การแสดงออกของตัวรับฮอร์โมนสเตียรอยด์ บนเนื้อเยื่อเต้านมสุนัขในสถานะต่างๆ ด้วยวิธีอิมมูนโนฮิสโตเคมี

นางสาว สุกัญญา มณีอินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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IMMUNOHISTOCHEMICAL STUDY OF CANINE MAMMARY STEROID RECEPTORS IN VARIOUS MAMMARY CONDITIONS

Miss Sukanya Manee-in

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
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สุกัญญา มณีอินทร์ : การแสดงออกของตัวรับฮอร์โมนสเตียรอยด์ บนเนื้อเยื่อเต้านมสุนัข ในสถานะต่างๆ ด้วยวิธีอิมมูนโนฮิสโตเคมี (IMMUNOHISTOCHEMICAL STUDY OF CANINE MAMMARY STEROID RECEPTORS IN VARIOUS MAMMARY CONDITIONS) อ.ที่ปรึกษา : รศ.น.สพ. ดร. สุดสรร ศิริไวทยพงศ์, อ.ที่ปรึกษาร่วม: รศ.น.สพ. ดร. ชัยณรงค์ โลหชิต, ผศ.สพ.ญ.ดร.ศยามณ ศรีสุวัฒนาสกุล 63หน้า

การศึกษานี้มีวัตถุประสงค์เพื่อ ศึกษาการแสดงออกของตัวรับฮอร์โมน เอสโตรเจน อัลฟา และโปรเจสเตอโรน บนเนื้อเยื่อเต้านมปกติของสุนัข ในระยะต่างๆของวงรอบการเป็นสัด การแสดงออกของตัวรับฮอร์โมนบนเนื้อเยื่อเต้านมปกติ และเนื้องอกเต้านม ในสุนัขตัวเดียวกัน และศึกษาผลของการฝัง จี เอ็น อาร์ เอช อโกนิส (เดสโรรีลิน) ที่มีต่อการแสดงออกของตัวรับฮอร์โมน โดยแบ่งเป็น 3 การทดลอง

การทดลองที่ 1 แบ่งกลุ่มสุนัขเป็น 2 กลุ่มการทดลอง กลุ่มที่ 1 เก็บตัวอย่างเนื้อเยื่อเต้านมปกติจากสุนัขจำนวน 24 ตัว จำแนกตามระยะการเป็นสัด 4 ระยะ ได้แก่ โปรเอสตรัส เอสตรัส ไดเอสตรัส และ แอนเอสตรัส กลุ่มละ 6 ตัว กลุ่มที่ 2 เก็บตัวอย่างเนื้อเยื่อเต้านมปกติจากสุนัขจำนวน 5 ตัว แต่ละตัวเก็บตัวอย่างในแต่ละระยะการเป็นสัด ได้แก่ แอนเอสตรัส โปรเอสตรัส เอสตรัส ไดเอสตรัสช่วงต้น ไดเอสตรัสช่วงกลาง และไดเอสตรัสช่วงท้าย ตรวจการแสดงออกของตัวรับฮอร์โมน เอสโตรเจน อัลฟา และโปรเจสเตอโรน ด้วยวิธี avidin-biotin-peroxidase complex (ABC) และคำนวณคะแนนของตัวรับฮอร์โมน ผลการศึกษาพบว่า คะแนนของตัวรับฮอร์โมนทั้งสองชนิดมีค่าต่ำที่สุดในระยะไดเอสตรัส ในทั้งสองกลุ่มการทดลอง พบคะแนนของตัวรับฮอร์โมนทั้งสองชนิดมีค่าสูงในระยะโปรเอสตรัส และเอสตรัส และพบคะแนนของตัวรับฮอร์โมนโปรเจสเตอโรนสูงในระยะแอนเอสตรัส ของทั้งสองกลุ่มการทดลอง พบความสัมพันธ์เชิงลบระหว่างตัวรับฮอร์โมนเอสโตรเจน อัลฟา รวมถึงตัวรับฮอร์โมนโปรเจสเตอโรน กับระดับฮอร์โมนโปรเจสเตอโรนในซีรั่ม จาก ผลการศึกษาครั้งนี้พบว่า การแสดงออกของตัวรับฮอร์โมนทั้งสองชนิดถูกควบคุมด้วยฮอร์โมนเอสโตรเจน และโปรเจสเตอโรนซึ่งมีระดับเปลี่ยนแปลงตามวงรอบการเป็นสัด

การทดลองที่ 2 ทำการเก็บตัวอย่างเนื้อเยื่อเต้านมปกติ และเนื้องอกเต้านม จากสุนัขตัวเดียวกันที่เป็นเนื้องอกเต้านม จำนวน 12 ตัว ตรวจการแสดงออกของตัวรับฮอร์โมน เอสโตรเจน อัลฟา และโปรเจสเตอโรน ด้วยวิธี avidin-biotin-peroxidase complex (ABC) และคำนวณคะแนนของตัวรับฮอร์โมน ผลการศึกษาพบว่า การแสดงออกของตัวรับฮอร์โมนทั้งสองชนิดในเนื้อเยื่อเต้านมปกติ และเนื้องอกเต้านม ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ พบความสัมพันธ์เชิงบวกระหว่างตัวรับฮอร์โมนเอสโตรเจน อัลฟา และ ตัวรับฮอร์โมนโปรเจสเตอโรน ทั้งในเนื้อเยื่อเต้านมปกติ และในเนื้องอกเต้านม จากผลการศึกษาครั้งนี้พบว่า การควบคุมการแสดงออกของตัวรับฮอร์โมนทั้งสองชนิด ในเนื้อเยื่อเต้านมปกติ และเนื้องอกเต้านม ของสุนัขตัวเดียวกันน่าจะมีกลไกการควบคุมแบบเดียวกัน และสามารถใช้การแสดงออกของตัวรับฮอร์โมนทั้งสองชนิดในการพยากรณ์ผลการตอบสนองต่อการใช้ฮอร์โมนในการรักษาเนื้องอกเต้านมในสุนัข

การทดลองที่ 3 แบ่งสุนัขเป็น 2 กลุ่ม กลุ่มละ 12 ตัว กลุ่มที่ 1 งยาหลอก (placebo) ใต้ผิวหนัง กลุ่มที่ 2 ฝังฮอร์โมน จี เอ็น อาร์ เอช อะโกนิส (เดสโรรีลิน) ใต้ผิวหนัง เก็บตัวอย่างเนื้อเยื่อเต้านมก่อนทำการฝัง และสัปดาห์ที่ 1, 2 และ 12 หลังจากการฝัง ตรวจการแสดงออกของตัวรับฮอร์โมน เอสโตรเจน อัลฟา และโปรเจสเตอโรน ด้วยวิธี avidin-biotin-peroxidase complex (ABC) และคำนวณคะแนนของตัวรับฮอร์โมน ผลการศึกษาพบว่า คะแนนของตัวรับฮอร์โมนทั้งสองชนิดมีค่าสูงสุดที่สัปดาห์ที่ 2 หลังจากการฝัง เมื่อเปรียบเทียบระหว่างกลุ่มพบว่าคะแนนของตัวรับฮอร์โมนเอสโตรเจน อัลฟา ที่สัปดาห์ที่สองหลังจากการฝัง ในกลุ่มที่ 2 สูงกว่ากลุ่มที่ 1 อย่างมีนัยสำคัญทางสถิติ คะแนนของตัวรับฮอร์โมนโปรเจสเตอโรน ก่อนการฝังและสัปดาห์ที่ 1 หลังจากการฝัง ในกลุ่มที่ 1 สูงกว่า กลุ่มที่ 2 อย่างมีนัยสำคัญทางสถิติ จากผลการศึกษาครั้งนี้พบว่า เดสโรรีลิน มีผลต่อการแสดงออกของตัวรับฮอร์โมนทั้งสองชนิด และการแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดอัลฟา และ โปรเจสเตอโรนหลังจากการฝังเดสโรรีลินนั้นมีแบบแผนคล้ายกัน โดยมีการเพิ่มการแสดงออกของตัวรับฮอร์โมนในช่วงแรกหลังจากการฝังในขณะที่มีการกระตุ้นการทำงานของรังไข่ และลดการแสดงออกของตัวรับฮอร์โมนหลังได้รับการกระตุ้นอย่างต่อเนื่องเป็นเวลานาน

ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์
สาขาวิชา วิทยาการสืบพันธุ์สัตว์
ปีการศึกษา 2550

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4675952531 : MAJOR THERIOGENOLOGY

KEY WORD: ESTROGEN RECEPTOR ALPHA (ERα) / PROGESTERONE RECEPTOR (PR) / IMMUNOHISTOCHEMISTRY / MAMMARY / CANINE

SUKANYA MANEE-IN: IMMUNOHISTOCHEMICAL STUDY OF CANINE MAMMARY STEROID RECEPTORS IN VARIOUS MAMMARY CONDITIONS. THESIS ADVISOR: ASSOC. PROF. SUDSON SIRIVAIDYAPONG, PhD, THESIS COADVISOR: ASSOC. PROF. CHAINARONG LOHACHIT, Dr. med. vet., ASST. PROF. SAYAMON SRISUWATANASAGUL, MSc, PhD , 65 pp.

The objective of the studies were to evaluate immunolocalization of ERα and PR in mammary gland of bitch in different stages of the estrus cycle (EXP 1), to evaluate the correlation between immunolocalization of ERα and PR in normal mammary tissue and mammary tumor in the same dogs (EXP 2) and to evaluate the effect of deslorelin on ERα and PR on canine mammary tissues (EXP 3).

EXP. 1. In the cross-sectional study, mammary tissues were collected from 24 different mixed breed bitches at 4 different stages of the estrous cycle which were; proestrus, estrus, diestrus and anestrus. For longitudinal study, mammary tissues were collected from 5 beagle bitches and at 6 different estrous stages for each bitch which were; anestrus, proestrus, estrus, early diestrus, mid diestrus and late diestrus. The expressions of ERα and PR were evaluated by ABC method and ERα and PR scores were calculated. The lowest scores of ERα and PR were found at diestrus in both groups. During estrus and proestrus the ERα and PR scores were significantly high. High score of the PR during anestrus was also observed in both groups. For the correlation with the levels of ovarian steroid hormone, the negative correlation between ERα and PR scores, and serum progesterone levels were found. This finding indicated that the expression of the ERα and PR was under the regulation of the ovarian steroid hormones which changed during different stages of the estrous cycle.

EXP. 2. Twelve tumoral and contralateral normal mammary tissues, both determined by histology, were surgically obtained from 12 dogs. The expressions of ERα and PR were evaluated by avidin-biotin-peroxidase complex (ABC) method and ERα and PR scores were calculated. The expressions of ERα and PR between normal and tumoral mammary tissues were not significantly different which suggested similar regulation of both steroid receptor expressions between normal and tumoral mammary gland within the same bitch. A positive correlation was found between the number of the ERα and the PR in both normal and tumoral mammary tissues which may indicate that either ERα or PR could be used as a prediction of hormonal therapy parameter in dogs.

EXP. 3. Twenty four bitches during anestrus were selected and devided into 2 groups, twelve bitches were implanted with placebo (untreated group) and the rest were implanted with 10 mg deslorelin (treated group). Mammary tissues were collected at 1 week before and 1, 2 and 12 weeks after implantation. The expression of ERα and PR were evaluated by ABC method and ERα and PR scores were calculated. The significantly highest score of ERα and PR were found at 2 week after implantation. When compared between groups, ERα in treated group was significantly higher than in untreated group at 2 weeks after implantation, PR score in untreated group were significantly higher than in treated group at before and 1 week after implantation. This finding demonstrated that deslorelin has the effects on steroid receptor expression in canine mammary tissue. Moreover, similar pattern on the expression of ERα and PR was found after deslorelin implantation, in which ERα and PR were up-regulated in the stimulation stage and down-regulated in the quiescent stage.

Department of Obstetrics Gynaecology and Reproduction

Field of study Theriogenology

Academic year 2007

Student's signature.....

Advisor's signature.....

Co-advisor's signature.....

