# การแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดอัลฟ่า และโปรเจสเตอโรนที่คอมดลูกของสุนัขที่มีปัญหามดลูกอักเสบมีหนอง ชนิดคอมดลูกเปิด และคอมดลูกปิด

**นางสาวปาณิสรา คุณกิตติ**



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# THE EXPRESSIONS OF OESTROGEN RECEPTORS ALPHA AND PROGESTERONE RECEPTORS IN THE CERVIX OF DOGS UNDERGONE OPEN- AND CLOSED-CERVIX PYOMETRA

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ปาณิสรา คุณกิตติ : การแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดอัลฟ่า และโปรเจสเตอโรน ที่คอมดลูก ของสุนัขที่มีปัญหามดลูกอักเสบมีหนอง ชนิดคอมดลูกเปิด และคอมดลูกปิด (THE EXPRESSIONS OF OESTROGEN RECEPTORS ALPHA AND PROGESTERONE RECEPTORS IN THE CERVIX OF DOGS UNDERGONE OPEN- AND CLOSED-CERVIX PYOMETRA) อ. ที่ปรึกษา: รศ.สพ.ญ.ดร.เกวลี ฉัตรดรงค์, อ. ที่ปรึกษาร่วม: ผศ.สพ.ญ.ดร.ศยามณ ศรีสุวัฒนาสกุล, 46 หน้า.

วิทยานิพนธ์นี้มีวัตถุประสงค์เพื่อ ศึกษาการแสดงออกของตัวรับฮอร์โมนเอสโตรเจน อัลฟ่า และตัวรับฮอร์โมนโปรเจสเตอโรน บนเนื้อเยื่อคอมดลูกชั้นต่างๆ ของสุนัขที่มีปัญหามดลูกอักเสบทั้งชนิดคอมดลูกเปิด และคอมดลูกปิด ด้วยวิธีอิมมูโนฮิสโตเคมี เก็บตัวอย่างเนื้อเยื่อคอมดลูกจากสุนัขเพศเมียจำนวน 49 ตัว โดยแบ่งออกเป็นสามกลุ่ม ได้แก่ กลุ่มควบคุม (จำนวน 8 ตัว) เก็บตัวอย่างคอมดลูกจากสุนัขเพศเมียสุขภาพแข็งแรงและอยู่ในระยะแอนเอสตรัส กลุ่มที่มีปัญหามดลูกอักเสบ แบ่งออกเป็นสองกลุ่มคือ มดลูกอักเสบชนิดคอมดลูกเปิด (มีของเหลวไหลออกมาจากช่องคลอด) จำนวน 22 ตัว และมดลูกอักเสบชนิดคอมดลูกปิด จำนวน 19 ตัว เก็บตัวอย่างเนื้อเยื่อคอมดลูกในสารละลาย 4 เปอร์เซ็นต์พาราฟอร์มัลดีไฮน์ นำไปผ่านกรรมวิธีทำสไลด์ และตรวจการแสดงออกของตัวรับฮอร์โมนด้วย ด้วยวิธี avidin-biotin-peroxidase complex (ABC) ผลการศึกษาพบว่า ที่เนื้อเยื่อชั้นต่างๆของคอมดลูกของสุนัขที่มีปัญหามดลูกอักเสบชนิดคอมดลูกเปิดและปิดไม่พบความแตกต่างของการแสดงออกของตัวรับฮอร์โมนเอสโตรเจน อัลฟ่า พบความแตกต่างของการแสดงออกของตัวรับฮอร์โมน เอสโตรเจน อัลฟ่าระหว่างกลุ่มควบคุมและกลุ่มที่เป็นมดลูกอักเสบ ($P<0.01$) ในกลุ่มที่คอมดลูกเปิดมีแนวโน้มของการแสดงออกของตัวรับฮอร์โมนเอสโตรเจน อัลฟ่าสูงกว่ากลุ่มที่คอมดลูกปิด การแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรนในเนื้อเยื่อชั้นต่างๆของคอมดลูกพบว่าส่วนมากไม่พบความแตกต่าง ยกเว้นที่ชั้นเยื่อบุ ที่การแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรนในกลุ่มที่คอมดลูกปิดมีคะแนนการแสดงออกมากกว่ากลุ่มที่คอมดลูกเปิดอย่างมีนัยสำคัญ จากผลการศึกษาครั้งนี้ไม่พบว่าตัวรับฮอร์โมนเอสโตรเจน และตัวรับฮอร์โมนโปรเจสเตอโรนมีความสัมพันธ์ต่อความแตกต่างของลักษณะคอมดลูกในสุนัขที่มีปัญหามดลูกอักเสบ ถึงแม้ว่าฮอร์โมนจะไม่ได้มีผลโดยตรง แต่อาจมีปัจจัยอื่นร่วมกับฮอร์โมนที่อาจส่งผลต่อความแตกต่างของลักษณะของคอมดลูกสุนัขในรายที่มีปัญหามดลูกอักเสบ

ภาควิชาสูติศาสตร์ เธนุเวชวิทยา และวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต.....ปาณิสรา คุณกิตติ.....

สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ลายมือชื่ออาจารย์ที่ปรึกษา.....

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PANISARA KUNKITTI: THE EXPRESSIONS OF OESTROGEN RECEPTORS ALPHA AND PROGESTERONE RECEPTORS IN THE CERVIX OF DOGS UNDERGONE OPEN- AND CLOSED-CERVIX PYOMETRA. THESIS ADVISOR: ASSOC. PROF. KAYWALEE CHATDARONG, Ph.D., THESIS COADVISOR: ASST. PROF. SAYAMON SRISUWATANASAGUL, Ph.D., 46 pp.

The aims of the study were to investigate the immunolocalizations of oestrogen receptors alpha (ERα) and progesterone receptors (PR) in different cervical tissue compartment of dog undergone open and closed-cervix pyometra, to study histopathology of the cervix in open and closed-cervix pyometra and to evaluate the correlation between serum ovarian steroid hormones levels and the expression of ERα and PR. Cervical tissues samples were collected from 49 bitches, cervical tissues at anoestrus stage were represented as control group (n=8), pyometra was divided into two groups; open-cervix pyometra (characterized by the presence of a vaginal discharge) (n=22) and closed-cervix pyometra (n=19). The samples were fixed in 4% paraformaldehyde, embedded in paraffin and immunohistochemical stained for ERα and PR by avidin-biotin-peroxidase complex (ABC) method. The total ERα scores in the cervical tissues in all compartments in open-cervix pyometra and closed-cervix pyometra groups were not significantly difference. The significant difference of total ERα score was found between pyometra and the control groups. The ERα total scores in open-cervix pyometra group tended to be higher than in closed-cervix pyometra group but they were not significantly difference. The total PR scores in the cervical tissues in most compartments were not shown significantly different between groups, except in the surface epithelium of the cervix that total PR score in closed-cervix pyometra was significantly higher than open-cervix pyometra. From this present study, oestrogen and progesterone receptors may not involve in the difference of cervical characteristic in canine pyometra, however the other factors in cooperation with the steroid hormone may involve in the difference of cervical characteristic in canine pyometra.

Department of Obstetrics Gynaecology and Reproduction Student's signature...ปานิสรา กุลกิจกิตติ์.....

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# CHAPTER I

# INTRODUCTION

Canine pyometra is a common uterine disease that usually occurs during metoestrus in middle to old age intact bitches (Borresen, 1975; De Bosschere et al., 2003). Bitches suffering from pyometra show a variety of symptoms associated with genital and extra-genital lesions. The etiology of the disease remains unclear. It is believed that an imbalance or abnormal responses to normal hormone levels affects the changes of structures and functions of epithelial cells at the endometrium and facilitates adherence, colonization and growth of bacteria (Hagman, 2004). The clinical signs of pyometra depend on stages of the disease but common signs are vaginal discharge secretion (in the cases that the cervix is open), dehydration, polydipsia, polyuria, lethargy, abdominal pain, anorexia and vomiting (Allen, 1992; Nelson and Feldman, 1986). Pathogenesis of the pyometra has been purposed by Dow (1959) suggesting that cystic-endometrial hyperplasia (CEH) is the initial phase of the pyometra before developing to become a cystic-endometrial hyperplasia pyometra complex (CEH-P). The pyometra can be classified into four groups based on histopathological examinations of the uteri: I) uncomplicated CEH, II) CEH with plasma cell infiltration, III) CEH with acute endometritis and IV) chronic endometritis (Dow, 1959). However, the recent histological and immunohistochemical studies have suggested that CEH and pyometra complex are not necessarily sequent events and it may develop independently (De Bosschere et al., 2001). Based on cervical characteristics, some studies classify pyometra into two groups; open-cervix pyometra and closed-cervix pyometra (Allen, 1992; Gobello et al., 2003). An obviously seen purulent vaginal discharge in the open-cervix pyometra results in an easier diagnosis compared to the closed-cervix pyometra (Allen, 1992; Smith, 2006). Pyometra diagnosis requires physical examination, hematology, radiography and ultrasonography. In the cases of closed-cervix pyometra, fluid is seen in the uterine horns as well as a marked increase in white cell counts in the uterine contents (Allen, 1992). Ovariohysterectomy is the definite

treatment of pyometra because the origin of the disease is removed, therefore, the recurrence is impossible. However, surgical treatment has its limitation, particularly in some cases that anesthesia is a risk or in cases that the owners refuse spaying (Trasch et al., 2003). In closed-cervix pyometra dogs; the use of hormones to increase uterine contraction i.e. prostaglandin F2alpha ($PGF_{2\alpha}$), oxytocin or oestrogen, are risky as it may induce uterine rupture (Kirihara et al., 2005; Smith, 2006). For these reasons, the medical treatment for pyometra has been introduced as an alternative treatment only in open-cervix pyometra. Recent study has reported that the administration of aglepristone (antiprogestin drug) alone can induce the opening of the cervix in the closed-cervix pyometra dogs and the treatment is more effective when combination treatment with clopostenol (Fieni, 2004). Therefore, the use of antiprogestin seems to be a safe alternative treatment, however the mechanism of changing the patency of the cervix is unknown.

The cervix works as a physical barrier of female reproductive tract, to prevent ascending infection from the external environment into the uterus by mucus secretion and constriction mechanism (Allen, 1992; Chatdarong et al., 2002). The anatomical changes of the cervix during the different stages of the oestrous cycle have been studied in many species (Re et al., 1995; Vermeirsch et al., 1999; Vesanen et al., 1992; Wang et al., 2000). In the bitches, the opening of the cervix has been observed during late-proestrus and oestrus when the oestrogen and progesterone ratio in blood circulation was high, whereas the closure of the cervix occurs when serum progesterone concentration is higher than 18.84 ng/ml (60 nmol/L) (Silva et al., 1995) implying that anatomical change of the cervix may be under the influence of sex steroid hormones, i.e. oestrogen and progesterone. While the patency of the cervix has been reported to change according to the stages of the oestrous cycle in the bitches (Silva et al., 1995), the mechanism of the control of cervical patency and the difference between open and closed-cervix pyometra in relation to sex steroid hormones has not been demonstrated in bitches with pyometra.

It is clear that biological responses to oestrogen and progesterone are mediated through their intracellular specific receptors in target cells (Brosen et al., 2004; Lantinga-van Leeuwen et al., 2000). Therefore, the presences of oestrogen receptors (ER) and

progesterone receptors (PR) may indirectly predict the mechanisms of hormone actions. Although earlier studies indicated that ER is detectable as two subtypes, oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ) (Kuiper et al., 1996). ERα is the dominating subtype in the uterus, oviduct, cervix and vagina in different species (Sar and Welsch, 1999; Saunders et al., 1997; Wang et al., 2000). The distribution of ERα varies in different tissues as well as in different cell types (Re et al., 1995; Rodriguez-Pinon et al., 2000; Sar and Welsch, 1999; Saunders et al., 1997; Thilander et al., 1990; Vesanen et al., 1992; Wang et al., 2000). PR is expressed as two distinct isoforms, PR-A and PR-B that are arises from the same gene (Conneely et al., 2003; Kastner et al., 1990). The expression of both isoforms has been reported in the uterus and cervical tissue in dogs (Srisuwatanasagul et al., 2005; Vermeirsch et al., 2000), pigs (Geisert et al., 1994) and human (Mote et al., 1999). In the cervix of dogs, the immunohistochemical expression of the PR has been demonstrated with the distribution in all tissue compartments (surface epithelium, stroma and myometrium of the cervix) (Vermeirsch et al., 1999).

