR4 expression in canine cervix may be due to the lack of MD2, the accessory molecule of TLR4 signaling that found previously in cultured epithelial cells derived from normal human vagina, endocervix and ectocervix (Fichorova et al., 2002; Nasu and Narahara, 2010). Furthermore, in the lower female reproductive tract, the cervix is exposed to a much broader variety of infectious microorganisms. The low expression of TLR4 in the cervix was reflective to the complex microorganisms associated with these tissues. Accordingly, the cervical tissues develop a mechanism for selectively response to pathogens while avoiding chronic inflammation due to immune responses to commensal bacteria. And, limiting levels of TLR4 may provide a mean of regulating sensitivity to bacterial components in the lower female reproductive tract (Pioli et al., 2004). Nevertheless, the expression of TLR4 in the surface epithelium of diestrous dogs was significantly increased in the cervix compared with the uterine horn and body. The reason may be due to the effect of progesterone dominance in this stage which may selectively regulated the expression of TLR4 in a distinct cell type as found in the endometrium in the previous study (Hirata et al., 2007). In addition, the expression of CD14 in human cervix was different from the endometrium that CD14 was found in the epithelial cells of the cervix and vagina (Herbst-Kralovetz et al., 2008; Nasu and Narahara, 2010). However the expression of accessory molecules of TLR4 should be further validated.

In conclusion, this is the first report of the expression of TLR2 and TLR4 in the endometrium of healthy and pyometra dogs. The presence of TLR2 TLR4 was demonstrated in the surface epithelium, glandular epithelium and stroma throughout the estrous cycle and in pyometra. This indicated the role of canine endometrium in immune surveillance on the one hand and in fundamental cellular process on the other hand in order to maintain the uterine homeostasis. The trend of the expression of TLR2 and TLR4 in the uterine horn and uterine body was similar when compared between layers and groups. These may be due to the both uterine horn and uterine body was classified as the upper part of the female reproductive tract which consists of the fallopian tubes and endometrium (Pioli et al., 2004). However, the intensity of the immunostaining was significant difference in some groups when compared between these two regions. The different levels of TLR2 and TLR4 expression related

to physiological changes in the distinct microenvironment of endometrium, leukocytes populations, cytokines and sex hormones. The finding of differential expression of TLR2 and TLR4 from this study indicated the relationship of TLR2, TLR4, innate immunity and the development of the uterine bacterial infection. Further investigation should be directed toward understanding the mechanisms and factors that control TLR2 and TLR4 expression in the canine endometrium in order to treat and prevent the bacterial infection effectively by modulate the TLR system and may leading to the development of the new adjuvants for the clinical application in the future.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

Current strategy: Canine uterine infection and the expression of TLR2 and TLR4 in healthy and infected canine uterus

Canine uterine infections are associated with bacteria (Baba et al., 1983) and have a detrimental effect on the dog. The most common pathologic condition in the uterus of the dog is pyometra (chronic purulent endometritis) (Kida et al., 2006). In this study, the bacterial species in the vagina and uterus were investigated in healthy dogs during the estrous cycle and dogs with pyometra. The results found that no healthy dogs in this study had a relationship between bacterial species in the vagina and uterus which may indicate that the immune surveillance in the female reproductive tract was critically influenced by the distinct microenvironment of each compartment of the female reproductive tract. In addition, the trend of the bacterial types presented in the vagina and uterus of healthy dogs were varied during estrous cycle. Hence, it was possible that the presence of bacteria in the vagina and uterus of healthy dogs was also influenced by the estrous cycle. However, in anestrous stage when the estradiol and progesterone levels were generally low, most of dogs in this stage were free of bacteria in the uterus. Thus, sex hormones that influenced the stage of the estrous cycle may not be only factor that indicated the presence or absence of bacterial species.

In dogs with closed-cervix pyometra, a different bacterial species from the vagina and uterus of the same dog was found and this was in which most of cases. This may be related to the different existence of microorganisms in the vagina and uterus and the many different processes involved in the clearance of bacteria (Watts et al., 1996). The innate immune defense system of the female reproductive tract is a complex and dynamic system comprising specific physical or physicochemical barriers, cellular effectors as well as bactericidal proteins and peptides (Mak et al., 2004) that may also be a reflex to the presence of bacteria in the vagina and uterus. Therefore, it has been noticed that the bacteria found in the vagina may not be an

initial cause of the uterine infection in dogs with closed-cervix pyometra. In dogs with opened-cervix pyometra, in most cases the same type of bacterial species was found between the vagina and uterus. This finding may suggest that bacterial species in the vaginas may a cause of the uterine infection due to the increasing amount of bacteria in the vagina that may flow through the cervical opening to the uterus or they may be found as a result of uterine drainage by the opening of the cervix from the uterine infection at the same time. Accordingly, for the eradication of bacteria in canine pyometra, bacterial swabs should be taken at infection site in order to choose a proper antimicrobial agent. Nevertheless, factors other than the type of bacteria, such as uterine immunity may influence the susceptibility to canine uterine infection.