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LIST OF ABBREVIATION

ABC	avidin-biotin-peroxidase complex
ATP	adenosine triphosphate
cm	centimeter
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
ERα	estrogen receptor alpha
FGF	fibroblast growth factor
GH	growth hormone
GnRH	Gonadotropin releasing hormone
hrs	hours
IGF-I	insulin-like growth factor-I
Ig G	Immunoglobulin G
kg	kilogram
M	Molar
min.	minute
mL	milliliter
mM	millimolar
ng	nanogram
PBS	phosphate buffer saline
PDGF	platelet-derived growth factor
pg	picogram
PR	progesterone receptor
SD	standard deviation
TGF-α	transforming growth factor-alpha
TGF-β	transforming growth factor-beta
µg	microgram
yrs	years
W	Watt

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Beside skin tumors, mammary tumors are the most common neoplasms in dogs (Fanton and Withrow, 1981). It is believed that mammary tumor depended on the endocrine status of the host (Schneider et al., 1969). Early ovariohysterectomy dramatically reduces the risk of mammary tumors in dogs, which was evidenced demonstrating by an increasing risk of the tumor developing to 0.5%, 8% to 26%, depending on whether the ovariohysterectomy is performed before the 1^{st}, 2^{nd} or any estrus thereafter, respectively (Schneider et al., 1969; Yamagami et al., 1996). Thus, estrogen and progesterone production during estrous cycles may play an important role in the pathogenesis of canine mammary tumors.

The ovarian hormones estrogen and progesterone exert their actions on target cells predominantly through the binding and activation of the estrogen receptor (ER) and progesterone receptor (PR) respectively (Brosens et al., 2004). More recently, steroid hormone receptors also have been detected in normal mammary tissue and mammary tumor (Donnay et al., 1995; Geraldes et al., 2000; Graham et al., 1999; Hamilton et al., 1977; MacEwen et al., 1982; Nieto et al., 2000). Analysis of the expressions of ER and PR has become accepted as a useful tool in prognosis and prediction of responses of hormonal therapy response in human breast cancer. In human breast cancer, a high content of ER in a tumor is associated with a good prognosis and allows hormonal treatment after surgical excision (Jensen and Desombre, 1997). However, information about ER and PR status and prognosis is not routinely available in veterinary medicine. However, in dogs, the presence of ER and PR also seems to relate to a good prognosis (Martin de las Mulas et al., 2004; Sartin et al., 1992).

Endocrine therapy has been widely used for treatment of breast cancer in women. Ovarian ablation was first used in the palliation of young women with metastatic

breast cancer in 1896 (Emens and Davidson, 2003). Until present, medical hormone ablation of various types is commonly used to treat human breast cancer. The purpose of hormonal therapy is to prevent further estrogen stimulation of breast cancer cells. This can be achieved by several different strategies, including blocking receptors with specific estrogen receptor modulators, such as tamoxifen a receptor antagonist; suppression of estrogen synthesis by aromatase inhibitors, or by use of Gonadotropin-releasing hormone (GnRH) analogues (Emens and Davidson, 2003; Santen et al., 1990).

GnRH agonists can decrease ovarian estradiol production indirectly by blocking of the hypothalamic-pituitary-ovarian axis. Previous study reported the result of using GnRH-agonist (Goserelin) on hormone-dependent canine mammary tumors that all the animals showed measurable response to treatment after 3 months (Lombardi et al., 1999). Suprelorin is a slow release and long acting form of deslorelin, a synthetic agonist of GnRH. Until the present work, no study has been done on the effect of deslorelin on the number of canine mammary steroid receptors.

The overall objective of the thesis is to study estrogen receptor alpha and progesterone receptor in normal and canine mammary tumor tissues.

1.2 Literature Review

1.2.1 Normal Canine Mammary Gland

Canine mammary glands are composed of 2 chains of mammary gland. Each chain consists of 5 glands including cranial thoracic, caudal thoracic, cranial abdominal, caudal abdominal and inguinal mammary gland respectively. Cranial and caudal superficial epigastric arteries mainly supply to the mammary gland, axillary intercostals arteries and branch of internal thoracic arteries also supply to the first and the second glands. The third, fourth and fifth mammary glands are supplied from the segmental and circumflex iliac branch of deep caudal epigastric arteries and a branch of perineal and perivulvar arteries. With respect to lymphatic drainage, the first, second and third glands are drained to axillary lymph nodes, the fourth and fifth are drained to the superficial inguinal lymph node. The third mammary gland may be drained to both lymph nodes (Hoffer, 1974).

1.2.2 Canine Mammary Gland Tumor

1.2.2.1 Etiology The cause of mammary tumors has not been completely established, but the influence of hormonal, viral, genetic and dietary influences have been studied. A viral component has not been demonstrated in dogs (Mann, 1984). Instead, there does appear to be a genetic influence on mammary tumors. Pure bred dogs are at greater risk than mixed breeds (Perez Alenza et al., 2000). Dietary fat may play a role in mammary carcinogenesis. High-fat diets in women and dogs seem to correlate with increased risk of mammary tumors (Perez Alenza et al., 2000; Sonnenschein et al., 1991). Estrogen and progesterone may play an important role in the pathogenesis of canine mammary tumors indicated by the protective effect of ovariohysterectomy. This hypothesis was reinforced by long-term administration of synthetic progestins can induced mammary gland disease (Concannon et al., 1981). Furthermore, growth hormone (GH) has been postulated as being involved in the pathogenesis of canine mammary tumors. In the dog growth hormone overproduction can be induced by long acting synthetic progestin. Increase levels of growth hormone have been observed in pituitaries of dogs with mammary tumor (El Etreby et al., 1980). Growth hormone results in acromegaly, characterized by overgrowth of soft tissue and bone. Spontaneous syndromes of growth hormone excess have been reported to occur; in elderly female dogs acromegaly and insulin resistance may develop in conjunction with the luteal phase of estrus cycle (Selman et al., 1994). Mammary tumors can produce growth factors that potentially could modulate their own proliferation in an autocrine fashion (i.e., transforming growth factor-alpha (TGF-α) and transforming growth factor-beta (TGF-β)) or with a paracrine mechanism (i.e., epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I)) (Donnay et al., 1996; Mol et al., 1997). The expression of EGF receptor in mammary tissues is related to the action of estrogen and progesterone and to the presences of functional receptors for estrogen (ER) and progesterone (PR) (Stewart et al., 1990).

1.2.2.2 Incidence, Type Canine mammary tumors can be benign or malignant, in which 40% - 50% of these tumors are malignant (Misdorp, 2002).

The classification of canine mammary tumors is mainly based on a histopathological diagnosis. Hampe and Misdorp (1974) have classified types of canine mammary tumors into 6 groups, followed the WHO classification of human breast tumors. These include carcinoma, sarcoma, carcinosarcoma (malignant mixed tumour), benign or apparently benign tumours, unclassified tumour and benign or apparently benign dysplasias. Mammary tumors in canine were classified as complex when both secretory and myoepithelial cells were present and as simple when only one of these types of cell was present (Hampe and Misdorp, 1974).

1.2.2.3 Treatment of Canine Mammary Tumors There are various methods for treatment of mammary tumors aimed at removing tumor mass and preventing recurrence or metastasis of the tumors.

Surgical Treatment

At the present surgical treatment remains the method of choice for canine mammary tumor therapy. The type of surgical treatment can be divided into 5 methods (Fanton and Withrow, 1981) as

1. Lumpectomy, which remove only tumor mass
2. Simple or local mastectomy, which remove tumor mass and the affected gland
3. Regional mastectomy which is based on venous and lymphatic drainage. Lymph node removal has usually been a matter of individual preference
4. Complete unilateral mastectomy
5. Complete bilateral mastectomy

Chemotherapy

The use of adjuvant chemotherapy has become common practice in human breast cancer therapy (Perlow and Holland, 1984). Chemotherapy is offered to patients who have high risk to the metastasis and is recommended in case of ER and PR negative. Antracyclines such as doxorubicin and epirubicin have been considered to be among the most active available agents to treat human breast cancer (Perlow and Holland, 1984).

Chemotherapy is also used in dogs with malignant mammary tumors, combined with surgical treatment (adjuvant chemotherapy) (Hahn et al., 1992). The chemotherapeutic drugs used in canine mammary tumors treatment consist of

doxorubicin, cyclophosphamide, mitoxantrone and 5-fluorouracil (Hahn et al., 1992; Theilen et al., 1987).

Endocrine Therapy

Human breast cancer is a typical hormone-dependent tumor, and various endocrine therapies have been employed for its treatment (Owen, 1979). To block estrogen action, selective ER modulators, such as tamoxifen and raloxifen, bind to the ERα, leading to attenuation of estrogen-responsive genes (Santen et al., 1990). To block estrogen synthesis, ovarian ablation can be achieved surgically or pharmacologically using goserelin or leuprolide in premenopausal women, which decrease ovarian estradiol production indirectly by impinging on the hypothalamic-pituitary-ovarian axis (Emens and Davidson, 2003). In case of patients who respond to the anti-estrogens but disease later progresses on, aromatase inhibitors should be used as a second-line therapy. Aromatase is a key enzyme of estrogen synthesis, which converts androgens to estrogen in peripheral adipose tissues. Antiaromatase agents inhibit aromatase activity, resulting in decreased estrogen production in breast tissues especially in postmenopausal women (Santen et al., 1990).

Endocrine therapy is not widely used in veterinary practice. Tamoxifen has a use as an antiestrogen at some target sites in the dog and may yet prove to be tumor static for mammary tumors. However, its use should be restricted to spayed bitches, and even in these animals the high incidence of estrogenic side effects means that it is unlikely to become as widely used as it is in women (Morris et al., 1993). Lombardi et al., (1990) demonstrated that the GnRH agonist goserelin administered as a subcutaneous implantation every 21 days for 12 months, exerted an inhibitory action on the growth of hormone-dependent canine mammary tumors, as all the animals showed measurable response to treatment after 3 months. An agonist initially stimulates the hormone secretion, but will subsequently cause a down-regulation of GnRH receptors resulting in the inhibition of luteinizing hormone production upon continuous exposure. The GnRH agonist goserelin has been demonstrated to have an effect on hormone dependent mammary tumor due to their ability to suppress estrogen and progesterone. Moreover, goserelin may have direct inhibitory effect on the proliferation of the tumor cells by interfering with the stimulatory action of specific stimulus which was epidermal growth

factor (EGF) and non specific stimulus such as ATP (*in vitro* studies) (Pagnini et al., 2002).

1.2.3 Action of Ovarian Steroid Hormones on Normal Mammary Gland

The mammary glands of most mammalian species are not fully developed and functioned at birth. Between birth and puberty, the growth of this structure is isometric in relation to the rest of the body, but at puberty under the influence of ovarian and pituitary hormones the gland undergoes the first phase of allometric growth. The second phase of allometric growth in the mammary gland occurs during pregnancy (Anderson and Clarke, 2004; Shyamala et al., 2002). In all species, the mammary glands are composed of various cell types, such as epithelium that are embedded in a fat pad, which is targeted for the proliferation of mammary epithelial cells(Anderson and Clarke, 2004). Estrogen and progesterone are major mitogens for the normal mammary epithelium, they also act indirectly via secretion of paracrine factors to stimulate cell cycle progression and, hence, proliferation. Steroid hormones (estrogen and progesterone) are small lipophilic molecules that enter target cells and nuclei primarily by diffusing through the plasma and nuclear membrane. In the nucleus they encounter receptors which bind their cognate ligands with high affinity and specificity(Anderson and Clarke, 2004; Brosens et al., 2004; Weihua et al., 2003).

1.2.3.1 Estrogen Mammary gland is a primary target tissue for estrogen. Estrogen stimulates the ductal growth of the mammary gland during puberty. Estradiol administration to ovariectomized adult mice can induce increasing of DNA synthesis in the mammary epithelial cells and antiestrogen treatment in prepubertal mice that still undergoing ductal growth contributed inhibition of DNA synthesis of the epithelial cells of the terminal end buds (Shyamala, 1997).

1.2.3.2 Progesterone Progesterone is a mammogenic hormone, that has growth promoting activity in mammary gland. The major developmental role of progesterone in the normal mammary gland has been postulated to be the formation of lobulo-alveolar structures during pregnancy in preparation for milk secretion (Graham and Clarke, 1997). Administration of progesterone to ovariectomized mice can increase

DNA synthesis in mammary epithelial cells, which is associated with the end buds and ducts (Shyamala, 1997).