The differences of steroid hormone receptors among different tissue compartments may indicate the different roles of the tissues under the influence of each steroid hormone. Therefore, the study of the expression of ERα and PR in the cervix of dogs undergone open-cervix pyometra and closed-cervix pyometra may lead to a better understanding in the mechanism of cervical patency between open-cervix pyometra and closed-cervix pyometra. Moreover, the findings from this study may also lead to the application of hormone receptor blockers for the purpose of pyometra treatment. Therefore, the present study aims to evaluate the expression of ERα and PR in the cervical tissues of bitches undergone open-cervix pyometra and closed-cervix pyometra.

# Aims of this study

1. To investigate the distribution of ERα and PR in different cervical tissue compartments of dogs undergone closed-cervix pyometra and open-cervix pyometra.

2. To study histopathology of the cervix in closed and open-cervix pyometra dogs.

3. To study correlation between oestrogen and progesterone concentrations in the serum and the expression of ERα and PR in the cervix of dogs undergone closed-cervix pyometra and open-cervix pyometra.

# CHAPTER II

# LITERATURE REVIEW

## 1. Canine pyometra

Canine pyometra is characterized by accumulation of purulent secretion in the uterine lumen of intact bitches (Hagman, 2004). It is a common reproductive disease that results in infertility and systemic illness. The disease usually occurs in the luteal phase of the oestrous cycle. Pyometra may occurs in young to middle-aged bitches but it is more common in older bitches (Blendinger and Bostedt, 1991; Borresen, 1975; Fransson, 2003; Nelson and Feldman, 1986; Noakes et al., 2001b; Sandholm et al., 1975).

### 1.1. Incidence

There are many factors that increase the incidence of pyometra in bitches; ie. age, breed, exogenous hormones and parity, Nulliparous bitches seem to have a higher risk of developing pyometra than primiparous and multiparous bitches (Niskanen and Thrusfield, 1998). In a group of colony-raised Beagles in Japan, pyometra was frequently observed in 8 – 11 years old dogs with the average age of onset around 9.36 $\pm$ 0.38 years old (Fukuda, 2001). In Sweden, the animal insurance data demonstrated 23.24% of all bitches developing pyometra before 10 years old (Egenvall et al., 2000). Bernese mountain dog, Rottweiler, Rough-haired collie, Cavalier King Charles Spaniel and Golden retriever are listed as predisposed breeds (Egenvall et al., 2001). Moreover, Niskanen and Thrusfield (1998) found that the administration of oestrogen is able to increase the risk for pyometra.

### 1.2. Pathogenesis

The etiology of canine pyometra remains unclear (Blendinger and Bostedt, 1991; Noakes et al., 2001a). It is believed that the pathogenesis of pyometra is complicated by numerous factors, including hormonal imbalance, bacterial infection and individual sensitivity to bacterial and inflammatory products (Hagman, 2004). Previous investigations on pyometra demonstrated that oestrogen and progesterone

concentrations are involved in the pathogenesis of pyometra and believed that CEH is the initial phase in the development of pyometra (Dow, 1959; Noakes et al., 2001a). The exact etiology of CEH is not known. The repeated and prolonged response of endometrium to progesterone during long luteal phase leads to hyperplasia and hypersecretion of endometrial glands. Moreover, the progesterone effect can be enhanced by priming the endometrium with oestrogen influence (De Bosschere et al., 2001; De Cock et al., 1997; Smith, 2006). CEH may contains sterile fluid in the uterine lumen which is defined as mucometra or hydrometra depending on the viscosity of the fluid observed. However, if secondary bacterial infection occurs, the accumulation of pus in the uterine lumen will present which consequently developes to pyometra (Fransson, 2003). Therefore, the secondary bacterial infection is accepted as a cause of pyometra and leading to the concept of "CEH – pyometra complex" (CEH-P) (Dow, 1959). The CEH-P is categorized into 4 groups based on the histopathological examination of the uteri: (I) uncomplicated CEH, which was a CEH of the endometrium with no evidence of inflammation, (II) CEH with plasma cell infiltration, (III) Acute endometritis, an acute inflammation along with CEH and (IV) chronic endometritis, which was difficult to recognize under clinical signs. However, based on the histological and immunohistochemical observations, it has been suggested that pyometra and CEH can develop independently (De Bosschere et al., 2002; De Bosschere et al., 2001).

Alternatively, pyometra can be classified into two groups based on the cervical characteristic: open-cervix pyometra and closed-cervix pyometra. A remarkable clinical sign of the open-cervix pyometra is the presence of purulent vaginal discharge which is not observed in the closed-cervix pyometra. It has been suggested that the clinical signs of closed-cervix pyometra is more likely to be severe than open-cervix pyometra (Borresen, 1975; Dow, 1959) because the pus can not be drained out for closed-cervix pyometra, resulting in an enlarged uterus and abdominal distension. Moreover, some bacteria can produce and release toxins which are absorbed into the blood circulation (Allen, 1992), therefore bitches with closed-cervix pyometra often become severely ill very rapidly.

Most of the bitches with pyometra exhibit their clinical signs during the dioestrus stage of the cycle (Borresen, 1975; Noakes et al., 2001b; Smith, 2006). However, in the

case of closed-cervix pyometra, the bitches often present clinical signs at a later stage of the disease. A variety of symptoms such as anorexia, vomiting, polidipsia, polyuria, lethargy, fever or hypothermia, abdominal distension and elevated heart and respiratory rates are observed in the bitches with pyometra (Borresen, 1975; Hagman, 2004). The diagnosis of pyometra is usually made by performing a thorough history and complete physical examinations (Biddle and Macintire, 2000). The suitable techniques used for diagnosis of the pyometra are ultrasonography and radiography (Bigliardi et al., 2004). However, pregnancy under-40-days can be possibly misinterpreted as pyometra on the radiography (Allen, 1992). Canine pyometra can reflect an abnormal or normal hematology and blood chemistry profiles. Leucocytosis with neutrophilia and left shift in the differential white blood cell count is always observed (Borresen, 1975; Dow, 1959; Sandholm et al., 1975). Moreover, in many cases, a pre-renal azotemia with hyperproteinemia and hyperglobulinemia can also be presented (Arnold et al., 2006; de Schepper et al., 1987).

1.3. Treatment

Ovariohysterectomy is a treatment of choice for pyometra dog as it can prevent the recurrence of the disease (Fransson, 2003; Noakes et al., 2001b). However, surgical treatment has its limitation particularly when the pyometra is usually complicated with other systemic illness which in risk of anesthesia or in cases that the owners expect for a future of dog breeding. For these reasons, an alternative treatment such as medical treatments is introduced (Blendinger and Bostedt, 1991; Trasch et al., 2003). The advantages of non-surgical treatment are that the fertility can be maintained and the risks of anesthesia and surgery are avoided (Trasch et al., 2003). There have been several reports of successful medical treatment by using antibiotic in combination with compounds promoting uterine contraction (Kirihara et al., 2005; Trasch et al., 2003) such as prostaglandin (Kirihara et al., 2005; Meyers-Wallen et al., 1986) and antiprogestin (Gobello et al., 2003).

It is commonly known that pyometra is a disease occurs in dioestrus stage (Noakes et al., 2001b; Sandholm et al., 1975) Therefore, the luteolytic drug seems to be useful for treatment of canine pyometra. The pharmacological effects of $PGF_{2\alpha}$ are luteolysis and myometrial contraction, that promote expulsion of uterine contents and

enhance cervical relaxation (Johnston et al., 2001). However, the recurrence of the disease after treatment and the adverse side effects of $PGF_{2\alpha}$ such as hypersalivation, vomiting, diarrhea, panting, defecation and body temperature drop cannot be avoided (Gilbert et al., 1989; Meyers-Wallen et al., 1986; Wheaton and Barbee, 1993). Most authors strongly recommend the use of $PGF_{2\alpha}$ just only in the cases of open-cervix-pyometra because uterine rupture or peritonitis after expulsion of purulent material in closed-cervix pyometra dog may be induced (Johnston et al., 2001; Wingfield, 2000).

In addition to $PGF_{2\alpha}$, an antiprogestin is also reported as a successful drug for the treatment of canine pyometra. Antiprogestins are synthetic steroids which competitively bind to the progesterone receptors. They have been reported to induce myometrial contractility and cervical relaxation (Blendinger et al., 1997; Hoffmann and Schuler, 2000). The anti-progestins, mifepristone (RU 486) and aglepristone (RU 534), have been used for experimental and clinical purposes of pregnancy termination and management of pyometra in the bitches (Gobello, 2006). A combination of prostaglandin and aglepristone has shown the effective results for the treatment of canine pyometra (Breitkopf et al., 1997; Fieni, 2004; Gobello et al., 2003). In the bitches with closed-cervix pyometra were treated with a combination of prostaglandin and aglepistone, 87% of the successful rate has been observed within 90 after treatment (Fieni, 2004). The opening of the cervix was observed after two administrations of aglepristone. With minimal side effects, the antiprogestin seems to be superior to the $PGF_{2\alpha}$ for pyometra treatment (Johnston et al., 2001).

**2. The canine cervix**

The cervix of the bitch located in conjunction between vagina and uterus. The cervical canal lies obliquely dorsoventral direction, with the external os closed to the ventral area of the vaginal fornix and the internal os opened into the body of the uterus. The cervical canal of the bitch is short, comparing to other species (average 1.5 to 2 cm in length). Canine cervix can frequently be abdominally palpated during proestrus and oestrus (Johnston et al., 2001). The canine cervix, which separates the vagina from the uterus, works like a physiological barrier between the uterus and the outside

environment to prevent ascending infection by natural defense mechanism such as mucous and cervical constriction, in contrary, in the cases of closed-cervix pyometra, the closure of the cervix seems increase severity of the disease (Biddle and Macintire, 2000; Noakes et al., 2001b).

2.1 Cervical opening during oestrus cycle

The reports of a macroscopic of cervical softening during oestrus, histological evidence of increased leukocyte invasion, associated with connective tissues change, have been reported in bovine (Breeveld-Dwarkasing et al., 2003a). Several factors that can be involved with the induction of softening in the non pregnant cervix, such as the increased expression of interleukin-8 (IL-8) in ewes and rabbits (el Maradny et al., 1994; Mitchell et al., 2002), or changes of collagen content in cows (Breeveld-Dwarkasing et al., 2003b). Dynamics of cervical opening can be observed during the oestrous cycle in bitches (Silva et al., 1995) and queens (Chatdarong et al., 2002). In the bitches, the cervix has been observed as open on 2.6 ± 2.8 day before LH peak, which is concurrent with the day of the maximal value of oestrogen : progesterone ratio (46.71 ± 36.33 pg/ml (171.1 ± 133.1 pmol/L) and 0.63 ± 0.75 ng/ml (2.0 ± 2.4 nmol/L), respectively). The closure of the cervix occurs 6.7 ± 1.4 days after the LH peak, before the first sign of cytological dioestrus (Silva et al., 1995). These data may indicate that there is a relationship between hormonal profiles and cervical opening. The present of oestrogen and progesterone receptors in the cervix, may indicates that these steroid hormones are involved in the regulation of functional processes in the cervix during oestrous cycle (Silva et al., 1995).

2.2 Cervical softening during pregnancy and parturition

During the pregnancy, the cervix has to remain firm and closed until the fetus is fully developed. However, a change of cervical structure before parturition has been described. Cervical adaptations during pregnancy can be categorized to four phases (Ann Word et al., 2007). The initial phase is called the softening phase, which the cervix begins to be soft in its texture. The second phase is the cervical ripening characterized by a decrease in tissue collagen concentration and an increase in

hydrophilic glycosaminoglycans (Leppert, 1995). The third phase is the cervical dilatation phase characterized by a massive invasion of leukocytes, occuring during cervical dilatation at laboring. The forth phase of cervical remodeling occurs after parturition, rapid recovery of cervical structure involving resolution of inflammation, loss of tissue hydration, and re-formation of the dense connective tissue and structural integrity of the cervix (Word et al., 2007). A significant increasing of leucocytes in cervical tissues during parturition has been reported for several species (Owiny et al., 1995; Winkler et al., 1999; Yoshida and Manabe, 1990). Previous study in women, the degree of cervical dilatation appeared to be correlated with the extent of neutrophilic infiltration, which can produce collagenolytic enzymes such as metalloproteinases (collagenases). The increasing of collagenases enzyme lead to increases of collagen turnover and degradation of newly synthesized collagen, result in decrease collagen content in the cervical tissue (Cawston, 1996; Winkler et al., 1999). Recent study was focus on the relationship between cervical ripening and nitric oxide (NO), which produced by endometrial cells (Garfield et al., 1998). In rat cervix, the increasing of cervical NO was observed during the active stage of parturition (Buhimschi et al., 1996). Moreover NO seems to be a cause of cell death either by necrosis or apoptosis in the cervix. All of these changes are associated with the cervical ripening process (Yu et al., 1995). Regarding prostaglandin (PG), cyclooxygenase-2 (COX-2) is found in the cervix and sharply increases during labor or just before labor, which leads to a local increase of prostaglandin E2 (PGE2) in the cervix. The increase of PGE2 leads to increase in collagen degradation, increase in hyaluronic acid and increase in stimulation of IL-8 release (Marx et al., 2006).