Moreover, the uterine bacterial species commonly isolated from healthy dogs in this study were *Escherichia coli*, *Streptococcus spp*. and *Staphylococcus spp*. which is in accordance with previous studies (Baba et al., 1983; Watts et al., 1996; Hagman and Kühn, 2002). Interestingly, in dogs with pyometra, these bacteria especially *Escherichia coli* were also commonly isolated from this study and the previous studies (Grindlay et al., 1973; Sandlhom et al, 1975; Nomura, 1984; Bjurström, 1993; Wadås et al., 1996; Fransson et al., 1997; Hagman and Kühn, 2002). This may indicate that there is no direct relationship between these bacteria and clinical illness from uterine bacterial infection. Other factors may also have contributed to the infectious processes the cause of canine uterine infection.

The nature and course of uterine infections not only depends on the type of invading bacteria but also involved with the immune status of the host (Singh et al., 2008). The immune surveillance in the female reproductive tract is critically influenced by the interplay of many factors such as sex hormones, resident leukocyte population and the distinct microenvironment of the reproductive tract (Hart et al., 2009). Thereby, in the chapter III, the leukocytes were investigated in healthy dogs in different regions of the uterus at different stages of the estrous cycle.

Lymphocytes, macrophages, neutrophils and plasma cells were the leukocytes that found in the uterus of healthy dogs in this study. And, the number of these leukocytes has shown different during different stages of the estrous cycle. The number of total leukocytes in the endometrial stroma was increased in anestrous dogs which corresponded to the previous experiment in chapter II that in most of dogs, the

bacteria were absent in the uterus at this stage. This may emphasize the influence of uterine immunity besides the effect of sex hormones in the clearance of bacteria to maintain the sterile environment in canine uterus.

Furthermore, the number of leukocytes in the uterine horn was similar to the uterine body but different from the cervix. And, the trend of the number of leukocytes was increased in the upper part which may suppose to maintains the sterile environment (Horne et al., 2008) and then decreased in the lower part of the canine reproductive tract except only at estrous stage that found the low number of leukocytes in the surface epithelium of the endometrium conversely this stage contained the high number of leukocytes in the cervix. This finding corresponded to the previous experiment in chapter II that most of bacteria could be isolated from the uterus at this stage (60%) which may support the action of resident endometrial leukocytes in the uterine immune surveillance to protect the host form the bacteria invasion. Whereas, in the lower part of the canine female reproductive tract, bacterial contamination of the uterus usually occurs prior to diestrus when the cervix is open (Rietschel et al., 1982; McAnulty, 1983; Pretzer, 2008). To protect the uterus from the pathogens invasion at this stage the leukocytes should be accumulate in the cervix preparing for combat to the infection. This may indicate the increased number of leukocytes in the cervix at estrous stage in this study which indicated the protective mechanism of the immune system in canine reproductive tract. It has been noticeable that the sex hormones seemed influenced the presence of the uterine leukocytes. However, the results from this study indicated that the number of uterine leukocytes not only depends on the effect of sex hormones, but other parts of the uterine immune system may also involve with the influx of uterine leukocytes.

The immune system has traditionally been divided into innate and adaptive immunity. However, innate immunity is the most universal, the most rapidly acting and may also the most important type of immunity (Beutler, 2004). TLRs are key mediators and the cellular components of the innate immune system (Beutler, 2004; Wira et al., 2005; Horne et al., 2008). Besides, TLR2 and TLR4 are the best characterized of Gram-positive and Gram-negative bacteria respectively (Darville et al., 2003). The leukocytes are the main source of IFN-γ in the endometrium (Herath et al., 2006a; Hirata et al., 2007) which upregulates expression of TLR4 in endometrial

stromal cells (Hirata et al., 2005, 2007). This may support the finding in chapter III that the number of leukocytes in the endometrial stroma was higher than the surface epithelium in all stages. Furthermore, the leukocytes in the endometrial surface epithelium at estrus was lower than other stages which may related to the absent of TLR4 expression in the endometrial surface epithelium of estrous dogs. These results support the previous study which concludes that leukocytes and TLRs may coordinately protect the endometrium against microbial invasion (Hirata et al., 2007). In contrast, the number of leukocytes in the surface epithelium of the cervix from the previous experiment in chapter III was highest at this stage although, the expression of TLR2 and TLR4 were lowest in the surface epithelium of the cervix at estrus. The low expression of TLR4 in the uterus at estrus might prevent an unfavorable inflammatory response of the endometrium evoked by microbial contaminants with upcoming sperms (Friberg et al., 1987; Svenstrup et al., 2003; Hirata et al., 2007). Thus, it is essential that the upper reproductive tract epithelium has the capacity to recognize and respond to ascending pathogens while simultaneously avoiding a state of unnecessary inflammation that might disrupt the epithelial barrier (Nasu and Narahara, 2010). Whereas, the high number of leukocytes in the cervix may be one of the uterine immune mechanisms that response to the bacterial contamination during the time of cervical opening or mating.