1.2.4 Steroid Hormone Receptors

Steroid hormone receptors are members of the nuclear hormone receptors family. The biological and physiological effect of steroid hormones including estrogen and progesterone are mediated through their binding with intracellular receptors. Steroid receptors consist of 4 regions these are: 1) a variable N-terminal region (A/B region) contains AF-1 transcription activation domain that has the independent activation function; 2) a conserved DNA-binding domain (DBD) or C region which consists of two zinc finger motifs that mediate specific DNA binding; 3) a hinge D region which acts as a bridge between LBD and DBD; 4) C-terminal region (ligand binding domain; LBD or E/F domain) which is important for hormone binding, contains AF-2 that has ligand dependent activation (Anderson and Clarke, 2004; Brosens et al., 2004; Weihua et al., 2003).

1.2.4.1 Mechanism of Receptor Action

Steroid receptors have the specific capability of hormone binding, DNA binding and gene activation. In the absence of specific ligands, steroid receptors are inactive *in vivo*. The addition of hormone to cells or to hormone-deficient animals results in the rapid transformation of the inactive receptor to an active state. Before hormone activation, steroid receptors form large, oligomeric complex. These complexs are unable to bind DNA. This inactive state was proposed to be maintained by association of receptor with other proteins, called heat shock protein (HSP). The site of interaction on receptors with HSP is located in the C-terminal portion of the protein. When binding with the hormonal ligands, the HSP is dissociated from the receptors. The hormone-receptor complexes diffuse through nuclear membrane. Steroid hormones modulate transcription of target genes by their interaction with specific gene sequences. These sequences are known as hormone responsive or regulatory elements (HRE) (Anderson and Clarke, 2004).

1.2.4.2 Subtype of Receptor

Estrogen Receptor Estrogen receptor has two isoforms ERα and ERβ, which are encoded by different genes. They have 97% sequence similarity in DBD but differ in the transactivation domain (AF-1) of N-terminal and only 55% amino acid identity in the C-terminal LBD. Both isoforms can bind estradiol with high affinity. This suggested that the different sets of protein in the transcriptional complex may interact with 2 isoforms and direct them to specific target (Brosens et al., 2004).

The estrogen target tissues were divided into 2 groups, the classical estradiol target tissues including the uterus, mammary gland, placenta, liver, central nervous system (CNS), cardiovascular system, bone, which contain an abundance of ERα. The secondary target tissues are the prostate gland, testis, ovary, pineal gland, thyroid gland, parathyroid gland, adrenal gland, pancreas, gall bladder, skin, urinary tract, lymphoid and erythroid tissues, which have a high expression of ERβ (Weihua et al., 2003). The general mechanism of ERβ is thought to be similar to that of ERα, but in 1998, Keightley reported that two isoforms have opposite transcriptional responses to the same ligand in specific cell contexts. ERβ has weaker ability to activate transcriptional than ERα. ERα appears to be indispensable in mediating the growth-stimulation. Study in female ER-knockout mice found normal prepubertal development, but no pubertal growth (Bocchinfuso and Korach, 1997).

The presence of ERα in both normal canine mammary tissue and mammary tumors was reported (Donnay et al., 1995; Ger Ides et al., 2000; Graham et al., 1999; Hamilton et al., 1977; MacEwen et al., 1982; Nieto et al., 2000), the presence of ERβ was also found in normal and tumoral canine mammary tissues (Martin de las Mulas et al., 2004).

The expression of ERα is regulated by estrogen and progesterone. In uterine tissues of mammals estrogen has an up-regulation effect, whereas progesterone has a down-regulation effect (Vu Hai et al., 1977). In the mammary gland, the down-regulation effect of the estrogen on the expression of ERα was reported in Rhesus monkey (Cheng et al., 2005) but the stimulated effected on the expression of ERα of estrogen-depleted mouse was reported (Shyamala et al., 2002). Progesterone down-regulates the cytoplasmic and nuclear ER protein concentration, decreasing the active estrogen

concentration and antagonizing the action of ER at the molecular level (Clark et al., 1977).

Progesterone Receptor The PR also has 2 isoforms PR-A and PR-B, which are encoded by a single gene as a result of either different mRNAs. PR-B is longer than PR-A, it contains an additional 164 amino acids at N-terminal. Both of them bind progesterone and are transcriptionally active (Brosens et al., 2004). The activity of PR-A and PR-B are different. PR-B tends to be a stronger transactivator than PR-A in many cell types. PR-A has been shown to inhibit the action of ERα. However, there are also genes more efficiently activated by PR-A. The ratio of PR-A to PR-B has been reported to vary between different species. In canine mammary tissue, both PR-A and PR-B were found and the proportion of the two PR isoforms was reported as equal (Lantinga-van Leeuwen et al., 2000).

Synthesis of PR in mammary glands is under estrogen and progesterone regulation. In uterine tissue of the rodent and other species, estrogen increases the concentration of receptors through a mechanism that depends on synthesis of RNA and protein, while progesterone decreases the concentration of its own receptors (Vu Hai et al., 1977). Studies in mice mammary gland showed the level of PR expression is positively regulated by estrogen and down-regulated by progesterone (Shyamala, 1997).

1.2.4.3 Clinical Application of Steroid Receptors in Human Breast Cancer and Canine Mammary Gland Tumors

It is important to discriminate between hormone-dependent and hormone-independent tumors to determine whether endocrine therapies should be employed. Analysis of ER and PR has become accepted and useful tools in prognosis and prediction of hormonal therapy response in human breast cancer (Iwase et al., 2003; Pichon et al., 1980). ERα is highly expressed in premalignant breast lesion. The expression of ER is related to disease-free survival and overall survival. The patient who has ER positive tumor will have longer period of disease-free survival and overall survival. The PR, which is produced as a result of the action of ERα, is also a good predictive biological marker. Adjuvant hormonal therapy should be offered to all patients

with tumors expressing ERα and/or PR, as assessed by immunohistochemistry (Jensen and Desombre, 1997).

The prognostic value of ER and PR status in canine mammary gland tumors is not completely understood. Steroid hormone receptors also have been detected in normal mammary tissue and mammary tumors (Donnay et al., 1995; Geraldes et al., 2000; Graham et al., 1999; Hamilton et al., 1977; MacEwen et al., 1982; Nieto et al., 2000). From these earlier studies, different results of ERα and PR status in normal canine mammary tissues as well as mammary tumors were reported. However, the information about ER and PR status as well as hormonal therapy is not routinely available in veterinary medicine.

1.2.5 Immunohistochemistry for Determination of Steroid Receptor Proteins

Immunohistochemistry has become a useful tool for diagnosis and research of neoplastic and infectious disease in many fields of medical and veterinary science.

The basis of immunohistochemistry is the detection of antigens, which measure receptors in the tissue sections by using specific antibodies binding to antigen in tissue specimens. There are several immunohistochemical staining methods used in diagnostic laboratories. These are direct immunostaining that uses specific antibodies labeled with an enzyme to detect the antigen; indirect immunostaining that uses an enzyme-conjugated anti-immunoglobulin secondary antibody to detect binding of primary antibody. This is more sensitive and specific than the direct method (Haines and Chelack, 1991). In addition to the indirect method, the avidin-biotin complex (ABC) method is the most adaptable of this technique, which have high specificity and sensitivity. The principle of this technique is the detection of antigens in tissue by a specific primary antibody followed by a second antibody to immunoglobulin labeled with biotin, which bind to each primary antibody and amplified the visible signal produced from binding reaction. Then, tissue is exposed to preformed complex of avidin and biotin molecules that are labeled with a peroxidase enzyme. A colored reaction product forms on the slides at sites of antibody-enzyme complex binding. Finally, the color of reaction is developed by chromogen. This technique can be performed on formalin

fixed-paraffin embedded tissues and requires heating to retrieve the epitopes, which are masked by formalin fixative (Haines and Chelack, 1991; Ramos-Vara, 2005).

1.2.6 Deslorelin

Deslorelin is a synthetic GnRH agonist in a biocompatible, slow release subcutaneous implant. The chemical structure of deslorelin is

[(6-D-tryptophan-9-(N-ethyl-L-prolinamide)-10-deglycinamide)]GnRH

Normally, the pulsatile release of GnRH by the hypothalamus causes the production of gonadotropins by the pituitary, which then stimulates the release of steroid sex hormones by the ovary. GnRH analogues bind to the pituitary GnRH receptors more avidly than GnRH itself. Thus, the chronic administration of GnRH analogues results in the down-regulation of pituitary GnRH receptors, effecting a dramatic suppression of gonadotropin secretion and consequent loss of ovarian steroid production (Emens and Davidson, 2003).

Deslorelin was reported for control fertility on both male and female dogs (Trigg et al., 2001; Wright et al., 2001). Average plasma level of deslorelin following subcutaneous implantations to the dogs was more than 1 μg/day for period of more than 1 year (Trigg et al., 2001). Until present, no study has been done on the effect of deslorelin on the number of canine mammary steroid receptors.

The studies of sex hormone manipulation are important for hormone-dependent mammary tumors. For further understanding of the mechanism involved in canine mammary gland tumors, effect of ovarian steroid hormones on canine mammary tissues and effect of deslorelin on normal canine mammary tissue, in relation to ovarian steroid hormone receptors in the bitch, the expressions of the ERα and PR in various mammary tissue conditions were investigated by using immunohistochemical assay.

1.3 Objectives

1. To evaluate immunolocalization of ERα and PR in mammary gland of bitch in different stages of the estrus cycle.
2. To evaluate the correlation between immunolocalization of ERα and PR in normal mammary tissue and mammary tumor in the same dog.
3. To evaluate the effect of a GnRH agonist (deslorelin) administered by implantation, on ERα and PR on canine mammary tissues.

CHAPTER II

THE NUMBER OF ERα AND PR IN THE MAMMARY GLAND OF BITCHES DURING DIFFERENT STAGES OF ESTRUS CYCLE USING IMMUNOHISTOCHEMICAL ASSAY

2.1 Abstract

The objective of this study was to evaluate the expressions of estrogen receptors alpha (ERα) and progesterone receptors (PR) in normal mammary tissues of bitches at different stages of estrous cycle. Tissue samples were collected from 24 different mixed breed bitches at 4 different stages of estrous cycle which were; proestrus, estrus, diestrus and anestrus. For longitudinal study, mammary tissues were collected from 5 beagle bitches at 6 different stages of the estrous cycle for each bitch which were; anestrus, proestrus, estrus, early diestrus, mid diestrus and late diestrus. The expressions of ERα and PR were evaluated by avidin-biotin-peroxidase complex (ABC) method and ERα and PR scores were calculated. The negative correlation between ERα and PR scores and serum progesterone levels were found. The lowest scores of ERα and PR were found at diestrus in both groups. During estrus and proestrus the ERα and PR scores were significantly high. High score of the PR during anestrus was also observed in both groups. This finding indicated that the expression of the ERα and PR was under the regulation of the sex steroid hormones.

2.2 Introduction

The development of the mammary gland is influenced by numerous factors, principally estrogen and progesterone that interplay with the action of various regulatory factors (Bocchinfuso et al., 2000; Graham and Clarke, 1997; Shyamala, 1997). Estrogen and progesterone are essential for normal mammary gland growth and development. Studies in mice demonstrated that estrogen stimulates ductal growth during puberty, whereas progesterone is the major stimulator of mammary epithelial DNA synthesis and lobulo-alveolar development (Anderson and Clarke, 2004; Bocchinfuso and Korach,

1997; Shyamala, 1997). The effects of estrogen and progesterone are mediated via their respective receptors in the target tissues in which estrogen and progesterone play an important role in regulation of the receptors expression (Shyamala et al., 2002; Weihua et al., 2003). Previous studies in many species have reported that steroid receptors expression in different reproductive organs including mammary tissue is likely to be under the influence of steroid hormones, which are up-regulated by estrogen and down-regulated by progesterone (Graham and Clarke, 1997; Sukjumlong et al., 2005; Sukjumlong et al., 2003; Vermeirsch et al., 2000; Vermeirsch et al., 1999). The biochemical detection of the estrogen and progesterone receptor concentrations in bitches during the estrous cycle demonstrated that the estrogen receptor concentrations were high in the luteal phase (Donnay et al., 1995b). This finding differed from the immunohistochemical investigation in mammary glands of mice that reported the lowest concentration of the receptors during the luteal phase (Shyamala et al., 2002). The biochemical method for detection steroids receptor used the homogenized tissues, thus the result obtained from this technique may represent the receptor concentration from various types of specific tissue compartments. The immunohistochemical assay has several advantages with respect to others biochemical method, including 1) the use of routine histological methods for tissue fixation and processing; 2) the precise histological identification of tissue structures with ER and PR (Martin de las Mulas et al., 2002).