Sex steroid hormones have been demonstrated to be involved in cervical ripening. In rat cervix, reduction of oestrogen levels leads to increase cervical cell apoptosis (Ramos et al., 2002), whereas increasing of oestrogen stimulates eosinophilic invasion (Luque et al., 1998), and relaxin also promotes a widespread reorganization of collagen fibers (Luque et al., 1998). The role of progesterone in the ripening of the cervix and parturition, possibly acting through an increase in NO and PG productions. Failure of the cervix to ripen or prolongation of the pregnancy may be due to an increase or over

expression of progesterone receptors, or a decrease in the NO and PG production (Buhimschi et al., 1996; Marx et al., 2006).

### 3. Steroid hormone receptors

Steroid hormones are lipophilic molecules derived from cholesterol and synthesized in the adrenal cortex (glucocorticoid, mineralocorticoids and adrenal androgens), the testis (testicular androgens, oestrogen), the ovary and placenta (oestrogen and progestagens or progestins). Steroid hormones reach their target cells via the blood circulation and pass through the cell membrane by diffusion, where they are bound to their specific receptors (Beato and Klug, 2000). Steroid hormone receptors are member of the nuclear receptor superfamily. The structure of nuclear receptor proteins is single polypeptide chains that is composed of multiple functional domains (Beato and Klug, 2000), as followings;

- A variable $NH_2$-terminal region (A/B),
- A conserved DNA-binding domain (DBD) or C-region,
- A hinge D-region,
- A conserved E-region that accommodates the ligand-binding domain (LBD)

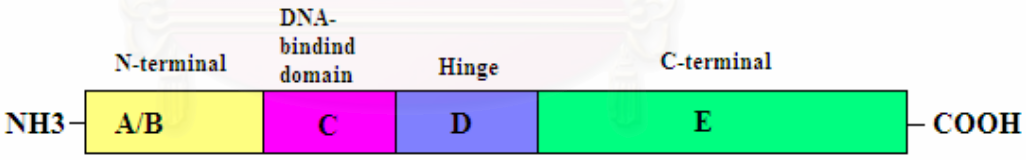




**Fig 1.** Structure of the nuclear receptor proteins. A/B represents the region N-terminal, C contains the DNA binding domain, D contains the Hinge region, E contains the ligand-binding domain (Shao and Brown, 2004).

**4. Determination of steroid receptor**

Several techniques have been developed to identify and quantify steroid receptor proteins in the tissue; immunohistochemistry, in situ hybridization, western blot, enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) (Al-Bader, 2006; Sun et al., 2001). Immunohistochemistry is considered a specific, sensitive and economic method for the determinations of oestrogen receptor and progesterone receptor (Huang et al., 2006). Moreover, Immunohistochemistry is able to investigate the quantification and distribution of these steroid receptors in different tissue compartments or cell types which is not possible for other methods. The principle of this technique is to detect the antigen in tissue section using specific antibodies (Aasmundstad et al., 1992).

The immunohistochemistry used in this present study is an avidin-biotin peroxidase complex (ABC) method. The avidin is a large glycoprotein, that can be labeled with peroxidase or fluorescein and it has a very high affinity for biotin. Biotin is a low molecular weight vitamin, which can be conjugated to a variety of biological molecules such as antibodies. In this present study a mouse monoclonal mouse-anti-human-oestrogen receptor alpha antibody clone 1D5 for ER$\alpha$ and a monoclonal mouse-anti-human-progesterone receptor antibody clone 10A9 for PR were used. The positive color was developed by using the chromagen which was 3,3'-diaminobenzidine, DAB (Boenisch et al., 2001).

**5. Actions of steroid hormones**

Ovarian steroid hormones (oestrogen and progesterone) exert their actions on the target cells through the binding and activation of their specific receptors (Lantinga-van Leeuwen et al., 2000; Tsai and O'Malley, 1994). The steroid hormone passes through the cell membrane by diffusion. Inside the target cells, the steroid hormone binds with a specific receptor in the cytoplasm, and then the hormone-receptor complex translocates into the nucleus and binds to the target gene. The steroid-receptor complex initiates synthesis of specific messenger ribonucleic acid (mRNA) from deoxyribonucleic acids (DNA) in the chromatin. The mRNA molecules then translocation to the cytoplasm where syntheses of new proteins occur. Therefore, the presence of hormone receptors

is as important as the amount of hormone to predict hormone actions (Vermeirsch et al., 2000a; Vermeirsch et al., 2000b). The biological response of the hormone is influenced by the amount of hormones available, the availability of receptor population, the dissociation rate of the hormone-receptor complex with the specific DNA site, and the replenishment of the receptor population (Rodriguez-Pinon et al., 2000).

### 6. Oestrogen receptors (ER)

ER exists in two different isoforms, ERα and ERβ (Kuiper et al., 1996). Although ERα and ERβ are similar in the structure, they differ in C-terminal ligand binding and in N-terminal transactivation domains (Kuiper et al., 1997). Both ER subtypes bind oestradiol-17β ($E_2$) with high affinity and specificity. The expression of both ER subtypes in different reproductive tissues has been studied in rat. The ERα is the dominant subtype in the uterus, oviduct, cervix and vagina, with the distribution varying in stroma and epithelium during the oestrous cycle, while in the ovary, the ERβ is the dominant subtype. ERβ levels in rat ovary are also variable during the oestrous cycle (Wingfield, 2000).

Early studies indicated that the ER were synthesized in the cytoplasm and translocated to the nucleus after binding to the hormone. Several nuclear proteins, including ER have been shown to shuttle continuously between the nucleus and the cytoplasm (Guiochon-Mantel et al., 1994). ER expression in the cervix and uterus have been characterized and quantified in human (Wang et al., 2001), ewe (Rodriguez-Pinon et al., 2000), cow (Breeveld-Dwarkasing et al., 2002; Vesanen et al., 1992), rat and bitch (Vermeirsch et al., 1999). In bitches, the expression of ERα were shown at the different stages of oestrous cycle (Dhaliwal et al., 1997; Vermeirsch et al., 2000b; Vermeirsch et al., 1999), during pregnancy (Vermeirsch et al., 2000b; Vermeirsch et al., 2002) and including in pyometra (De Bosschere et al., 2002; De Cock et al., 1997). During proestrus, the lower ERα score has been found in the stromal cells and smooth muscle cells. In contrast, the highest ERα score has been observed in every cell layers during metoestrus.

Previous studies on ERα in the different cell populations in the uterus of bitches with pyometra found that the uterus with pyometra has lower ERα expression than the

normal uterus (De Bosschere et al., 2003; De Bosschere et al., 2002; Ververidis et al., 2004). The low expression of ERα does not only indicate a decreased synthesis, but also a decreased activation of ERα in pyometra dog (Ververidis et al., 2004).

**7. Progesterone receptor**

Progesterone plays a major role in regulating growth, development and function of female reproductive tissues by stimulating or inhibiting the expression of specific genes (Clarke and Sutherland, 1990). The biological action of this steroid hormone is mediated by binding to the PR, which belongs to the nuclear receptor superfamily of ligand-activated transcription factors. In most mammalian species, two PR isoforms have been described; PR-A and PR-B. Both proteins are arised from the same gene (Conneely et al., 2003). Both forms bind to progesterone and transcriptionally active. The importance of PR was studied in knockout mice; which showed that ablation of PR-A results in severe abnormalities in ovarian and uterine functions leading to female infertility and ablation of PR-B does not affect ovarian, uterine and thymic responses to progesterone but results in reduced mammary ductal morphogenesis and alveologenesis during pregnancy (Conneely et al., 2003). The expression of progesterone receptor in reproductive tissues has been widely study in several species such as humans, rodents, cows, ewes, pigs, cats and dogs (Re et al., 1995; Rodriguez-Pinon et al., 2000; Thilander et al., 1990; Vesanen et al., 1992). In dogs, the expression of PR in the uterine tissues has been reported that PR was observed in different uterine tissue compartments (epithelial cells of the surface epithelium, the glandular ducts and the basal glands, endometrial stroma cells and myometrial smooth muscle cells (Vermeirsch et al., 2000a). The PR expression varies during different stages of the oestrous cycle (Vermeirsch et al., 2000a). The proportional and intensity scores of PR expression are highest during proestrus and decrease through oestrus to early metoestrus. These results are similar to that describe by Dhaliwal et al (1997). Many study in mammals have reported that PR is up-regulated by oestradiol-17β and down-regulated by progesterone (Bouchard, 1999; Johnston et al., 1985; Lessey and Gorell, 1981). The same pattern was found in uterus with pyometra (Ververidis et al., 2004). In canine pyometra, the expression of PR in connective tissue stroma and myometrium of

the uterine tissue were less striking than during anoestrus and there was absent of PR expression in the surface epithelium (De Bosschere et al., 2003).

Most of the studies of PR in cervical tissue have been done in human as many cervical diseases were severely harmful in human. However, the expression of PR in the cervical tissues has been described in rat (Ramos et al., 2000), bovine (Vesanen et al., 1992), ovine (Rodriguez-Pinon et al., 2000), guinea-pig (Rodriguez et al., 2003), equine (Re et al., 1995) and canine (Vermeirsch et al., 2000a). For the better understanding, the study of steroid receptors expression in cervical tissue was still needed in order to clarify the mechanisms of these proteins on their target tissues. In bitches, the expression of PR on the cervical tissue was determined by Vermeirsch et al (2000), the PR total scores were low in early metoestrus, increased in late metoestrus but decreased again to moderate values in anoestrus. They found that the high PR staining score of the cervix in late metoestrus were in contrast with uterine horn and body.

# CHAPTER III

# MATERIALS AND METHODS

## 1. Animals

Cervical tissues and blood sera were obtained from 49 bitches in anoestrus (n=8), bitches undergone open-cervix pyometra (n=22) and bitches undergone closed-cervix pyometra (n=19). All the bitches were subjected to ovariohysterectomize at the Obstetric and Gynaecology Unit, Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The data of age, breed and the history of contraceptive used were recorded. The bitches in anoestrus (control group) were 4 Mongrels, 1 French Bull dog, 1 St. Bernard, 1 Labrador retriever and 1 Poodle, aging between 8 months to 4 years old (average 26.37 $\pm$ 15.11 months). Anoestrus was defined by vaginal cytology in which more than 90% of parabasal cells were seen (Johnston et al., 2001), presence of inactive ovaries (no evidence of mature follicle and active corpus luteum) and serum progesterone levels less than 2 ng/ml (6.36 nmol/L). Pyometra was diagnosed by history taking, clinical signs, hematology and Ultrasonography. The bitches in open-cervix pyometra were 12 Mongrels, 2 Bangkaews, 2 Labrador retrievers, 3 Poodles, 1 St. Bernard, 1 Rottweiler and 1 Chi Hua Hua, aging between 1 to 12 years old (average 73.6 $\pm$ 34.0 months). The open-cervix pyometra was defined by the presence of vaginal discharge. The bitches in closed-cervix pyometra were 13 Mongrels, 2 Poodles, 1 Miniature pinscher, 1 Yorkshire Terrier, 1 Doberman and 1 Shih Tzu, aging between 9 months to 15 years (average 76.7 $\pm$ 39.5 months). The bitches undergone pyometra that classified as closed-cervix pyometra had showed no evidence of vaginal discharge.