The high levels of TLR4 expression was found in the endometrium of pyometra dogs when compared to healthy dogs which indicated the effect of the uterine defense mechanism against LPS of the Gram-negative bacteria. While, the expression of TLR2 was not highly expressed in the endometrium as found in TLR4. This result may indicate a protective role for TLR2 in canine uterine infection which can decrease the uterine pathology due to unfavorable inflammation and improve balance between protective and pathological inflammatory responses to maintain the uterine homeostasis (Si-Tahar et al., 2009). Interesting, the expression of TLR2 was absent in the endometrial stroma of healthy dogs at all stages. This may promote the infection from the invading pathogens once the epithelial cells have been destroyed by the pathogens especially Gram-positive bacteria. Moreover, the expression of TLR2 was absent in the stroma of the cervix at estrus. In this period, bacteria could enter to the uterus through the opening of the cervix easily. Consequently, it was possible that

the lack of TLR2 in the endometrium may be the related to the cause of canine uterine infection.

Another important role of TLR besides the regulation of immunity is maintaining tissue homeostasis by regulating tissue repair and regeneration. This study found that the physiological changes in the glandular epithelium by apoptosis at diestrus may be related to the higher expression of TLR4 in the glandular epithelium of the uterine horn at this stage from the present study.

Future direction: Clinical application of the TLR drugs

Antibiotic have been proven to be powerful tool in the control of infectious disease. However, the use of even very powerful antibiotic has been accompanied by the emergence of pathogens with multidrug resistance. Accordingly, the development of non-antibiotic agents may contribute to combat against invading pathogens (Kaisho and Akira, 2002). Knowledge about functions of TLRs and their ligands may lead to the development of new therapeutic approaches to treat a wide spectrum of diseases including infectious diseases (Akira, 2009) by interfering in the TLR activation cascade (Werling and Coffey, 2007). In addition, the TLRs may be involved in the pathogenesis of inflammation via the recognition of host products. So, reagents that inhibit the action of TLRs may work as anti-inflammatory drugs (Kaisho and Akira, 2002). Inhibitors of TLR responses or TLR antagonists may be effective for treatment of endotoxin shock as well as inflammatory diseases. Sepsis is a systemic response to infection caused by LPS from Gram-negative bacteria. More recently, TLR4 antagonists including Eritoran (E5564) and TAK-242 have been develop. These compounds may be promising for the treatment of sepsis (Akira, 2009). These new therapeutic drugs may be adjusted to treat the canine uterine infection in the future.

Conclusion

The study of canine uterine immunity and the expression of TLR2 and TLR4 in canine uterus was the beginning in the understanding of canine uterine infection and lead to clarify the one part of the innate immunity in canine uterus. This is the first report of the expression of TLR2 and TLR4 in the uterus of healthy and pyometra dogs. The presence of TLR2 and TLR4 was demonstrated in the surface epithelium, glandular epithelium and stroma of canine uterus throughout the estrous cycle and in pyometra. This indicated the role of canine uterus in immune surveillance on one hand and in fundamental cellular process on the other hand in order to maintain the uterine homeostasis. The different level of TLR2 and TLR4 expression seems to be related to physiological changes in the distinct microenvironment of the uterus, leukocytes populations and sex hormones which may have both direct and indirect effect on the expression of these TLRs. The finding of differential expression of TLR2 and TLR4 from this study indicated the relationship of the TLRs, innate immunity and the development of the uterine bacterial infection. Further investigation should be directed toward understanding the mechanisms and factors that control TLR2 and TLR4 expression in canine uterus in order to treat or prevent the uterine bacterial infection effectively by modulate the TLR system and may leading to the development of the new adjuvants for the clinical application in the future.

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APPENDIX

APPENDIX

List of publication and conferences

1. Chotimanukul, S. and Sirivaidyapong, S. 2011. Differential expression of Toll-like receptor 4 (TLR4) in healthy and infected canine endometrium. Theriogenology. 76: 1152-1161.
2. Chotimanukul, S. and Sirivaidyapong, S. 2011. The difference of bacterial species in vagina and uterus of healthy dogs. Thai J. Vet. Med. Suppl. 41: 168-169.
3. Chotimanukul, S. and Sirivaidyapong, S. 2011. The relationship of canine vaginal and uterine bacterial species in closed-cervix and opened-cervix pyometra. 1^{3th} Association of Institutions for Tropical and Veterinary Medicine (AITVM) Conference, Sofitel Centara Grand Hotel, Bangkok, Thailand, 23-26 August 2010, p184-186.
4. Sirivaidyapong, S and Chotimanukul, S. 2011. The investigation of bacteria in vagina and uterus of bitches with closed-cervix and opened-cervix pyometra. The 9th Chulalongkorn University Veterinary Annual Conference. Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 1 April 2010, p131.

VITAE

Sroisuda Chotimanukul was born on February 19th 1983 in Bangkok, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (DVM) with the 2nd honour from Faculty of Veterinary Science, Chulalongkorn University in 2006. In 2007, she received a scholarship from H.M. King Bhumibol Adulyadej's 72nd Birthday Anniversary Scholarship from Chulalongkorn University to perform a Ph.D. program of Theriogenology at Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her focus research is about canine female reproductive immunology.