For further understanding of the mechanism involved in mammary gland tumors, in relation to steroid hormone receptors in the bitch, the expression of ERα and PR in normal mammary glands; both in the same and in different bitches, during different stages of the estrous cycle were investigated by using immunohistochemical assay.

2.3 Materials and methods

2.3.1. Animals

The experiments were divided into two studies; cross-sectional and longitudinal studies. For cross-sectional study, twenty four healthy bitches of mixed breeding, aged between 1-5 years, free from mammary lesion, which referred to obstetrics and

gynaecology unit, Chulalongkorn University small animal teaching hospital for routine ovariohysterectomy (OVH). The bitches were divided into 4 groups which were categorized by stages of the estrous cycle (proestrus n=6, estrus n=6, diestrus n=6 and anestrus n=6). The stages of estrous cycle were confirmed by cytological examination of vaginal cytology (Feldman and Nelson, 1996), history data and serum progesterone level (less than 0.5 ng/mL for anestrus, 0.5-1 ng/mL for proestrus, more than 1 ng/mL for estrus and 15-60 ng/mL for diestrus) (Feldman and Nelson, 1996) which was collected before ovariohysterectomy. For longitudinal study, five healthy Beagle bitches, free from mammary lesion were used. The bitches were housed separately in indoor cages with outdoor runs, fed a commercial dry canine diet twice daily and given water *ad libitum*. The bitches were divided into 6 groups which were categorized by stages of estrus cycle (anestrus, proestrus, estrus, early diestrus, mid diestrus and late diestrus). The stage of diestrus was categorized into 3 stages in order to investigate the progesterone levels during diestrus.

2.3.2. Collection of samples

For cross-sectional study, twenty four samples of normal mammary tissues were obtained from each bitch during the process of routine ovariohysterectomy (OVH). For longitudinal study, normal mammary tissues were obtained at 6 different stages of estrus cycle for each bitch. In this study group, blood samples collection and vaginal smears were performed every 3 days to detect the stage of estrous cycle. In both study groups, approximately 1x1 cm of mammary samples were collected. Immediately after surgical excision, they were fixed in 4% paraformaldehyde up to 48 hours. Thereafter they were dehydrated, embedded in paraffin and 4 μm thick sections were cut from each block and mounted on Polysine™ slides (Menzel-Glazer, Germany). The sections were kept until the immunohistochemical procedure was performed.

2.3.3. Estradiol-17β and progesterone assays

Serum estradiol-17β concentrations were measured by chemiluminescent immunoassay system, using IMMULITE® Estradiol kit (Diagnostic Products Corporation, Los Angeles, CA, USA). The within-assay coefficients of variation ranged from 1.9 to 5.9 %. Serum progesterone concentrations were measured by using a commercial solid-phase progesterone radioimmunoassay (Coat-A-Count Progesterone kit™, Diagnostic

Products Corporation, Los Angeles, CA, USA). The progesterone standards used were 0, 0.1, 0.5, 2, 10, 20 and 40 ng/ml. The within-assay coefficients of variation ranged from 7 to 9 %.

2.3.4 Immunohistochemistry of estrogen alpha and progesterone receptors

The specimens were deparaffinized in xylene and rehydrated in graded alcohol. After washing with distilled water, they were subjected to high-temperature antigen retrieval by incubation with 0.01 M citrate buffer, pH 6.0 in a microwave at high power (750 W) 15 minutes (5 min. x 3) for PR, and 25 minutes (5 min. x 5) for ER. Additional citrate buffer was added if necessary between each heating in order to prevent slides from drying out. After cooling down at room temperature for 20 minutes, the slides were rinsed with 10 mM, pH 7.4 phosphate-buffered saline (PBS). The following procedures were performed at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes, then tissues were rinsed with PBS. Non – specific staining was eliminated by incubating the sections with normal horse serum for 30 minutes. Excess serum was removed and the sections were incubated with the specific primary mouse monoclonal antibody to ERα (Dako, clone 1D5, dilution 1:50) for 3 hours at 37°c and PR (Immunotech, clone 10A9, dilution 1:100) for 2 hours at room temperature all slides were placed in a humidity chamber. After primary antibody binding, the sections were washed in PBS and incubated with the secondary antibody, a biotinylated horse anti-mouse Ig G (Vectastain® ABC kit, Vector Laboratories, Inc., USA) in a dilution of 1:200 for 30 minutes. After slides were washed in PBS the sections were incubated for 30 minutes with a horseradish peroxidase avidin biotin complex (Vectastain® ABC kit, Vector Laboratories, Inc., USA). After a final wash with PBS, the color was developed with a freshly prepared solution of 3, 3' – diaminobenzidine (DAB kit, Vector Laboratories, Inc., USA). All sections were counterstained with Meyer's hematoxylin, dehydrated and mounted with glycerine gelatin for investigation under a light microscope.

The samples that were treated with non-immune serum, instead of the specific antibody, were run as negative controls. Normal canine uterus at estrous stage known to express ERα and PR was served as positive controls.

2.3.5 Evaluation of immunohistochemical data

The immunostaining for estrogen and progesterone receptors was assessed on the basis of visually estimated percentage of alveolar and tubular epithelial cells with positive nuclear staining (brown nuclei) by counting 1,000 cells in 10 to 20 fields per histological sections with nuclear staining for ERα and PR. The human score system was used. ERα score and PR score were calculated as $P_1 + (2 \times P_2) + (3 \times P_3)$ where P_1, P_2 and P_3 are the estimated percentages of positive nuclei with low (P_1), medium (P_2) and high (P_3) intensity of immunostaining color (Snead et al., 1993).

2.3.6 Statistical analysis

Data were handled and statistically analyzed using the SAS statistical package (version 9, SAS Institute, Inc., 2002, Cary, NC, USA). Normal distribution of residuals from the statistical models was tested using UNIVARIATE procedure option NORMAL. Differences in mean numbers of ERα score and PR score were tested using analysis of variance. General Linear Mixed model was used to compare least-squares means between stages when overall significance for that effect was found. Correlation between ERα and PR scores and serum level of estradiol-17β and progesterone were evaluated using Pearson correlation coefficients. A *P*-value ≤ 0.05 was considered statistically significant.

2.4 Results

2.4.1 Serum hormone levels

In cross-sectional study, the serum estradiol-17β and progesterone levels in all 24 bitches change according to the stages of the estrous cycle, which were demonstrated in Fig. 1A. The estradiol-17β level was high during proestrus and estrus, progesterone level was high during diestrus. The same pattern was also found in 5 bitches in the longitudinal study which was shown in Fig. 1B. In addition, highest progesterone level was found at mid diestrus while it was lower at the stage of late diestrus.

2.4.2 Immunohistochemistry

ERα and PR

The positive immunolocalization of the ERα and PR were found in the nuclei of alveolar and tubular epithelium of the normal canine mammary tissues (Fig. 2). In both cross-sectional and longitudinal studies, ERα and PR immunostaining score (mean ± SD) at different stages of the estrous cycle were shown in table 1 and 2 respectively.

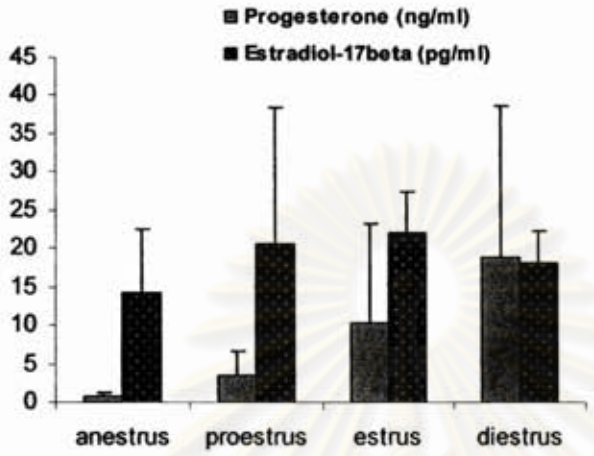

Fig. 1A. The levels of serum estradiol-17β and progesterone in bitches during different stages of estrous cycle (cross-sectional study), mean ± SD (n=24)

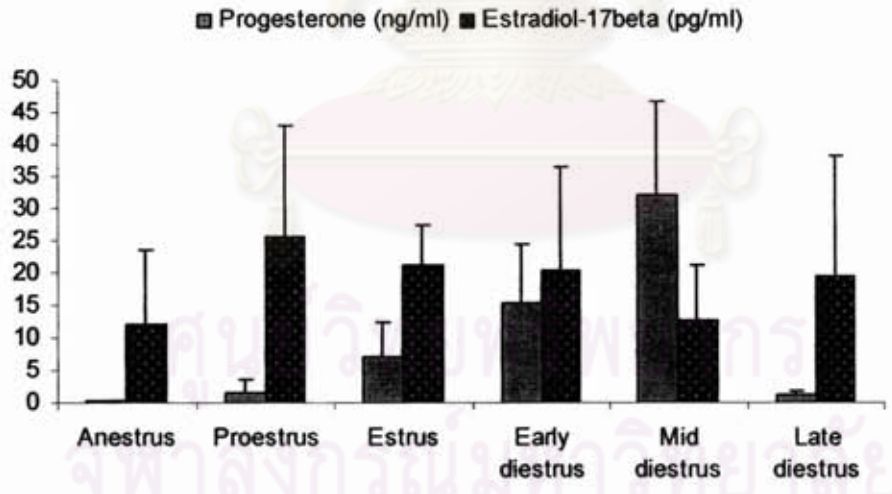

Fig. 1B. The levels of serum estradiol-17β and progesterone in bitches during different stages of estrous cycle (longitudinal study), mean ± SD (n=5)

Table 1. Cross-sectional study (n=24), ERα and PR scores of the canine mammary tissues at different stages of the estrous cycle (mean ± SD)

Stage of estrous cycle	ERα score	PR score
Proestrus	$62.28 \pm 13.75^{a,b}$	102.88 ± 38.78^{a}
Estrus	78.91 ± 15.20^{a}	110.38 ± 27.48^{a}
Diestrus	20.26 ± 16.62^{c}	23.80 ± 13.69^{b}
Anestrus	46.04 ± 12.08^{b}	95.38 ± 39.81^{a}
Overall significance	$P < 0.001$	$P < 0.001$

Mean (± SD) within the same column followed by the different superscript letters are significantly different ($P \leq 0.05$)

Table 2. Longitudinal study (n=5), ERα and PR scores of the canine mammary tissues at different stages of the estrous cycle (mean ± SD)

Stage of estrous cycle	ERα score	PR score
Proestrus	$63.37 \pm 15.06^{a,b,c}$	$103.06 \pm 45.03^{a,b}$
Estrus	$68.95 \pm 21.76^{a,b}$	122.29 ± 28.61^{a}
Early diestrus	72.08 ± 24.32^{a}	$98.51 \pm 17.61^{a,b}$
Mid diestrus	40.14 ± 11.50^{c}	58.18 ± 22.19^{b}
Late diestrus	$52.53 \pm 7.68^{a,b,c}$	66.96 ± 48.66^{b}
Anestrus	$47.78 \pm 12.24^{b,c}$	127.66 ± 18.11^{a}
Overall significance	$P < 0.05$	$P < 0.05$

Mean (± SD) within the same column followed by the different superscript letters are significantly different ($P \leq 0.05$)