## 2. Hormonal analyses

The blood samples were taken from cephalic vein prior to surgery. The sera were separated by centrifugation and stored at -20°C until assayed. The oestradiol-17$\beta$ concentration was determined by luminescence immunoassay (Immulite® Estradiol,

Diagnostic product cooperation, Los Angeles, CA, USA). The intra-assay coefficients of variation for oestradiol-17β were 1.98% at 0 pg/ml (0 pmol/L) and 5.94% at 42.8 pg/ml (157.07 pmol/L). The inter-assay coefficients of variation for oestradiol-17β were 20.11% at 0 pg/ml (0 pmol/L) and 20.10% at 42.5 pg/m (155.97 pmol/L). The progesterone was determined by a luminescence immunoassay (Immulite® Progesterone, Diagnostic product cooperation, Los Angeles, CA, USA) the intra-assay coefficients of variation for the progesterone concentration were 3.88% at 0.13 ng/ml (0.41 nmol/L) and 6.52% at 36.1 ng/ml (114.79 nmol/L). The inter-assay coefficients of variation for progesterone concentration were 3.83% at 0.13 ng/ml (0.41 nmol/L) and 16.33% at 36.1 ng/ml (114.79 nmol/L).

### 3. Tissues collection

Immediately after ovariohysterectomy, the ovaries were examined for their structure and recorded. The cervix was longitudinally cut to open into the lumen and fixed in 4% paraformaldehyde for 36-48 hrs. The cervical tissues were longitudinally embedded in paraffin blocks, cut into 5 μm thick, placed on coated slides (3-aminopropyl-triethoxysilane, minimum 98%) (SIGMA-ALDRICH, Germany) for immunohistochemical evaluation. Each longitudinal section consisted of two parts of the cervix which were cranial part (uterine part) of the cervix and caudal part of the cervix (vaginal part). The uterine part of cervix was defined by simple columnar epithelium. And the vaginal part of the cervix was defined by stratified squamous epithelium.

### 4. Immunohistochemical detection of ERα and PR

After the tissue sections were deparaffinized in xylene and rehydrated with graded alcohol, antigen retrieval was performed to enhance the reaction between antigen and antibody in a microwave at 750 Watt by immersing the slides in 0.01M citric buffer (pH 6) for 10 minutes (5 minutes 2 times). During microwaving, the level of citric buffer was monitored and added if necessary to prevent the sections from drying. Thereafter, the slides were allowed to cool for 20 minutes and rinsed in phosphate buffer saline (PBS). Endogenous peroxidase activity was blocked by incubation the sections for 10 minutes using 3% hydrogen peroxide in methanol at room temperature (RT). The

sections were then rinsed in PBS and incubated in a humidified chamber. To prevent non-specific reactions, the samples were incubated with normal horse serum (NHS) for 30 minutes at RT and further applied with the primary antibody. The immunohistochemical detection of ERα and PR was performed using a mouse monoclonal primary antibody (DAKO, clone 1D5, dilution 1:100) and a monoclonal mouse-anti-human-progesterone receptor antibody (Immunotech, clone 10A9, dilution 1:100), respectively. The incubation time for the primary antibody was 3 hrs at RT. The positive control for both ERα and PR was the tissue section of bitches' endometrium at the oestrus stage. The negative control was obtained by replacing the primary antibody to ERα and PR with PBS. After primary antibody binding, the sections were rinsed in a PBS and incubated with the secondary antibody, a biotinylated horse anti-mouse IgG (Vectastain® ABC kit, Vector Laboratories, Inc., USA) at the dilution of 1:200 for 30 minutes. After rinsing the sections was incubated in a horseradish avidin–biotin peroxidase complex (Vectastain® ABC kit, Vector Laboratories, Inc., USA) for 30 minutes. Then, a chromogen, 3,3'-diaminobenzidine (DAB kit, Vector Laboratories, Inc., USA) was added for 5 minutes to visualize the bounded enzyme (brown colour). All sections were counterstained with Mayer's hematoxylin followed by mounting in glycerine-gelatin before investigation.

**5. Classification of positively stained cells**

The expression levels of ERα and PR positive cells in two part of the uterine part of cervix and vaginal part of cervix were evaluated by the same person, using a light microscope at 400X magnification. Three different tissue compartments were observed: the surface epithelium (SE), connective tissue of the propria-submucosa layer (PS) and smooth muscle cells of the tunica-muscularis layer (M). Each tissue compartments was given an immunohistochemical total score, which consisted of the addition of an intensity score (I) and a proportional score (P) as described by Vermeirsch et al. (1999). The intensity score was defined as the intensity of the brown stained cell nuclei. The proportional score was defined as the percentage of the brown stained cell nuclei in the tissue compartments. The staining intensity (of all cells) was averaged and scored as; I0; absent staining, I1, weak staining, I2, moderate staining and I3,strong staining. The

proportion a scores were classified in to six levels based on the percentage of positive nuclei; no positive nuclei (P0), less than 1% positive nuclei (P1), 1-9 % positive nuclei (P2), 10 – 32% positive nuclei (P3), 32 – 65% positive nuclei (P4) and more than 65% positive nuclei (P5).

**6. Infiltration of inflammatory cells**

Ten sections of the cervical tissue were randomly sampled from each group (control, open-cervix pyometra and closed-cervix pyometra) and were stained with hematoxylin and eosin. The inflammatory cells (lymphocytes, neutrophils, macrophages and plasma cells) were quantified in five microscopic fields (781.25 $\mu m^2$) of each compartment under a light microscope at 400X magnification.

**7. Statistical analysis**

The statistical analyses were performed using the Statistical Analysis Systems software (Version 9, SAS Institute Inc., 2002, Cary, NC). For each bitch and tissue compartments, the mean value of positive score (proportional score, intensity score and total score) of ER$\alpha$ and PR was calculated and used for statistical analysis. One-way ANOVA was performed, for each tissue compartments using general linear model (GLM) procedure. The Spearman rank correlation test was used for determination of correlations between the immunohistochemical scores and the serum hormone concentrations. The p-values $\leq 0.05$ was regarded as statistically significant.

For the results of inflammatory cells, the differences in mean scores of inflammatory cell between groups were analysed with One-way ANOVA, The p-values $\leq 0.05$ was considered as statistically significance.

# CHAPTER IV

# RESULTS

## 1. General observations

In all bitches, the history of contraceptive drugs usage was recorded. In the control group, none of the bitch has been treated with contraceptive drugs. Six bitches (27.27%) in open-cervix pyometra group and ten bitches (52.63%) in closed-cervix pyometra have been treated with contraceptive drugs. The hematological and blood biological profile which were checked before operation showed that most bitches in both open-cervix and closed-cervix pyometra presented leukocytosis with neutrophilia and left shift in the differential of white blood cell count.

## 2. Serum hormone level

The hormonal levels of serum oestradiol-17β and progesterone are show in Fig. 1 as a mean of each experimental group. The higher levels of serum oestradiol-17β were found in the control group than in the open-cervix pyometra groups ($P<0.05$). The levels of serum oestradiol-17β in the control group were 23.48 ± 9.66 pg/ml (86.17 ± 35.45 pmol/L) (range 12.1 to 38.8 pg/ml (44.40 ± 142.39 pmol/L); n=10), whereas in open-cervix pyometra and closed cervix pyometra were 9.0 ± 9.8 pg/ml (33.03 ± 35.96 pmol/L) (range 0 to 35.2 pg/ml (0 to 129.18 pmol/L) ; n=22) and 14.8 ± 15.54 pg/ml (54.31 ± 57.03 pmol/L) (range 0 to 42.5 pg/ml (0 to 155.97 pmol/L) ; n=19) respectively.

Mean ± SD of serum progesterone ($P_4$) levels were 0.56 ± 0.38 ng/ml (2.01 ± 1.39 nmol/L) (range 0.04 to 1.3 ng/ml (0.14 to 4.77 nmol/L); n=8) in control group, 8.2 ± 8.5 ng/ml (30.09 ± 31.19 nmol/L) (range 1.5 to 36.1 ng/ml (5.50 to 132.48 nmol/L) ; n=22) in open-cervix pyometra group and 10.6 ± 8.7 ng/ml (38.90 ± 31.92 nmol/L) (range 0.31 to 26.4 ng/ml (1.13 to 96.88 nmol/L) ; n=19) in closed-cervix pyometra group. In the control group, the lower of serum progesterone level were found when compare with open and closed-cervix pyometra ($P<0.05$).

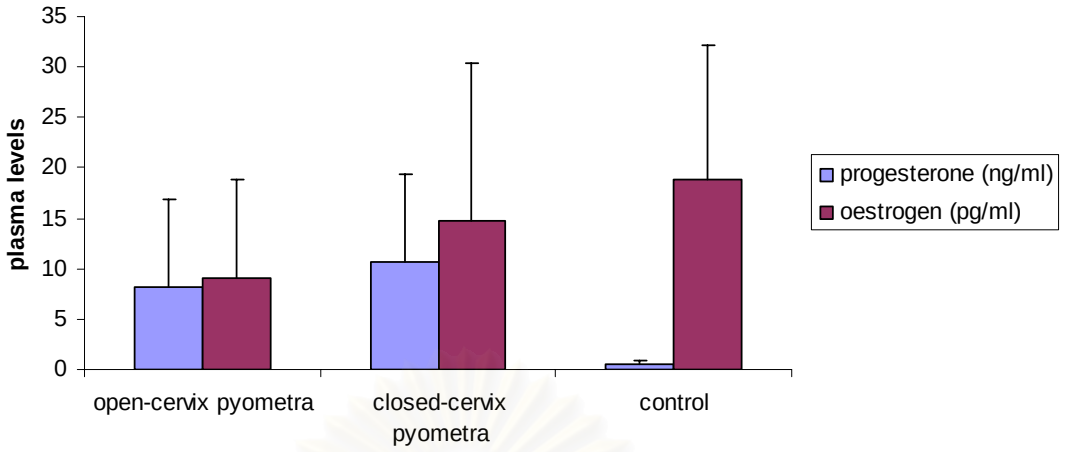


Fig.2 serum levels of oestradiol-17β and progesterone (mean ± SD) in open-cervix pyometra (n=22), closed-cervix pyometra (n=19) and anoestrus bitches (n=8).

### 3. Histopathology

The histopathological studies were separately determined in two parts of the cervix, the vaginal part and the uterine part. The histopathology of the vaginal part of the cervix was not different among the groups, whereas, the major changes were seen in the uterine part of the cervix. In the uterine part of the cervix in both pyometra groups, the cervical gland showed cystic dilatation containing large amounts of neutrophils and secretion. In both vaginal part and uterine part of the cervix, the infiltration of inflammatory cells (neutrophils, plasma cells, macrophage and lymphocytes) were observed in all tissue compartments, especially in epithelial and propria submucosa layers, whereas it is rarely observed in the muscular layer.

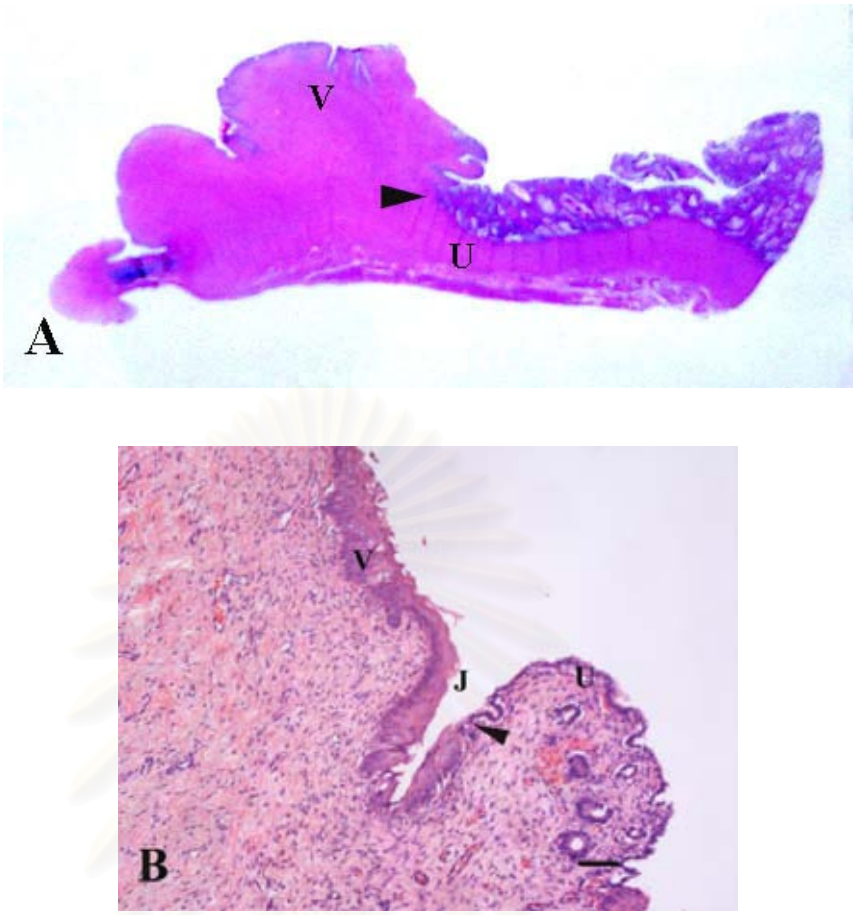


Fig 3. Histopathology of the canine cervix, A, longitudinal section showed the vaginal part of the cervix (V), the uterine part of the cervix (U) and squamocolumnar junction (arrow head). B, the histology of canine cervix showed stratified squamous epithelium of the vaginal part of the cervix (V) and simple columnar epithelium of the cervix (U)and squamocolumnar junction (arrow head), scale bar = 100μm.

**4. Infiltration of inflammatory cells**

The numbers of inflammatory cells (neutrophils, plasma cells, macrophage and lymphocytes) per 781.25 $mm^2$ (five fields of ocular reticule) were analysed under the light microscope at a magnification of 400x. The results showed a significant difference between the control and pyometra groups ($P<0.01$), whereas no significant difference was observed between open-cervix pyometra and closed-cervix pyometra (Fig. 4).

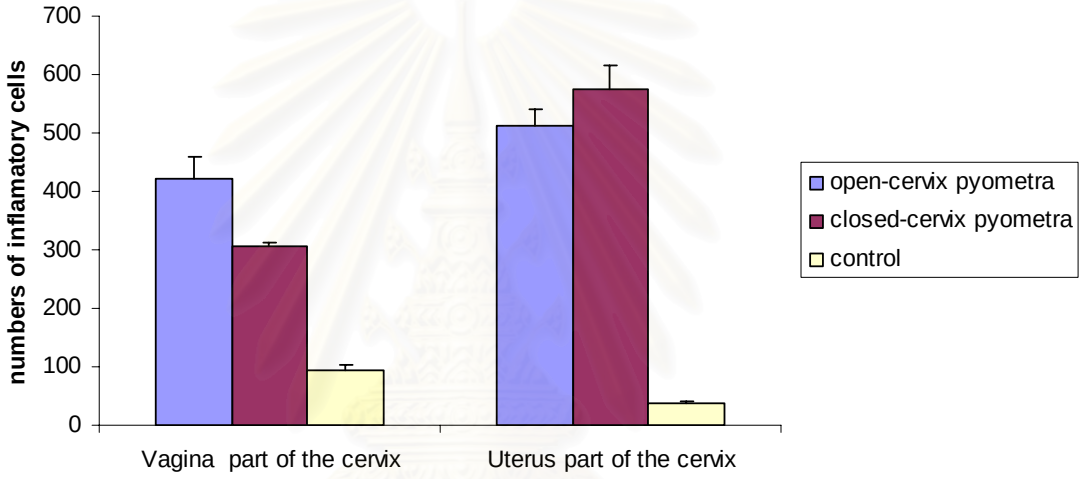


**Fig 4.** Numbers of inflammatory cells (neutrophils, plasma cells, macrophage and lymphocytes) within the cervical tissues in open-cervix pyometra, closed-cervix pyometra and anoestrus (control) groups.

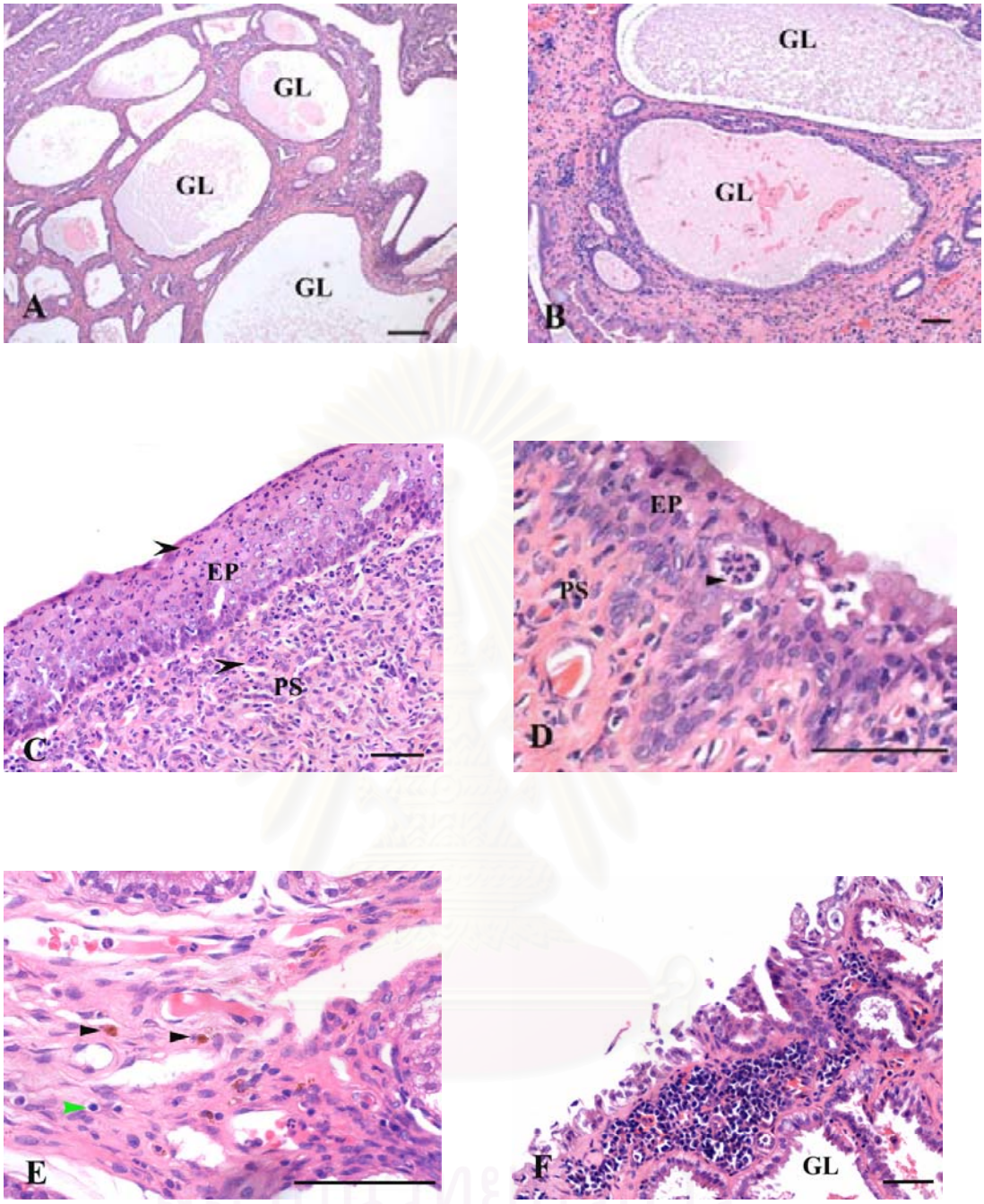


Fig. 5. Histopathology of the cervix in bitches developing pyometra, A, B: cervical gland showed cystic dilatation (GL) contained large amounts of neutrophils and secretion, C: open-cervix pyometra: inflammatory cells infiltrate in vaginal epithelium and propria sumucosa, neutrophil in epithelium and propria submucosa (black arrowhead), D: closed-cervix pyometra: inflammatory cells infiltrate in vaginal epithelium and propria sumucosa, E; Propria submucosa, macrophages contains brownish pigment granules (black arrowheads), lymphocyte (green arrowhead), F; lymphocyte accumulate in propria submucosa, scale bar = 100μm

**5. Oestrogen receptor alpha**

The total score of ERα in both open-cervix pyometra and closed-cervix pyometra were significantly lower than in the control group in all compartments ($P<0.01$), but it was not significantly different between open-cervix pyometra and closed-cervix pyometra. However, the ERα total score in the open-cervix pyometra group tended to be higher than the closed-cervix pyometra group (Table 1). Regarding ERα proportional score, significantly lower score was found for the muscular layer of vaginal part in closed-cervix pyometra compared with open-cervix pyometra ($P<0.05$).

When considered between uterine part and vaginal part of the cervical tissue, the ERα total score was significantly lower in the surface epithelium of uterine part in both groups of pyometra ($P<0.05$). For a comparison of ERα total score among the tissue compartments, the ERα total score in the vaginal part of both open-cervix pyometra and closed-cervix pyometra was not different among different cervical tissue compartment, whereas in the uterine part, lower ERα total score was always found in the surface epithelium compared to the tunica muscularis of both open-cervix pyometra and closed-cervix pyometra ($P<0.05$).

**Table 1**. The total score (intensity and proportional score) of ERα immunohistochemical staining in uterine and vaginal part of cervical tissue present in each group.

| Type | Uterine part of cervix | | | Vaginal part of cervix | | |
|---|---|---|---|---|---|---|
| | SE | PS | M | SE | PS | M |
| Control | 5.12±1.24 [a] | 6.12±1.64 [a] | 6.25±0.70 [a] | 4.50±2.0 [a] | 6.62±1.18 [a] | 6.12±1.24 [a] |
| Open-cervix pyometra | 0.95±1.52 [b] | 2.09±1.65 [b] | 2.54±2.15 [b] | 2.18±1.73 [b] | 3.04±1.29 [b] | 3.13±1.67 [b] |
| Closed-cervix pyometra | 0.57±1.38 [b] | 1.57±1.74 [b] | 2.31±1.73 [b] | 2.00±1.97 [b] | 2.10±1.59 [b] | 2.26±1.72 [b] |
| Overall significant | *P*<0.01 | *P*<0.01 | *P*<0.01 | *P*<0.01 | *P*<0.01 | *P*<0.01 |

SE, surface epithelium

PS, propria submucosa

M, tunica muscularis

NS, not significant.

Mean (±SD) within the same column followed by the different superscrip letters. are significantly different (*P*<0.05)