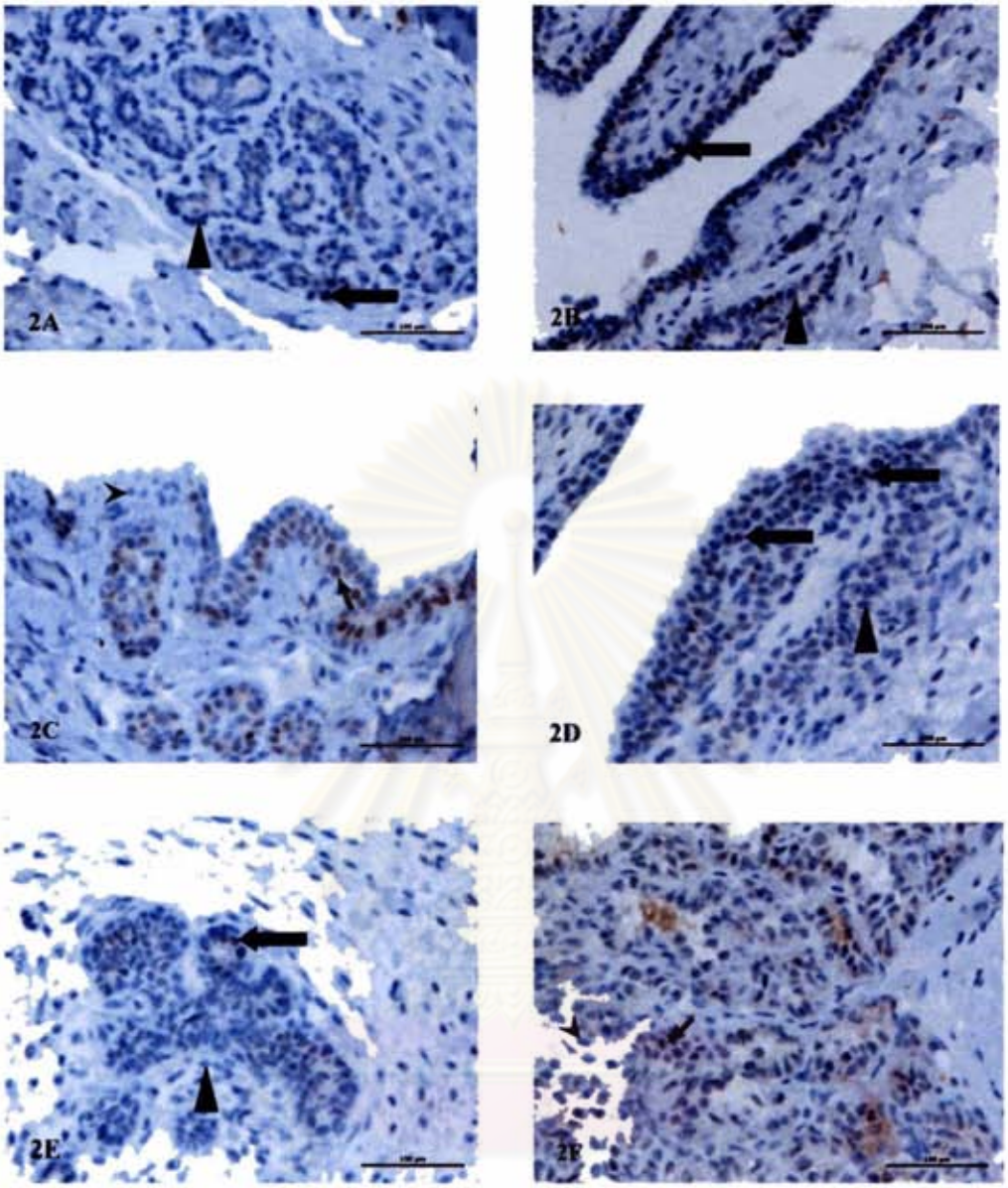

Fig. 2 ERα immunostaining in normal canine mammary tissue during different stages of estrous cycle, anestrus (2A), proestrus (2B), estrus (2C), early diestrus (2D), mid diestrus (2E) and late diestrus (2F). Arrow head and black arrow show, respectively, negative and positive immunostaining cells.

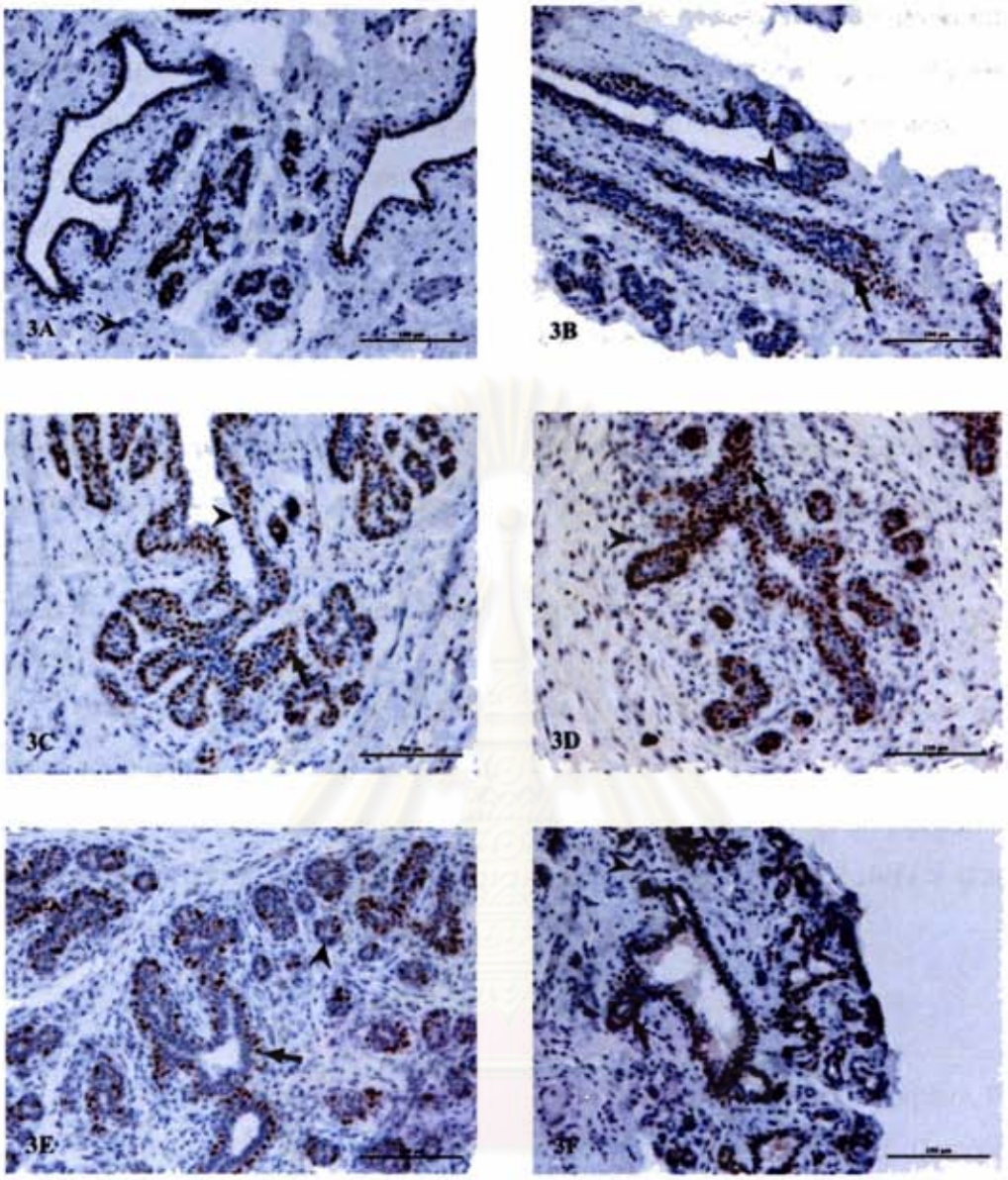

Fig. 3 PR immunostaining in normal canine mammary tissue during different stages of estrous cycle, anestrus (3A), proestrus (3B), estrus (3C), early diestrus (3D), mid diestrus (3E) and late diestrus (3F). Arrow head and black arrow show, respectively, negative and positive immunostaining cells.

The significantly high score of the ERα positive nuclear stained cells in cross-sectional group were shown at proestrus and estrus, whereas the significantly lowest score were found during diestrus. At anestrus, the ERα score was significantly higher than at the diestrus but significantly lower than in estrus. In the longitudinal group, the lowest ERα immunostaining score was found at mid diestrus which was significantly lower than early diestrus and estrus. Moreover highest score was found at early diestrus but it is not significantly different to proestrus and estrus.

The score of the PR positive cells in the cross-sectional group was lowest at diestrus, which was significantly different to the others. In the longitudinal group, the lowest score was observed at mid diestrus and late diestrus with significantly different to estrus and anestrus.

2.4.3 Correlation between serum estradiol-17β and progesterone, and ERα and PR scores

The significant correlation was found between the ERα score and PR score (positive correlation, $P \leq 0.01$).

The negative correlations were found between the levels of serum progesterone and ERα score and PR score, though not significant (r = -0.37, P = 0.07 and r = -0.38, P = 0.07 respectively).

2.5 Discussion

In the present study, by using immunohistochemistry, the steroid receptors, ERα and PR in both cross-sectional and longitudinal studies were evaluated. Comparing between these two studies, the results of ERα and PR score in normal bitch mammary gland were similar, which indicated that variation among individual bitch during the different stages of the estrous cycle may not have a significant effect on the study of steroid receptor by immunohistochemistry. However, to investigate more details on the presence of the ERα and PR at different progesterone levels during diestrus, the stage of diestrus should be divided into 3 stages as early diestrus, mid diestrus and late diestrus as shown in the longitudinal study.

The high ERα score at proestrus, estrus and the low ERα score in normal mammary tissue at diestrus were observed in both groups. This may be related to the

serum estradiol-17β and progesterone levels as suggested by the studies in normal human breast tissue that the expression of ER was detected more in follicular phase than in luteal phase (Soderqvist et al., 1993). The same as in other reproductive tissue of mammals such as uterus , in which estradiol has up-regulation effect, whereas progesterone has down-regulation effect (Dhaliwal et al., 1997; Galabova-Kovacs et al., 2004; Sukjumlong et al., 2005; Sukjumlong et al., 2003; Vermeirsch et al., 2000; Vermeirsch et al., 1999; Vu Hai et al., 1977). In addition, in the longitudinal study which divided diestrus into 3 stages according to the levels of serum progesterone, it was shown that during early diestrus, ERα score was still high which confirmed the up-regulation by estradiol which was still high at that period. On the other hand, during mid diestrus when progesterone level was highest, the lowest ERα score in normal bitch mammary gland was observed. These results on the bitch mammary gland confirmed the down-regulation effect by high levels of progesterone as suggested by other earlier studies human breast tissue (Battersby et al., 1992) and in canine uterine tissues (Dhaliwal et al., 1997; Vermeirsch et al., 2000; Vermeirsch et al., 1999). Moreover, it was supported by the results of the correlation between ERα and progesterone levels from the present study that progesterone levels was likely to be negatively correlated with ERα score in the bitch mammary gland, though it was not significantly different.

In the present study, the lowest PR score was observed during diestrus and mid diestrus in cross-sectional group and longitudinal group respectively. This may due to the increasing progesterone levels as suggested by the study in mice which demonstrated the down-regulation of PR in mammary glands during pregnancy as the results from the antagonistic effect of progesterone on estrogen action (Shyamala, 1997). Moreover, the high PR score was found at anestrus, may be the influence of low progesterone levels and it may indicate the sensitivity for estrogens and progesterone in a sexual quiescent stage that react to decrease progesterone concentration by an increase PR expression (Vermeirsch et al., 2000). Similary to ERα, the correlation between PR score and serum progesterone level is likely to be negative correlated and that the positive correlation was also found between ERα and PR score in the present study.

When compared the present investigation with the earlier biochemical study of ERα and PR in the bitch mammary gland and uterus (Donnay et al., 1995b), the results are not in agreement. The earlier study by Donnay et al. (1995b) demonstrated the significantly higher ER concentration in the mid luteal phase, and low PR concentration in the early luteal phase, but during the follicular phase the PR concentrations were quite constant in the mammary gland. The difference may be explained by the different method using for detection of the steroid receptors. As the biochemical assay used the homogenized mammary tissues, therefore the concentration of the receptors obtained from this technique may be the results of steroid receptors from various tissue types in addition to mammary epithelial cells.