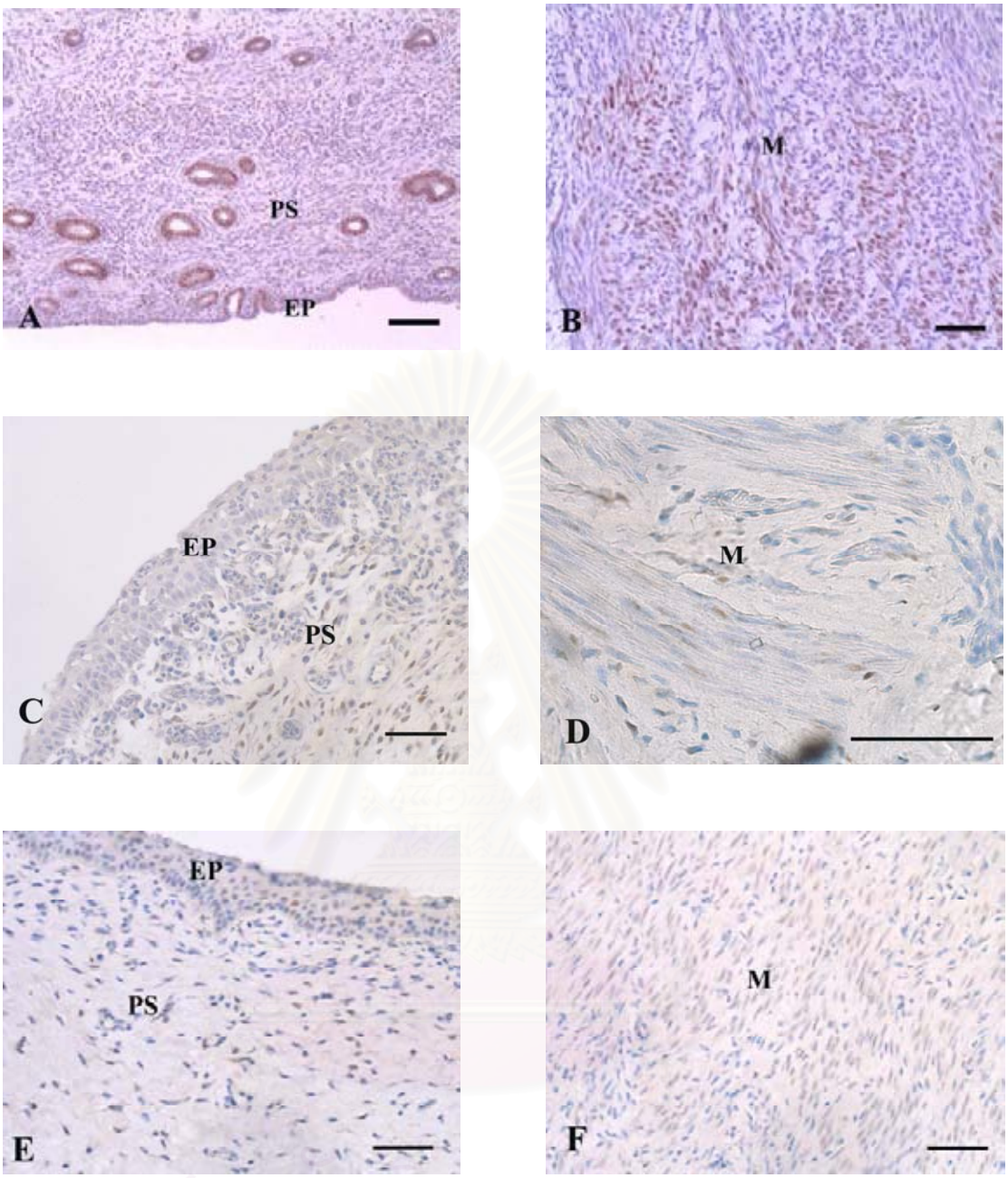


Fig 6. Immunohistochemical staining of ERα in different tissue compartments of the canine cervix; the epithelium layer (EP), propria submucosa layers (PS) and muscular layer (M), The brown nuclei cells represent the positive immunohistochemical staining, control group (A and B), closed-cervix pyometra (C and D), open-cervix pyometra (E and F), scales bar 100 μm

**6. Progesterone receptor**

The total score of PR in the cervical tissues in most compartments did not show significant difference between experimental groups (control, open-cervix and closed cervix pyometra), except in the surface epithelium. In the surface epithelium of the uterine part, the lowest PR score was observed in open-cervix pyometra group ($P<0.05$) and the highest PR score was found in control group ($P<0.05$). In the surface epithelium of the vaginal part, PR score in control group was significantly higher than pyometra groups ($P<0.05$) (Table 2).

Regarding the PR intensity score, in the epithelial layer of the vaginal and uterine part, higher of PR intensity score was observed in the control group compare with both pyometra groups with significant difference between control group and open-cervix pyometra ($P<0.05$). For the proportional score, in the epithelial layer of the vaginal and uterine part, the highest of PR proportional score was found in control group when compared with both pyometra groups.

The comparison of PR total score between uterine and vaginal part showed that the PR total scores of the surface epithelium of the vaginal part were higher than the uterine part in open-cervix groups ($P<0.05$). The difference of PR total score among tissue compartments were also found in both uterine part and vaginal part of cervical tissues that the PR total score of surface epithelium was always significantly lower than in the submucosal and muscular layer ($P<0.05$).

**Table 2**. The total score (intensity and proportional score) of PR immunohistochemical staining in uterine and vaginal part of cervical tissue present in each group.

| Type | Uterine part of cervix | | | Vagina part of cervix | | |
|---|---|---|---|---|---|---|
| | SE | PS | M | SE | PS | M |
| Control | 4.37±2.06[a] | 6.37±1.68 [a] | 6.25±1.03[a] | 5.37±1.50[a] | 5.87±1.45 [a] | 6.50±1.06[a] |
| Open-cervix pyometra | 1.59±1.89[b] | 5.40±1.14[a] | 6.36±1.09[a] | 2.95±1.78[b] | 5.81±1.62[a] | 5.81±1.62[a] |
| Closed-cervix pyometra | 2.63±1.89[c] | 5.26±1.66[a] | 6.00±1.85[a] | 3.31±1.56[b] | 5.57±1.80[a] | 5.31±1.70[a] |
| Overall significant | *P*<0.05 | NS | NS | *P*<0.05 | NS | NS |

SE, surface epithelium

PS, propria submucosa

M, tunica muscularis

NS, not significance.

Mean (±SD) within the same column followed by the different superscript letters are significantly different (*P*<0.05)

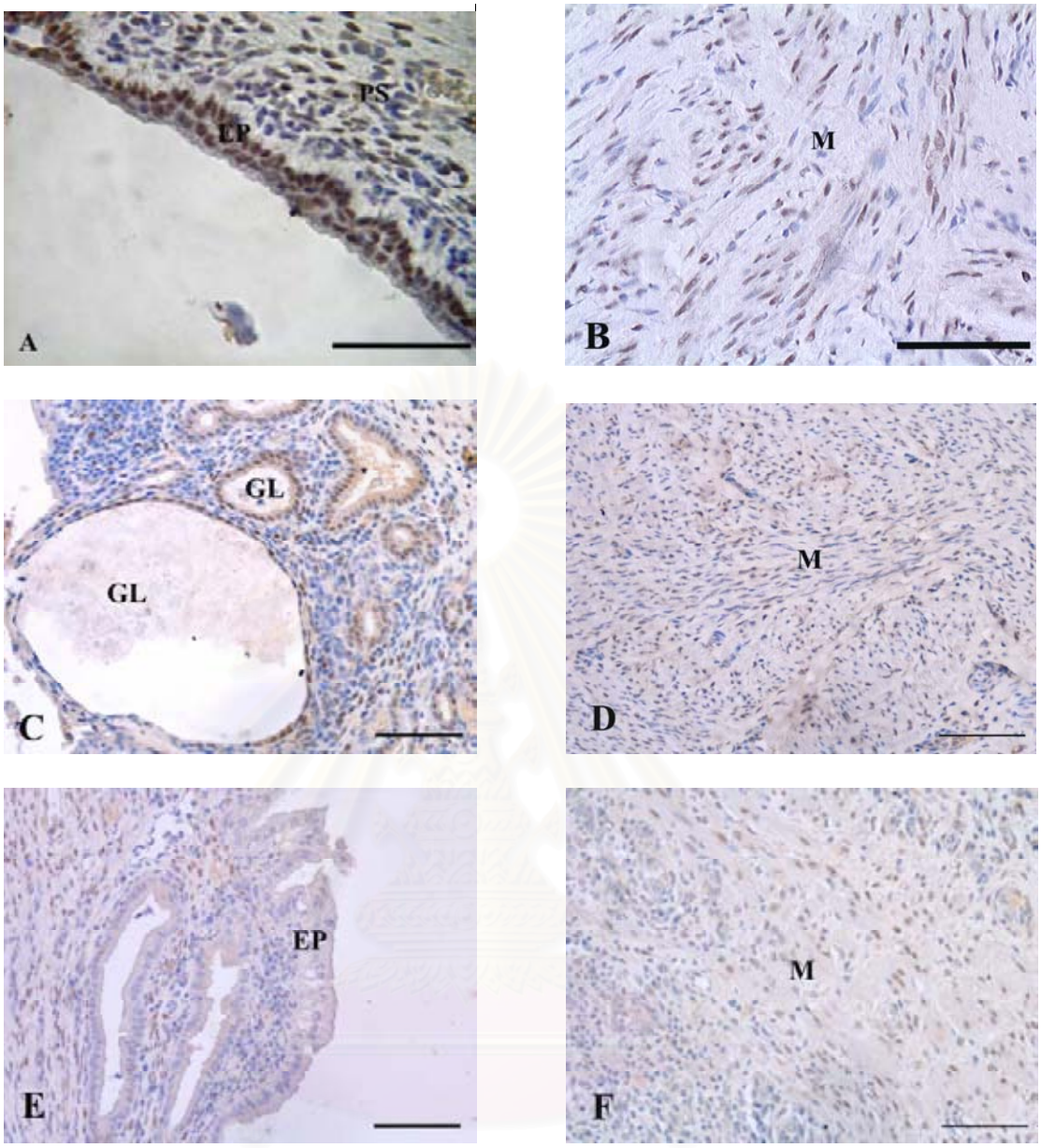


Fig 7. Immunohistochemical staining of PR in different tissue compartments of the canine cervix. The brown nuclei cells represent the positive immunohistochemical staining of PR,: the epithelium layer (EP), propria submucosa layers (PS), cervical Gland (GL) and muscular layer (M), control group (A and B), closed-cervix pyometra (C and D), open-cervix pyometra (E and F), scale bar 100 μm

**7. Relations of serum hormones and expression of hormone receptors**

For closed-cervix pyometra, negative correlations were found in vaginal part between serum progesterone levels and:

- levels of PR immunostaining in muscular layer of the vaginal part ($P < 0.05$).
- levels of ERα immunostaining in propria submucosa of the vaginal part ($P<0.05$).

- For the correlation with the serum levels of oestradiol-17β, negative correlations were observed in closed-cervix pyometra group between the serum level of oestradiol-17β and:

- levels of ERα immunostaining in propria submucosa layer of the uterine part ($P<0.05$).
- levels of PR immunostaining in epithelial layer of the vaginal part ($P < 0.05$).

For control and open-cervix pyometra groups, there were no significant correlation between oestradiol-17β and progesterone levels and the levels of ERα and PR immunostaining in the cervical tissue.

# CHAPTER V

## Discussion

In the present study, the control group has showed the higher serum oestradiol-17β concentrations than the open-cervix pyometra groups ($P<0.05$), while no significant difference between open and closed-cervix pyometra groups was found. The fluctuation of the serum oestradiol-17β level may be a result of the follicular development waves that has been reported in the bitches throughout the anoestrus stage (Feldman and Nelson, 1996). Therefore, the high oestradiol-17β concentrations could be found during anoestrus in the bitch.

The significantly higher serum progesterone levels in the pyometra groups compared to that in the control group in this study reinforced the evidence that most of canine pyometra occur in dioestrous stage, when the blood progesterone levels are high (Nelson and Feldman, 1986; Noakes et al., 2001).

In the present study, the histopathological evaluation was done in two separate parts of the cervix which were uterine parts and vaginal parts. As the study of the dog cervix morphology was rare, therefore longitudinal section of the cervical canal was used in the present study in order to distinguish the difference of epithelial morphology between vaginal and uterine part of the cervix. The histopathology of the uterine part of the cervix in the cases of pyometra were similar to that demonstrated in the previous study, that has been described as severe cystic endometrial hyperplasia and accumulation of large amounts of inflammatory cells (neutrophils, plasma cells, macrophages and lymphocytes) in epithelial and propria submucosa layers (De Bosschere et al., 2001). In the vaginal part, the invasive of inflammatory cells was similar to the uterine part of the cervix. Moreover, the numbers of inflammatory cells in cervical tissues were counted. The results showed a significant difference between the control and the pyometra groups ($P<0.01$) The invasion of the inflammatory cells has been suggested as the cause of collagen degradation and lead to cervical ripening which was prior process of cervical opening (Cawston, 1996; Winkler et al., 1999). However, in

the present study the inflammatory cells may not play a role in cervical dilatation in the cases of canine pyometra since no significant difference in number of inflammatory cells was observed between the open-cervix pyometra and closed-cervix pyometra groups.

In the present study, ERα and PR were observed in all compartments (epithelium layer, propria submucosa layer and tunica muscularis layer) of the cervical tissue of bitches developing open-cervix pyometra and closed-cervix pyometra. This finding is similar to the earlier studies that the positive immunostainings of ERα and PR have been demonstrated in all tissue compartments of the normal canine cervix (Vermeirsch et al., 2000; Vermeirsch et al., 1999), vagina (Vermeirsch et al., 2002) and uterus (Vermeirsch et al., 2000; Vermeirsch et al., 1999).

The significant higher ERα total score was observed in all compartments of the cervical tissue in the control group than in the open and closed-cervix pyometra groups. This finding was in agreement with the previous studies in the canine uterus. The immunohistological staining for ERα in the uterus with pyometra has been demonstrated as being lower than in the normal uterus (De Bosschere et al., 2002; De Cock et al., 1997). Moreover, the similar result was found when ERα in uterus with pyometra was determined by biochemical method (Ververidis et al., 2004). This finding supported the hypothesis that down-regulation effect of P4 on the ERα in uterine tissue of dogs (De Bosschere et al., 2002; Lessey et al., 1981; Vermeirsch et al., 1999) and the low expression of ERα may indicate a decreasing in synthesis or decreasing in the activation of ER in the pyometra bitches (Ververidis et al., 2004). However, the total score of the ERα in the open-cervix pyometra and closed-cervix pyometra did not differ ($P<0.01$, but the ERα proportional score was lower in the muscular layer of the vaginal part in the closed-cervix pyometra than the open-cervix pyometra ($P<0.05$). As ERα may involve with muscular activities and contraction (Langendijk et al., 2002; Mesiano et al., 2002), therefore higher ERα in the muscular layer may indicate higher muscular contraction to release pus from the vagina of open-cervix pyometra.

In most tissue compartments, the total PR scores in the pyometra groups were not significantly different to the control group except that in the epithelial layer of the uterine part and vaginal part of cervix, in which the total PR score in the open and closed-cervix pyometra groups was significantly lower than the control group ($P<0.05$).

When comparing with the results from the earlier studies in pyometra uterus, the similar pattern is observed. The lower PR expression has been shown in epithelial layer of the pyometra uteri than in the normal uteri of the bitches, while the higher PR expression was found in stroma and myometrium of the uterus with pyometra than in normal uterine (De Bosschere et al., 2002; Srisuwatanasagul et al., 2005). This may indicated the loss of down-regulation in propria submucosa and tunica muscularis layers in cases of pyometra (Srisuwatanasagul et al., 2005). When comparing the tissue compartments, different staining score was observed in epithelial layer. The PR total score in the epithelial layer of all studied groups showed significantly lower than the other compartments ($P<0.05$). It could be explained that the epithelium layer may response to the progesterone differently from the propria submucosa and tunica muscularis.

In open-cervix pyometra, there was no correlation between oestradiol-17β and progesterone levels and the levels of ERα and PR immunostaining, whereas in closed-cervix pyometra, the negative correlation between serum progesterone levels and the PR immunostaining was found in the muscular layer of vaginal part ($P<0.05$) and the negative correlations between serum progesterone levels and ERα immunostaining were found in propria submucosa of the vaginal part ($P<0.05$). These correlations are similar to earlier studies in normal bitches that there is a down regulation of ERα and PR under the influence of rising serum progesterone levels (Clarke and Sutherland, 1990; Dhaliwal et al., 1997; Lessey and Gorell, 1981; Vermeirsch et al., 1999; Vesanen et al., 1992). The differences in the correlation of steroid receptor and hormonal level may have a role in the characteristic of the cervix in cased of open and closed cervix pyometra but further study was needed to clarify this hypothesis.

Earlier studies described about the opening of the cervix that it usually occurs during proestrus, oestrus and parturition (Allen, 1992; Word et al., 2007). Steroid hormones, oestrogen and progesterone, have been demonstrated to be involved in cervical opening (Garfield et al., 1998; Silva et al., 1995). During oestrous period the opening of the canine cervix was found and relationship between hormonal profile and cervical opening was reported (Silva et al., 1995). In rat cervix, during parturition, increasing oestrogen leads to increase collagenase activity and cervical cell apoptosis. Whereas, progesterone promote cervical ripening through an increase in nitric oxide and

prostaglandin production (Buhimschi et al., 1996; Luque et al., 1998; Marx et al., 2006; Ramos et al., 2000).

In conclusion, from the present study, the difference between open and closed cervix pyometra was found only in the tunica muscularis of vaginal part of the cervix. Therefore, this finding may suggest that steroid receptors, ER$\alpha$ and PR may not involve in the difference of cervical characteristic in canine pyometra except that in the tunica muscularis of the vaginal part. However, other factors besides steroid receptors may have a role in the difference of cervical characteristic which lead to the difference between closed and open cervix pyometra or that the steroid hormones may involve in the cervical characteristic in other aspects.

# REFERENCES


Aasmundstad, T. A., Haugen, O. A., Johannesen, E., Hoe, A. L., & Kvinnsland, S. 1992. Oestrogen receptor analysis: correlation between enzyme immunoassay and immunohistochemical methods. J Clin Pathol, 45(2): 125-129.

Al-Bader, M. D. 2006. Estrogen receptors alpha and beta in rat placenta: detection by RT-PCR, real time PCR and Western blotting. Reprod Biol Endocrinol, 4: 13.

Allen, W. E. 1992. Anatomy of the Bitch's Reproductive Tract. Fertility and Obstetrics in the Dog, 1-3. London: Blackwell Scientific Publication.

Arnold, S., Hubler, M., & Reichler, I. 2006. Canine Pyometra: New Aproaches to an Old Disease. Paper presented at the World Small Animal Veterinary Congress WSAVA Year:

Beato, M. & Klug, J. 2000. Steroid hormone receptors: an update. Hum Reprod Update, 6(3): 225-236.

Biddle, D. & Macintire, D. K. 2000. Obstetrical emergencies. Clin Tech Small Anim Pract, 15(2): 88-93.

Bigliardi, E., Parmigiani, E., Cavirani, S., Luppi, A., Bonati, L., & Corradi, A. 2004. Ultrasonography and cystic hyperplasia-pyometra complex in the bitch. Reprod Domest Anim, 39(3): 136-140.

Blendinger, K. & Bostedt, H. 1991. [The age and stage of estrus in bitches with pyometra. Statistical inquiry and interpretive study of the understanding of variability]. Tierarztl Prax, 19(3): 307-310.

Blendinger, K., Bostedt, H., & Hoffmann, B. 1997. Hormonal state and effects of the use of an antiprogestin in bitches with pyometra. J Reprod Fertil Suppl, 51: 317-325.

Boenisch, T., Farmild, A. J., Stead, R. H., Welcher, R., Harvey, R., & Atwood, K. N. 2001. Staining Methods. In T. Boenisch (Ed.), Handbook Immunochemical Staining Methods, 3rd ed. Clifornia: Copyright DAKO Corporation.

Borresen, B. 1975. Pyometra in the dog -- a pathophysiological investigation. I. The pyometra syndrome, a review. Nord Vet Med, 27(10): 508-517.

Bouchard, P. 1999. Progesterone and the progesterone receptor. J Reprod Med, 44(2 Suppl): 153-157.

Breeveld-Dwarkasing, V. N., de Boer-Brouwer, M., Mostl, E., Soede, N. M., van der Weijden, G. C., Taverne, M. A., & van Dissel-Emiliani, F. M. 2002. Immunohistochemical distribution of oestrogen and progesterone receptors and tissue concentrations of oestrogens in the cervix of non-pregnant cows. Reprod Fertil Dev, 14(7-8): 487-494.

Breeveld-Dwarkasing, V. N., de Boer-Brouwer, M., te Koppele, J. M., Bank, R. A., van der Weijden, G. C., Taverne, M. A., & van Dissel-Emiliani, F. M. 2003a. Regional differences in water content, collagen content, and collagen degradation in the cervix of nonpregnant cows. Biol Reprod, 69(5): 1600-1607.

Breeveld-Dwarkasing, V. N., te Koppele, J. M., Bank, R. A., van der Weijden, G. C., Taverne, M. A., & van Dissel-Emiliani, F. M. 2003b. Changes in water content, collagen degradation, collagen content, and concentration in repeated biopsies of the cervix of pregnant cows. Biol Reprod, 69(5): 1608-1614.

Breitkopf, M., Hoffmann, B., & Bostedt, H. 1997. Treatment of pyometra (cystic endometrial hyperplasia) in bitches with an antiprogestin. J Reprod Fertil Suppl, 51: 327-331.

Brosen, J. J., Tullet, J., Varshochi, R., & Lam, E. W. F. 2004. Steroid receptor action. Best Practice & Research Clinical Obstetrics and Gynecology, 256-283. London: Elsevier.

Buhimschi, I., Ali, M., Jain, V., Chwalisz, K., & Garfield, R. E. 1996. Differential regulation of nitric oxide in the rat uterus and cervix during pregnancy and labour. Hum Reprod, 11(8): 1755-1766.

Cawston, T. E. 1996. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. Pharmacol Ther, 70(3): 163-182.

Chatdarong, K., Kampa, N., Axner, E., & Linde-Forsberg, C. 2002. Investigation of cervical patency and uterine appearance in domestic cats by fluoroscopy and scintigraphy. Reprod Domest Anim, 37(5): 275-281.

Clarke, C. L. & Sutherland, R. L. 1990. Progestin regulation of cellular proliferation. Endocr Rev, 11(2): 266-301.

Conneely, O. M., Mulac-Jericevic, B., & Lydon, J. P. 2003. Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms. Steroids, 68(10-13): 771-778.

De Bosschere, H., Ducatelle, R., Vermeirsch, H., Van Den Broeck, W., & Coryn, M. 2001. Cystic endometrial hyperplasia-pyometra complex in the bitch: should the two entities be disconnected? Theriogenology, 55(7): 1509-1519.

De Bosschere, H., Ducatelle, R., Vermeirsch, H., Simoens, P., & Coryn, M. 2002. Estrogen-alpha and progesterone receptor expression in cystic endometrial hyperplasia and pyometra in the bitch. Anim Reprod Sci, 70(3-4): 251-259.

De Bosschere, H., Ducatelle, R., & Tshamala, M. 2003. Uterine oestrogen and progesterone receptor expression in experimental pyometra in the bitch. J Comp Pathol, 128(2-3): 99-106.

De Cock, H., Vermeirsch, H., Ducatelle, R., & De Schepper, J. 1997. Immunohistochemical analysis of estrogen receptors in cystic-endometritis-pyometra complex in the bitch. Theriogenology, 48(6): 1035-1047.

de Schepper, J., van der Stock, J., Capiau, E., & de Cock, I. 1987. Renal injury in dogs with pyometra. Tijdschr Diergeneeskd, 112 Suppl 1: 124S-126S.

Dhaliwal, G. K., England, G. C., & Noakes, D. E. 1997. Immunocytochemical localization of oestrogen and progesterone receptors in the uterus of the normal bitch during oestrus and metoestrus. J Reprod Fertil Suppl, 51: 167-176.

Dow, C. 1959. The cystic hyperplasia-pyometra complex in the bitch. J Comp Pathol, 69: 237-250.

Egenvall, A., Bonnett, B. N., Olson, P., & Hedhammar, A. 2000. Gender, age, breed and distribution of morbidity and mortality in insured dogs in Sweden during 1995 and 1996. Vet Rec, 146(18): 519-525.

Egenvall, A., Hagman, R., Bonnett, B. N., Hedhammar, A., Olson, P., & Lagerstedt, A. S. 2001. Breed risk of pyometra in insured dogs in Sweden. J Vet Intern Med, 15(6): 530-538.

el Maradny, E., Kanayama, N., Halim, A., Maehara, K., Sumimoto, K., & Terao, T. 1994. Interleukin-8 induces cervical ripening in rabbits. Am J Obstet Gynecol, 171(1): 77-83.

Feldman, E. C. & Nelson, R. W. 1996. Canine Female Reproduction. CANINE and FELINE ENDOCRINOLOGY and REPRODUCTION, 2nd ed., 525-664. Philadelphia: W.B.SAUNDERS COMPANY.

Fieni, F. 2004. Treatment of Metritis/Pyometras Complex in The Bitch with Antiprogestins Associated or not with Prostaglandins. Paper presented at the 5th International Symposium on Canine and Feline Reproduction Year: 69-71.

Fransson, B. A. 2003. Systemic Inflammatory Response in Canine Pyometra. Department of Small Animal Clinical Sciences Faculty Uppsala.

Geisert, R. D., Pratt, T. N., Bazer, F. W., Mayes, J. S., & Watson, G. H. 1994. Immunocytochemical localization and changes in endometrial progestin receptor protein during the porcine oestrous cycle and early pregnancy. Reprod Fertil Dev, 6(6): 749-760.

Gilbert, R. O., Nothling, J. O., & Oettle, E. E. 1989. A retrospective study of 40 cases of canine pyometra-metritis treated with prostaglandin F-2 alpha and broad-spectrum antibacterial drugs. J Reprod Fertil Suppl, 39: 225-229.

Gobello, C., Castex, G., Klima, L., Rodriguez, R., & Corrada, Y. 2003. A study of two protocols combining aglepristone and cloprostenol to treat open cervix pyometra in the bitch. Theriogenology, 60(5): 901-908.

Gobello, C. 2006. Dopamine agonists, anti-progestins, anti-androgens, long-term-release GnRH agonists and anti-estrogens in canine reproduction: a review. Theriogenology, 66(6-7): 1560-1567.