For the results of correlation, the present study demonstrates that the ERα and PR scores were more likely to be negative correlated with the serum progesterone level, this finding was in agreement with previous study in human breast tissue that indicated the expression of ERα was down-regulated by progesterone (Battersby et al., 1992). Moreover, the immunohistochemical study of ERα and PR in macaques mammary tissue during menstrual cycle demonstrated higher PR expression in the follicular phase than in the luteal phase (Stute et al., 2004). In contrast, the expression of ERα in mammary tissue of macaques at follicular phase was negatively correlated with serum estradiol concentrations (Stute et al., 2004). Therefore, this finding may indicate that the expression of receptors protein in canine mammary tissues may depend on the level of circulating progesterone more than estradiol-17β, which reinforced by the significantly higher ERα and PR score during anestrus than diestrus, when the estradiol-17β levels were similarly low, but progesterone level was high only during diestrus. Moreover, the positive correlation between ERα score and PR score that found in the present study supported the hypothesis that the presence of PR is indicated that ER was function. Since PR was identified as an ER-regulated gene product (Graham and Clarke, 1997; Osborne et al., 2005).

In the mammary gland, the study in primate indicated that the ERα protein levels were down-regulated during follicular phase when estradiol levels increase (Cheng et al., 2005). Conversely, this present study found that the serum estradiol-17β levels were not down-regulated the ERα score as shown by high level of estradiol-17β during

proestrus and estrus and no negative correlation found between ERα score and estradiol levels. This finding may be due to the different of the menstrual cycle and different in the circulating level of estradiol-17β in different species, in which estradiol-17β in primate species could increase up to 100-350 pg/mL during the follicular phase (Cheng et al., 2005).

In conclusion, the present study reported the different finding of steroid receptors protein expression in canine mammary tissues, which differed from the previous biochemical investigation in bitches. The results showed that ERα and PR were regulated by ovarian steroid hormones, which may be up-regulated by estradiol-17β and down-regulated by progesterone. The same pattern of the ERα and PR were demonstrated in both cross-sectional and longitudinal studies. Moreover, it was shown that the expression of ERα and PR in canine mammary tissues was negatively correlated to the levels of progesterone than correlated with estradiol-17β levels. However, the presence of steroid hormone receptors by immunohistochemical detection can not indicate their functional state or binding activity. Therefore, the further study on the receptors mRNA should be performed to investigate the functional state of the receptors.

CHAPTER III

THE NUMBER OF ERα AND PR IN THE MAMMARY GLANDS OF BITCHES WITH AND WITHOUT TUMOR MASS USING IMMUNOHISTOCHEMICAL ASSAY

3.1 Abstract

The objectives of this study were to evaluate the expressions of estrogen receptors (ERα) and progesterone receptors (PR) in histologically normal and tumoral canine mammary tissues within the same bitch and to correlate between the scores of both receptors and duration of tumor growth. Twelve tumoral and contralateral normal mammary tissues, both determined by histology were obtained from 12 dogs by surgical excision. The expressions of ERα and PR were evaluated by avidin-biotin-peroxidase complex (ABC) method and ERα and PR scores were calculated. The expressions of the ERα and the PR between normal and tumoral mammary tissues were not significantly different. The PR expression and duration of tumor growth showed a significantly inverse correlation. A positive correlation was found between the number of the ERα and the PR in both normal and tumoral mammary tissues which may indicate that either ERα or PR could be used as a prediction of hormonal therapy parameter in dogs. However, the information about ERα and PR status as well as hormonal therapy is not routinely available in veterinary medicine and it appears that half of the mammary tumors from the present study are hormone-independent tumors (ERα and PR negative). Therefore, the treatment of canine mammary tumor by hormonal therapy should be carefully considered.

3.2 Introduction

Next to skin tumors, mammary tumors are the most common neoplasms in dogs, in which approximately 40-50% of these tumors are malignant (Fanton and Withrow, 1981). It can be cause of death when the tumor cells are spread to other organs. The evidence of mammary tumors is thought to be depended on the endocrine status of the host (Yamagami et al., 1996). Estrogen and progesterone may play an important role in the pathogenesis of canine mammary tumors (Fanton and Withrow, 1981; MacEwen, 1990; Misdorp, 2002). The ovarian steroid hormones, estrogen and progesterone exert their actions on target cells predominantly through the binding and activation of the estrogen receptor (ER) and progesterone receptor (PR) (Brosens et al., 2004). There are two receptors for estrogen, estrogen receptor alpha and beta (ERα and ERβ respectively) (Martin de las Mulas et al., 2004). In female, ERα is abundant in classical estrogen target tissue including mammary gland, while ER-β is highly expressed in non-classical estrogen target tissue such as prostate epithelial, ovarian follicle, (Weihua et al., 2003). Analysis of the expressions of ERα and PR has become accepted and useful tools in prognosis and prediction of hormonal therapy response in human breast cancer (Snead et al., 1993). Regarding ERα and PR positive breast tumor, it indicated that the tumor would be depended on estrogen for growth and should be response to hormonal therapy (Jensen and Desombre, 1997). On the other hand, ERα and PR negative tumor would be less dependent on estrogen therefore it would be less responsive to hormonal therapy (Osborne et al., 2005). In dogs, steroid hormone receptors also have been detected in normal mammary tissue and mammary tumors (Donnay et al., 1995; Geraldes et al., 2000; Graham et al., 1999; Hamilton et al., 1977; MacEwen et al., 1982; Nieto et al., 2000). From these earlier studies, different results of ERα and PR status in normal canine mammary tissues as well as mammary tumors were reported. Regarding immunohistochemitry, the available data on steroid receptors of normal mammary and tumoral tissue was done by collecting samples from different bitches. As endocrine status may vary among individual bitch and it can be the important effects on the expression of steroid hormone receptors in mammary tissues (Donnay et al., 1995b). Moreover, regarding immunoshistochemichal technique, there are no previous reports

on the detection of ERα and PR in normal and tumoral mammary tissues within the same bitch. Therefore, the objectives of this study were to evaluate the differences between immunohistologically ERα and PR expression in normal mammary tissue and mammary tumor from the same bitch and to determine the relation between ERα and PR scores and the duration of tumor growth.

3.3 Materials and Methods

3.3.1. Animals

A total of 12 adult intact bitches of various breeds bearing mammary tumors were included in this study. All bitches were referred to obstetric and gynaecology unit, Chulalongkorn University small animal hospital for treatment of mammary tumor by surgical excision. In this present study, only adenoma and adenocarcinoma types were selected to represent for benign and malignant tumors respectively. Moreover, in order to avoid the different endocrine status among individual bitch, mammary tumor and normal mammary tissues were collected from the same bitch.

3.3.2. Collection of samples

Both mammary tumors and contralateral normal mammary tissues from the same bitch were obtained during the surgical procedure. Immediately after surgical excision, they were fixed in 4% buffer paraformaldehyde up to 48 hrs. Thereafter, they were dehydrated, embedded in paraffin and 4 μm thick sections were cut from and placed on Polysine™ slides (Menzel-Glazer, Germany). One section was stained with hematoxylin and eosin, and the other sections were used for immunohistochemistry.

3.3.2 Histological examination

Sections of each tumor, stained with hematoxylin and eosin, were evaluated microscopically and classified using the diagnostic criteria proposed by the World Health Organization classification of tumor in domestic animals (Hampe and Misdorp, 1974).

3.3.3 Immunohistochemistry of estrogen and progesterone receptors

The specimens were deparaffinized in xylene and rehydrated in graded alcohol. After washing with distilled water, they were subjected to high-temperature antigen retrieval by incubation with 0.01 M citrate buffer, pH 6.0 in a microwave at high power

(750 W) for 15 minutes (5 min. x 3). Additional citrate buffer was added if necessary between each heating in order to prevent slides from drying out. After cooling down at room temperature for 20 minutes, the slides were rinsed with 10 mM, pH 7.4 phosphate-buffered saline (PBS). The following procedures were performed at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes, then tissues were rinsed with PBS. Non – specific staining was eliminated by incubating the sections with normal horse serum for 30 minutes. Excess serum was removed and the sections were incubated with the specific primary mouse monoclonal antibody to ERα (Novocastra NCL-ER-LH2, clone CC4-5, dilution 1:50) and PR (Immunotech, clone 10A9, dilution 1:100) for 2 hours in a humidity chamber. After primary antibody binding, the sections were washed in PBS and incubated with the secondary antibody, a biotinylated horse anti-mouse Ig G (Vectastain® ABC kit, Vector Laboratories, Inc., USA) in a dilution of 1:200 for 30 minutes. After slides were washed in PBS the sections were incubated for 30 minutes with a horseradish peroxidase avidin biotin complex (Vectastain® ABC kit, Vector Laboratories, Inc., USA). After a final wash with PBS, the color was developed with a freshly prepared solution of 3, 3' – diaminobenzidine (DAB kit, Vector Laboratories, Inc., USA). All sections were counterstained with Mayer's hematoxylin, dehydrated and mounted with glycerine gelatin for investigation under a light microscope.

The samples that were treated with non-immune serum, instead of the specific antibody, were run as negative controls. Normal canine uterus at estrous stage known to express ERα and PR was served as positive controls.

3.3.4 Evaluation of immunohistochemical data

The immunostaining for estrogen and progesterone receptors was assessed on the basis of visually estimated percentage of neoplastic cells with positive nuclear staining (brown nuclei) by counting 1,000 cells in 10 to 20 fields per histological sections with nuclear staining for ERα and PR. The human score system was used. ERα score and PR score were calculated as $P_1 + (2 \times P_2) + (3 \times P_3)$ where P_1, P_2 and P_3 are the estimated percentages of positive nuclei with low (P_1), medium (P_2) and high (P_3) intensity of immunostaining color(Snead et al., 1993)

3.3.5 Statistical analysis

The differences between the expressions of histologically ERα and PR on normal mammary tissues and mammary tumors were evaluated using Paired T-test and correlation between ERα and PR scores and duration of tumor growth within the groups of bitch were evaluated using Spearman's rank correlation coefficient. A probability of error (P value) ≤ 0.05 was selected as significant.

3.4 Results

Of the 12 tumors, 4 were histologically malignant (adenocarcinoma) and 8 were benign (adenoma). All of tumor masses located on the caudal mammary glands of both sites (left and right 3rd, 4th and 5th). The age number and site of mammary samples of the bitches are shown in table 3 with ERα score and PR scores in table 4. In general, ERα and PR immunostaining were localized in the nuclei of normal, benign and malignant epithelial cells (Figs. 4 and 5). In the negative controls, all nuclei were negative.