Hagman, R. 2004. New Aspects of Canine Pyometra Studies on Epidemiology and Pathogenesis. Doctoral thesis. Small Animal Clinical Sciences Faculty Swedish University of Agricultural Sciences.

Hoffmann, B. & Schuler, G. 2000. Receptor blockers - general aspects with respect to their use in domestic animal reproduction. Anim Reprod Sci, 60-61: 295-312.

Huang, Z., Zhu, W., Meng, Y., & Xia, H. 2006. Development of new rabbit monoclonal antibody to progesterone receptor (Clone SP2): no heat pretreatment but effective for paraffin section immunohistochemistry. Appl Immunohistochem Mol Morphol, 14(2): 229-233.

Johnston, S. D., Kiang, D. T., Seguin, B. E., & Hegstad, R. L. 1985. Cytoplasmic estrogen and progesterone receptors in canine endometrium during the estrous cycle. Am J Vet Res, 46(8): 1653-1658.

Johnston, S. D., Root Kustritz, M. V., & Olson, P. N. S. 2001a. Disorders of the Canine Uterus and Uterine Tubes (Oviducts). In R. Kersey (Ed.), Canine anf Feline Theriogenology, 1st ed., 206-224. Philadelphia: Saunders.

Johnston, S. D., Root Kustritz, M. V., & Olson, P. N. S. 2001b. The Canine Estrus Cycle. In R. Kersey (Ed.), Canine anf Feline Theriogenology, 1st ed., 16-31. Philadelphia: Saunders.

Kastner, P., Bocquel, M. T., Turcotte, B., Garnier, J. M., Horwitz, K. B., Chambon, P., & Gronemeyer, H. 1990. Transient expression of human and chicken progesterone receptors does not support alternative translational initiation from a single mRNA as the mechanism generating two receptor isoforms. J Biol Chem, 265(21): 12163-12167.

Kirihara, N., Naganawa, A., Hori, T., Kawakami, E., & Tsutsui, T. 2005. Influence of a PGF2alpha-analogue, etiproston tromethamine, on the functional corpus luteum of dogs. J Vet Med Sci, 67(1): 1-6.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., & Gustafsson, J. A. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A, 93(12): 5925-5930.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., & Gustafsson, J. A. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology, 138(3): 863-870.

Langendijk, P., Bouwman, E. G., Soede, N. M., Taverne, M. A., & Kemp, B. 2002. Myometrial activity around estrus in sows: spontaneous activity and effects of estrogens, cloprostenol, seminal plasma and clenbuterol. Theriogenology, 57(5): 1563-1577.

Lantinga-van Leeuwen, I. S., van Garderen, E., Rutteman, G. R., & Mol, J. A. 2000. Cloning and cellular localization of the canine progesterone receptor: co-

localization with growth hormone in the mammary gland. J Steroid Biochem Mol Biol, 75(4-5): 219-228.

Leppert, P. C. 1995. Anatomy and physiology of cervical ripening. Clin Obstet Gynecol, 38(2): 267-279.

Lessey, B. A. & Gorell, T. A. 1981. Nuclear progesterone receptors in the beagle uterus. J Steroid Biochem, 14(7): 585-591.

Luque, E. H., Munoz de Toro, M. M., Ramos, J. G., Rodriguez, H. A., & Sherwood, O. D. 1998. Role of relaxin and estrogen in the control of eosinophilic invasion and collagen remodeling in rat cervical tissue at term. Biol Reprod, 59(4): 795-800.

Marx, S. G., Wentz, M. J., Mackay, L. B., Schlembach, D., Maul, H., Fittkow, C., Given, R., Vedernikov, Y., Saade, G. R., & Garfield, R. E. 2006. Effects of progesterone on iNOS, COX-2, and collagen expression in the cervix. J Histochem Cytochem, 54(6): 623-639.

Mesiano, S., Chan, E. C., Fitter, J. T., Kwek, K., Yeo, G., & Smith, R. 2002. Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. J Clin Endocrinol Metab, 87(6): 2924-2930.

Meyers-Wallen, V. N., Goldschmidt, M. H., & Flickinger, G. L. 1986. Prostaglandin F2 alpha treatment of canine pyometra. J Am Vet Med Assoc, 189(12): 1557-1561.

Mitchell, S. E., Robinson, J. J., King, M. E., McKelvey, W. A., & Williams, L. M. 2002. Interleukin 8 in the cervix of non-pregnant ewes. Reproduction, 124(3): 409-416.

Mote, P. A., Balleine, R. L., McGowan, E. M., & Clarke, C. L. 1999. Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. J Clin Endocrinol Metab, 84(8): 2963-2971.

Nelson, D. E. & Feldman, E. C. 1986. Pyometra. Veterinary Clinical of North America Small Animal Practice, 561-576. Philadelphia: WB Saunders.

Niskanen, M. & Thrusfield, M. V. 1998. Associations between age, parity, hormonal therapy and breed, and pyometra in Finnish dogs. Vet Rec, 143(18): 493-498.

Noakes, D. E., Dhaliwal, G. K., & England, G. C. 2001a. Cystic endometrial hyperplasia/pyometra in dogs: a review of the causes and pathogenesis. J Reprod Fertil Suppl, 57: 395-406.

Noakes, D. E., Parkinson, T. J., England, G. C. W., & Arthur, G. H. 2001b. Cystic endometrial hyperplasia and pyometra. In G. H. Arthur (Ed.), Arthur's Veterinary Reproduction and Obstetrics, eight ed., 650-655. London: W.B. Saunders.

Owiny, J. R., Gilbert, R. O., Wahl, C. H., & Nathanielsz, P. W. 1995. Leukocytic invasion of the ovine cervix at parturition. J Soc Gynecol Investig, 2(4): 593-596.

Ramos, J. G., Varayoud, J., Kass, L., Rodriguez, H., Munoz de Toro, M., Montes, G. S., & Luque, E. H. 2000. Estrogen and progesterone modulation of eosinophilic infiltration of the rat uterine cervix. Steroids, 65(7): 409-414.

Ramos, J. G., Varayoud, J., Bosquiazzo, V. L., Luque, E. H., & Munoz-de-Toro, M. 2002. Cellular turnover in the rat uterine cervix and its relationship to estrogen and progesterone receptor dynamics. Biol Reprod, 67(3): 735-742.

Re, G., Badino, P., Novelli, A., Di Renzo, G. F., Severino, L., De Liguoro, M., & Ferone, M. R. 1995. Distribution of cytosolic oestrogen and progesterone receptors in the genital tract of the mare. Res Vet Sci, 59(3): 214-218.

Rodriguez, H. A., Kass, L., Varayoud, J., Ramos, J. G., Ortega, H. H., Durando, M., Munoz-De-Toro, M., & Luque, E. H. 2003. Collagen remodelling in the guinea-pig uterine cervix at term is associated with a decrease in progesterone receptor expression. Mol Hum Reprod, 9(12): 807-813.

Rodriguez-Pinon, M., Tasende, C., Meikle, A., & Garofalo, E. G. 2000. Estrogen and progesterone receptors in the ovine cervix during the postpartum period. Theriogenology, 53(3): 743-750.

Sandholm, M., Vasenius, H., & Kivisto, A. K. 1975. Pathogenesis of canine pyometra. J Am Vet Med Assoc, 167(11): 1006-1010.

Sar, M. & Welsch, F. 1999. Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. Endocrinology, 140(2): 963-971.

Saunders, P. T., Maguire, S. M., Gaughan, J., & Millar, M. R. 1997. Expression of oestrogen receptor beta (ER beta) in multiple rat tissues visualised by immunohistochemistry. J Endocrinol, 154(3): R13-16.

Silva, L. D., Onclin, K., & Verstegen, J. P. 1995. Cervical opening in relation to progesterone and oestradiol during heat in beagle bitches. J Reprod Fertil, 104(1): 85-90.

Smith, F. O. 2006. Canine pyometra. Theriogenology, 66(3): 610-612.

Srisuwatanasagul, K., Sukjumlong, S., Sirivaidyapong, S., & Pienchop, S. 2005. Immunohistochemical study of Progesterone receptor (PR) expression between pyometra and normal uterus at dioestrus in the bitch. Thai J. Vet. Med, 35(2): 57-64.

Sun, C. F., Wu, T. L., Tsao, K. C., & Wu, J. T. 2001. Development of two ELISA for estrogen and progesterone receptor with sufficient sensitivity for fine needle aspirate and core biopsy. J Clin Lab Anal, 15(3): 138-143.

Thilander, G., Eriksson, H., Edqvist, L. E., & Rodriguez-Martinez, H. 1990. Variations in oestradiol and progesterone receptor levels in the porcine myometrium and cervix during pregnancy and parturition. Zentralbl Veterinarmed A, 37(5): 329-338.

Trasch, K., Wehrend, A., & Bostedt, H. 2003. Follow-up examinations of bitches after conservative treatment of pyometra with the antigestagen aglepristone. J Vet Med A Physiol Pathol Clin Med, 50(7): 375-379.

Tsai, M. J. & O'Malley, B. W. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem, 63: 451-486.

Vermeirsch, H., Simoens, P., Lauwers, H., & Coryn, M. 1999. Immunohistochemical detection of estrogen receptors in the canine uterus and their relation to sex steroid hormone levels. Theriogenology, 51(4): 729-743.

Vermeirsch, H., Simoens, P., Hellemans, A., Coryn, M., & Lauwers, H. 2000a. Immunohistochemical detection of progesterone receptors in the canine uterus and their relation to sex steroid hormone levels. Theriogenology, 53(3): 773-788.

Vermeirsch, H., Simoens, P., & Lauwers, H. 2000b. Immunohistochemical detection of the estrogen receptor-alpha and progesterone receptor in the canine pregnant uterus and placental labyrinth. Anat Rec, 260(1): 42-50.

Vermeirsch, H., Van Den Broeck, W., Coryn, M., & Simoens, P. 2002a. Immunolocalization of sex steroid hormone receptors in the canine uterine tube

and their relation to sex steroid hormone concentrations. Reprod Fertil Dev, 14(3-4): 241-250.

Vermeirsch, H., Van den Broeck, W., Coryn, M., & Simoens, P. 2002b. Immunohistochemical detection of androgen receptors in the canine uterus throughout the estrus cycle. Theriogenology, 57(9): 2203-2216.

Ververidis, H. N., Boscos, C. M., Stefanakis, A., Saratsis, P., Stamou, A. I., & Krambovitis, E. 2004. Serum estradiol-17 beta, progesterone and respective uterine cytosol receptor concentrations in bitches with spontaneous pyometra. Theriogenology, 62(3-4): 614-623.

Vesanen, M., Isomaa, V., Alanko, M., & Vihko, R. 1992. Bovine uterine, cervical and ovarian androgen receptor concentrations. Correlation with estrogen and progesterone receptor concentrations. Acta Vet Scand, 33(4): 379-386.

Wang, H., Eriksson, H., & Sahlin, L. 2000. Estrogen receptors alpha and beta in the female reproductive tract of the rat during the estrous cycle. Biol Reprod, 63(5): 1331-1340.

Wang, H., Stjernholm, Y., Ekman, G., Eriksson, H., & Sahlin, L. 2001. Different regulation of oestrogen receptors alpha and beta in the human cervix at term pregnancy. Mol Hum Reprod, 7(3): 293-300.

Wingfield, W. E. 2000. Reproductive emergencies. Veterinary emergency medicine secrets, 2nd ed., 364-366. Philadelphia: Henley&Belfus INC.

Winkler, M., Oberpichler, A., Tschesche, H., Ruck, P., Fischer, D. C., & Rath, W. 1999. Collagenolysis in the lower uterine segment during parturition at term: correlations with stage of cervical dilatation and duration of labor. Am J Obstet Gynecol, 181(1): 153-158.

Word, R. A., Li, X. H., Hnat, M., & Carrick, K. 2007. Dynamics of cervical remodeling during pregnancy and parturition: mechanisms and current concepts. Semin Reprod Med, 25(1): 69-79.

Yoshida, Y. & Manabe, Y. 1990. Different characteristics of amniotic and cervical collagenous tissue during pregnancy and delivery: a morphologic study. Am J Obstet Gynecol, 162(1): 190-193.

Yu, S. Y., Tozzi, C. A., Babiarz, J., & Leppert, P. C. 1995. Collagen changes in rat cervix in pregnancy--polarized light microscopic and electron microscopic studies. Proc Soc Exp Biol Med, 209(4): 360-368.

# VITAE

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