Table 3. General information of 12 bitches in the study

No.	Age (yrs)	Growth duration (month)	site	Diameter (cm.)	Histological type
1	8	6	R 3,4	15	Adenoma
2	6	1	R 5	2	Adenoma
3	14	2	R 4,5	10	Adenoma
4	10	12	R 5	10	Adenocarcinoma
5	10	12	L 4	20	Adenocarcinoma with lymphatic metastasis
6	8	4	R 5	13	Adenoma
7	3	3	L 5	7.5	Adenoma
8	8	12	L 4,5	7.5	Simple tubulopapillary adenocarcinoma
9	10	12	R 3	1.5	Simple tubulopapillary adenocarcinoma
10	2	0.5	L 4	6	Adenoma
11	6	2	L 3-5	1.5	Adenoma
12	5	12	R 3-5	6.25	Adenoma

F = female, R = right side, L = left side

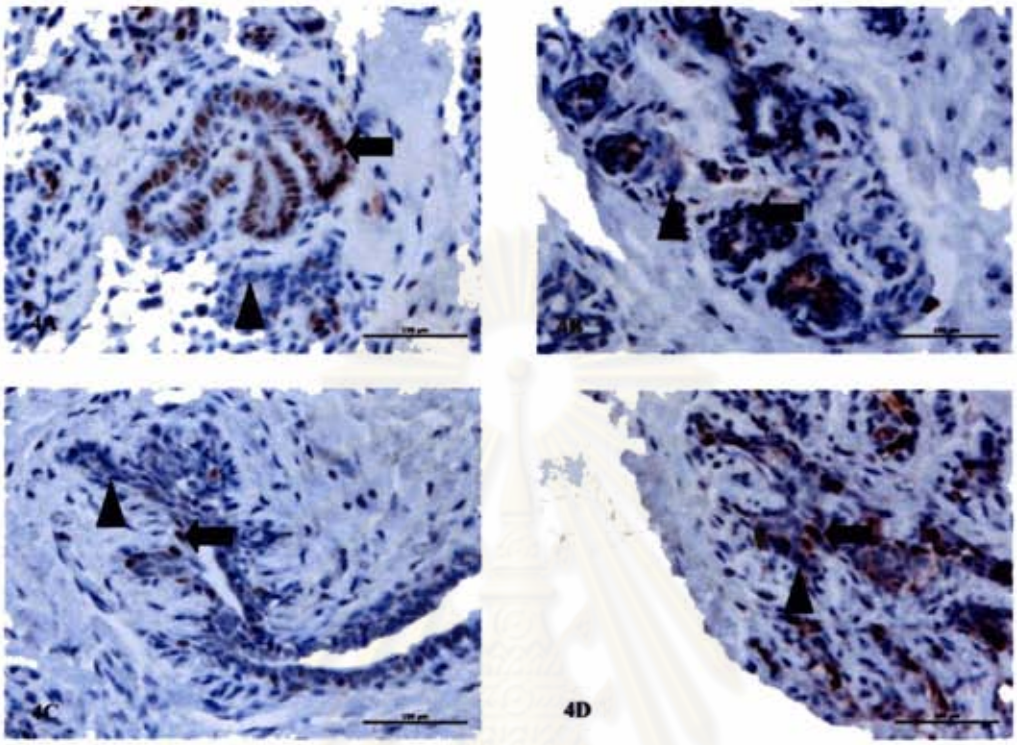

Fig. 4 Immunostaining in canine mammary tissue within the same bitch, 4A and 4B represent ERα immunostaining in normal mammary tissue and benign tumoral tissue, respectively. 4C and 4D represent PR immunostaining in normal mammary tissue and benign tumoral tissue, respectively. Arrow head and arrow show, respectively, negative and positive immunostaining cells.

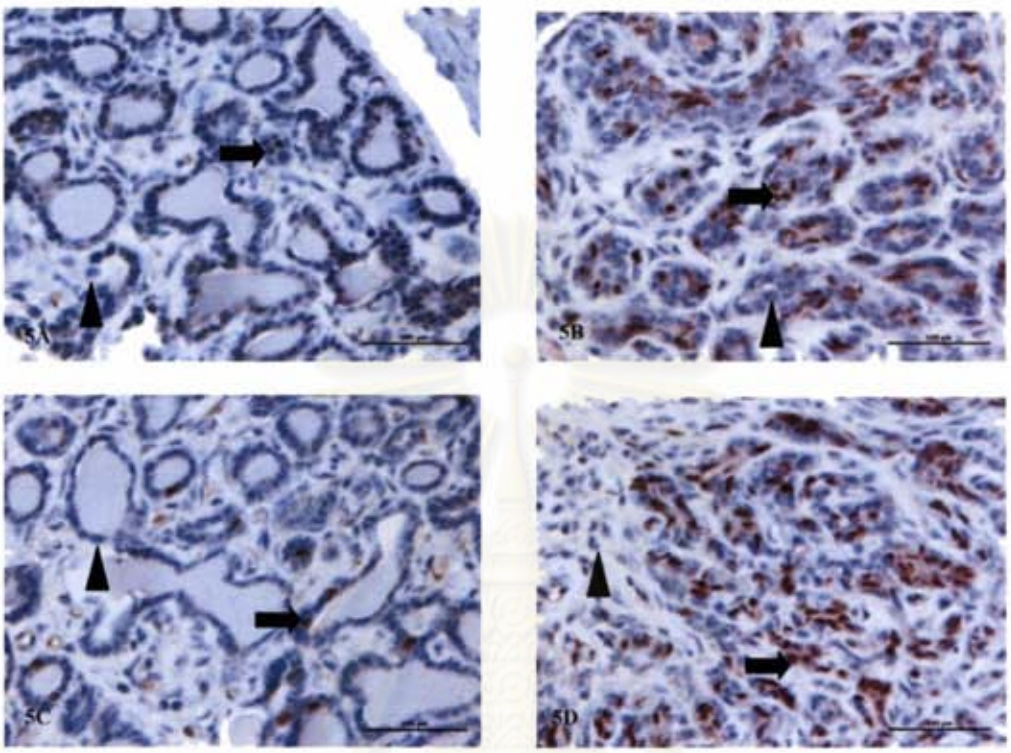

Fig. 5 Immunostaining in canine mammary tissue within the same bitch, 5A and 5B represent ERα immunostaining in normal mammary tissue and malignant tumoral tissue, respectively. 5C and 5D represent PR immunostaining in normal mammary tissue and malignant tumoral tissue, respectively. Arrow head and arrow show, respectively, negative and positive immunostaining cells.

Table 4. Estrogen receptor (ERα) and progesterone receptor (PR) scores in histologically normal and tumoral mammary tissues in the same dog

No.	Normal mammary tissue		Mammary tumor tissue	
	ERα score	PR score	ERα score	PR score
1	43.9	43.4	0.0	0.0
2	0.0	0.0	0.0	0.0
3	30.7	137.2	28.9	16.2
4	53.0	12.1	110.4	117.2
5	49.7	40.2	0.0	0.0
6	0.0	0.0	0.0	0.0
7	17.1	18.6	13.7	57.0
8	0.0	0.0	0.0	0.0
9	0.0	153.5	0.0	98.6
10	0.0	46.3	0.0	38.5
11	0.0	0.0	6.4	13.3
12	0.0	0.0	0.0	0.0

The positive percentages of ERα and PR in normal and tumoral mammary tissues were shown in table 5. The positive percentages of ERα and PR in normal mammary tissues were higher than mammary tumors, though they were not significantly different.

The ERα and PR scores were positively correlated (Spearman's coefficient ($P \leq 0.01$) in both normal and tumoral mammary tissues. The expressions of ERα and PR were not significantly correlated to the duration of tumor growth and tumor size. However, regarding the groups of benign and malignant tumors separately, PR score significantly decreased with increased duration of tumor growth ($P \leq 0.05$) in malignant tumors, but not with ERα score. In malignant tumors, ERα and PR score decreased when tumor size increased, though not significant.

Table 5. Summary of ERα and PR in normal mammary tissues and mammary tumors from 12 bitches

Mammary tissues	ERα	PR
Normal mammary tissues		
N	12	12
% Positivity	41.67	58.33
Mean ± SD	16.20 ± 21.93	37.61 ± 53.55
Range	0 – 53	0 – 153
Mammary tumors		
N	12	12
% Positivity	33.33	50.00
Mean ± SD	13.28 ± 31.82	28.40 ± 41.51
Range	0 – 110	0 – 117

3.5 Discussion

All of the mammary tumors in this investigation are on the posterior mammary glands, this finding is reinforced that canine mammary tumors increased in frequency from anterior to posterior gland (Mann, 1984; Misdorp, 2002).

The presence of ERα and PR in normal and tumoral canine mammary tissues was detected by using specific monoclonal antibodies directed against human protein. Immunoreactivity was recognized in normal tissues as well as benign and malignant tumors in which both ERα and PR were localized mainly in the nuclear area of alveolar and tubular epithelial cells. Similar immunostaining results have been found in previous studies (Geraldes et al., 2000; Nieto et al., 2000). Moreover, by using biochemical assay, earlier studies have reported the similar results that ERα and PR could be detected in both normal and tumoral canine mammary tissues (Donnay et al., 1995; Elling and Ungemach, 1983; Hamilton et al., 1977; MacEwen et al., 1982; Rutteman et al., 1988; Sartin et al., 1992).

The expressions of the ERα and the PR in normal and tumoral mammary tissues in this study were not significantly different, which suggested similar regulation of both steroid receptor expressions in those tissues within the same bitch. In accordance, Millanta et al. (2005) reported similar results that the immunohistochemical detection of ERα and PR in canine normal mammary tissue and benign mammary tumor from different dogs are not significantly different, but they are significantly higher than in carcinoma. Conversely, Donnay et al. (1995b) found that mean ER concentrations were significantly higher in normal than tumoral tissue, but PR concentrations were significantly lower in normal than in tumor. The discordance may be attributable to tumor heterogeneity and different method used for detection of these steroid hormone receptors. In veterinary practice, in case of mammary tumors, the first recommendation is to remove tumor masses and the affected glands (Mann, 1984). After surgical excision of the tumors, the receptor status of the remained mammary glands may alter due to the influence of ovarian steroid hormones. For further investigation of the steroid receptors from these mammary glands, therefore, ovariohysterectomy concurrently performed with tumor removal may be of useful to eradicate the effect of ovarian steroid hormone on the expression of the receptor.

From the present study, it seems that normal mammary glands have higher mean percentage of ERα as well as PR than mammary tumors from the same bitch though not significant. Our results confirm other earlier study which reported a decrease in ERα levels from healthy to neoplastic mammary tissues (Millanta et al., 2005). However, the lack of statistical significance from the present study could be related to the high number of ERα and PR negative results in the normal mammary tissue studied. In contrast, Geraldes et al. (2000) reported that all benign tumors in dogs analysed contained PR while high percentage of normal as well as mammary tumors from the present study were PR negative. The difference between these may attribute to different hormonal status of the bitches investigated as suggested by Donnay et al. (1995b). Moreover, it appears that half of the mammary tumors from the present study are hormone-independent tumors (ERα and PR negative). Therefore, the treatment of canine mammary tumor by hormonal therapy should be carefully considered. Besides ovarian steroid hormones, oestrogen and progesterone, it has been reported that other growth

factors such as epidermal growth factor, insulin liked growth factor-I and transforming growth factor (Donnay et al., 1996; Misdorp, 2002; Mol et al., 1997) as well as high-fat diet may influence on the pathogenesis of mammary gland tumors (Perez Alenza et al., 2000; Sonnenschein et al., 1991).

A positive correlation was found between the number of the ERα and the PR in both normal and tumoral mammary tissues. This was in agreement with Donnay et al. (1995b) and could be related to induction of PR by estrogen binding to the ER (Elling and Ungemach, 1983; Horwitz and McGuire, 1978). In human breast cancer, the analysis of the expressions of ERα and PR has become accepted and useful tools in prognosis and prediction of hormonal therapy (Iwase et al., 2003; Pichon et al., 1980). It was shown that a high content of ER in a tumor is associated with a better prognosis and allows hormonal treatment after surgical excision.

In dogs, the presence of ER and PR also seem to relate to a better prognosis (Sartin et al., 1992), therefore, the positive correlation between ERα and PR from the present study may indicate that either ERα or PR could be used as a prediction of hormonal therapy parameter in dogs. In human breast cancer all patients that have ERα and/or PR positive should be received hormonal therapy (Jensen and Desombre, 1997). Moreover, PR was defined as an ER-regulated gene product; therefore the presence of PR may indicate that the ER is function. Therefore, both ERα and PR positive tumor would response to hormonal therapy more than the ERα positive tumor without PR (Osborne et al., 2005). However, the information about ER and PR status as well as hormonal therapy is not routinely available in veterinary medicine.

From the present study, PR score and duration of tumor growth showed a significant inverse correlation. This finding reinforces the hypothesis that the development of breast cancer is pararelled by a progressive decrease in hormone dependence. The progression towards malignancy in spontaneous canine mammary tumor is accompanied by a decrease in hormone steroid dependency and an increase in autonomous growth (Rutteman et al., 1988; Schmitt, 1995).

Four bitches from the study did not demonstrate either ERα or PR expression at all, this may due to several factors such as the lack of steroid receptors, the undetected

receptors by monoclonal antibody but might still be function or non-functional status of the receptors.

In conclusion, the present study showed that both normal and tumoral mammary tissues from bitches have heterogeneous expression of steroid receptors, ERα and PR. The results from this study may be used for the further investigation of the recurrence of tumors in the non-removal mammary gland and also useful to select the appropriate therapy for canine mammary tumors after surgical treatment. However, hormonal therapy could be applied with cautious in dog mammary tumors as half of the mammary tumors investigated from the present study may be categorized as hormone-independent tumors (ERα negative/PR negative) and further studies about hormonal therapy for mammary gland tumors should be investigated in veterinary medicine.

CHAPTER IV

THE EFFECT OF LONG-ACTING GnRH AGONIST (DESLORELIN) ON ERα AND PR IN CANINE MAMMARY TISSUE

4.1 Abstract

The objective of this study was to evaluate the expressions of estrogen receptors alpha (ERα) and progesterone receptors (PR) in normal mammary tissues of bitches before and after GnRH agonist, deslorelin implantation. Twenty four puberty bitches with no any pathological condition of the mammary gland, during anestrous stage were selected and divided into two groups: twelve bitches were implanted with placebo (placebo or control group); and the other twelve bitches were implanted with 9.4 mg deslorelin (deslorelin group). Mammary tissues were collected from all bitches before implantation and at 1, 2 and 12 weeks after implantation. The expressions of ERα and PR were evaluated by avidin-biotin-peroxidase complex (ABC) method and ERα and PR scores were calculated. The highest scores of both ERα and PR were found at 2 weeks after deslorelin implantation with significantly different to other stages. This finding indicated that deslorelin may effect the expression of ERα and PR by influenced on the hypothalamic-pituitary-ovarian axis, in which stimulated the expression when initial implantation and subsequently suppressed after sustained stimulation.

4.2 Introduction

Endocrine therapy has been widely used for treatment of breast cancer in women. Ovarian ablation was first used in the palliation of young women with metastatic breast cancer in 1896 (Santen et al., 1990). Until present, medical hormonal ablation of various types is commonly used to treat human breast cancer (Corbin, 1982; Emens and Davidson, 2003; Santen et al., 1990). The purpose of hormonal therapy is to prevent further estrogen stimulation of breast cancer cells. This can be achieved by several different strategies, including blocking the receptors with specific estrogen receptor modulators such as tamoxifen which are estrogen receptor antagonist. Other strategies

of hormonal therapy are also used which are suppression of estrogen synthesis by aromatase inhibitors, or by use of luteinizing hormone-releasing hormone (LHRH) analogues (Emens and Davidson, 2003; Santen et al., 1990). Regarding tamoxifen, which was used as an anti-estrogen at some target sites in the dog, may yet prove to be tumor-static for mammary tumors. However, its use will have to be restricted to spayed bitches, and even in these animals the high incidence of estrogenic side effects means that it is unlikely to become as widely used as it is in women (Morris et al., 1993).

Previous study about GnRH-agonist (Goserelin) on the growth of hormone-dependent canine mammary tumors showed that the inhibitory action of this GnRH agonist cause objective response to treatment after 3 months (Lombardi et al., 1999). This GnRH agonist initially stimulates the hormone secretion, but will subsequently cause a down-regulation of GnRH receptors resulting in the inhibition of luteinizing hormone production upon continuous exposure. Moreover, Goserelin inhibits the proliferation of the tumor cells by interfering with the stimulatory action of epidermal growth factor (EGF) (*in vitro* studies) (Pagnini et al., 2002) and decrease ovarian estradiol production indirectly by blocking of the hypothalamic-pituitary-ovarian axis. For Deslorelin, which is a slow release and long acting synthetic agonist of GnRH was reported for control fertility on both male and female dogs (Trigg et al., 2001; Wright et al., 2001). Average plasma level of deslorelin following subcutaneous implantations to the dogs was more than 1 μg/day for period of more than 1 year (Trigg et al., 2001). Until present, no study has been done on the effect of deslorelin on the number of canine mammary steroid receptors.

The studies of sex hormone manipulation are important for hormone-dependent mammary tumors. For further understanding of the effect of deslorelin implantation involved in canine normal mammary tissues, in relation to steroid hormone receptors, the expressions of ERα and PR in normal mammary glands before and after deslorelin implantation were investigated in the present study.

4.3 Materials and methods

4.3.1 Animals

Twenty four healthy anestrous bitches of mixed breeding, aged between 2-3 years, free from mammary lesions were used. The bitches were housed separately in indoor cages with outdoor runs, fed a commercial dry canine diet twice daily and given water *ad libitum*. The animals were divided into two groups; group 1 (placebo or control group), referred as placebo implantation, group 2 (deslorelin group), referred as deslorelin implantation. Blood samples were taken before the experiment from both groups to assess hematology and blood chemistry values, as well as estradiol and progesterone circulating levels. Stage of estrus cycle was also confirmed by cytological examination of vaginal cytology and serum progesterone level (Feldman and Nelson, 1996).

4.3.2 Drug administration and samples collection

The potent long-acting GnRH agonist "deslorelin" prepared as a biocompatible cylindrical implant (3.6 mm long x 2.3 mm in diameter) was developed, manufactured and supplied by Peptech Animal Health Pty Limited, NSW, Australia. Implants were manufactured by a proprietary method that involved extrusion of deslorelin with a matrix consisting principally of low-melting point lipids and biological surfactant. Each implant contained 4.7 mg of active ingredient deslorelin. Two implants (9.4 mg) were preloaded in a disposable syringe-like implanter and packed in an individual package. Implants were terminally sterilized by e-beam irradiation and then kept at $4^{\circ}C$ until use. In a real time *in vitro* dissolution system, these implants released doses of more than 1μg / day for period of more than 1 year (Trigg et al., 2001).

Blood sample were taken at 1 and 2 week after implantation, and every 2 week intervals for 3 months from all dogs to assess treatment tolerability and changes in hematology and blood chemistry values, as well as estradiol-17β and progesterone circulating levels.

Normal mammary tissues were obtained before implantation and at 1, 2 and 12 weeks after implantation by incisional biopsy under general anesthesia. Immediately after surgical excision, they were fixed in 4% paraformaldehyde up to 48 hours.

Thereafter they were dehydrated, embedded in paraffin and 4 μm thick sections were cut from each block and mounted on Polysine™ slides (Menzel-Glazer, Germany). The sections were used for immunohistochemistry.

4.3.3 Immunohistochemistry of estrogen and progesterone receptors

The specimens were deparaffinized in xylene and rehydrated in graded alcohol. After washing with distilled water, they were subjected to high-temperature antigen retrieval by incubation with 0.01 M citrate buffer, pH 6.0 in a microwave at high power (750 W) 15 minutes (5 min. x 3) for PR, and 25 minutes (5 min. x 5) for ERα. Additional citrate buffer was added if necessary between each heating in order to prevent slides from drying out. After cooling down at room temperature for 20 minutes, the slides were rinsed with 10 mM, pH 7.4 phosphate-buffered saline (PBS). The following procedures were performed at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes, then tissues were rinsed with PBS. Non – specific staining was eliminated by incubating the sections with normal horse serum for 30 minutes. Excess serum was removed and the sections were incubated with the specific primary mouse monoclonal antibody to ERα (Dako, clone 1D5, dilution 1:50) for 3 hours at 37°c and PR (Immunotech, clone 10A9, dilution 1:100) for 2 hours at room temperature all slides were placed in a humidity chamber. After primary antibody binding, the sections were washed in PBS and incubated with the secondary antibody, a biotinylated horse anti-mouse IgG (Vectastain® ABC kit, Vector Laboratories, Inc., USA) in a dilution of 1:200 for 30 minutes. After slides were washed in PBS the sections were incubated for 30 minutes with a horseradish peroxidase avidin biotin complex (Vectastain® ABC kit, Vector Laboratories, Inc., USA). After a final wash with PBS, the color was developed with a freshly prepared solution of 3, 3' – diaminobenzidine (DAB kit, Vector Laboratories, Inc., USA). All sections were counterstained with Mayer's hematoxylin, dehydrated and mounted with glycerine gelatin for investigation under a light microscope.

The samples that were treated with non-immune serum, instead of the specific antibody, were run as negative controls. Normal canine uterus at estrous stage known to express ERα and PR was served as positive controls.

4.3.4 Evaluation of immunohistochemical data

The immunostaining for estrogen and progesterone receptors were assessed on the basis of visually estimated percentage of alveolar and tubular epithelial cells with positive nuclear staining (brown nuclei) by counting 1,000 cells in 10 to 20 fields per histological sections with nuclear staining for ERα and PR. The human score system was used. ERα score and PR score were calculated as $P_1 + (2 \times P_2) + (3 \times P_3)$ where P_1, P_2 and P_3 are the estimated percentages of positive nuclei with low (P_1), medium (P_2) and high (P_3) intensity of immunostaining color(Snead et al., 1993).

4.3.5 Estradiol-17β and progesterone assays

Serum estradiol concentrations were measured by chemiluminescent immunoassay system, using IMMULITE® Estradiol kit (Diagnostic Products Corporation, Los Angeles, CA, USA). The within-assay coefficients of variation ranged from 1.9 to 5.9 %. Serum progesterone concentrations were measured by using a commercial solid-phase progesterone radioimmunoassay (Coat-A-Count Progesterone kit™, Diagnostic Products Corporation, Los Angeles, CA, USA). The progesterone standards used were 0, 0.1, 0.5, 2, 10, 20 and 40 ng/ml. The within-assay coefficients of variation ranged from 7 to 9 %.

4.3.6 Statistical analysis

Data were handled and statistically analyzed using the SAS statistical package (version 9, SAS Institute, Inc., 2002, Cary, NC, USA). Normal distribution of residuals from the statistical models was tested using UNIVARIATE procedure option NORMAL. Differences in mean numbers of ERα score and PR score were tested using analysis of variance. The statistical model included the fixed effects of stage (four groups: before implantation, 1 week after implantation, 2 weeks after implantation and 12 weeks after implantation) and group (two groups: placebo and deslorelin); the interaction between stage and group. General Linear Mixed model was used to compare least-squares means between stages when overall significance for that effect was found. Correlation between ERα and PR scores and serum level of estradiol-17β and progesterone were evaluated using Pearson's correlation coefficients. A *P*-value ≤ 0.05 was considered statistically significant.

4.4 Results

In all bitches, there was no allergic or inflammatory reaction observed at the site of implantation at any time of the study. The hematology and serum chemistry parameter tested every 2 weeks, for each bitch was in normal range. (data were not shown).

Before implantation, estrous sign was not found in all bitches in both groups. In deslorelin group, bloody vaginal discharge was observed at approximately 1 week after implantation. The estrous signs were still observed and continued until 2 to 3 weeks later.

4.4.1 Serum hormone concentrations

Before implantation, all 24 bitches in both deslorelin and placebo groups were in anestrus as classified by vaginal cytology and serum progesterone concentrations. The average serum progesterone concentration in deslorelin group was 0.23 $\pm$ 0.23 ng/ml, and in placebo group the average concentration was 0.22 $\pm$ 0.15 ng/ml.

In the deslorelin group, the serum estradiol-17β and progesterone levels in all 12 bitches were demonstrated in Fig. 6A. The serum estradiol-17β level was high before implantation and subsequently decreased after implantation. The serum progesterone level increased at 2 weeks after implantation and subsequently decreased at 12 weeks after implantation. In the placebo group, the serum estradiol-17β and progesterone levels in all 12 bitches changed according to the stages of the estrous cycle which were shown in Fig. 6B.

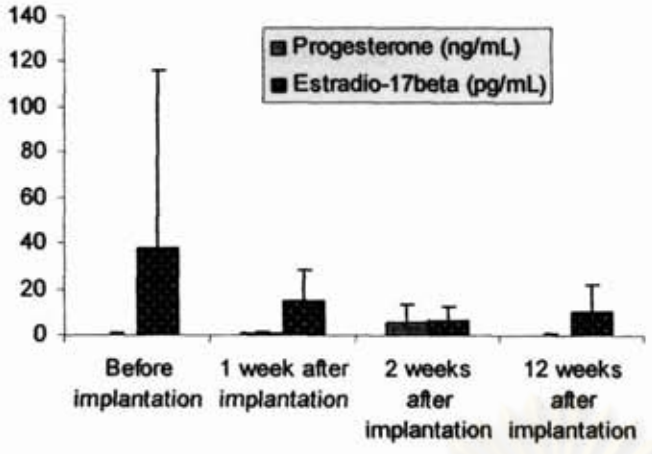

Fig. 6A The levels of serum estradiol-17β and progesterone in bitches during different stages of deslorelin implantation, mean ± SD

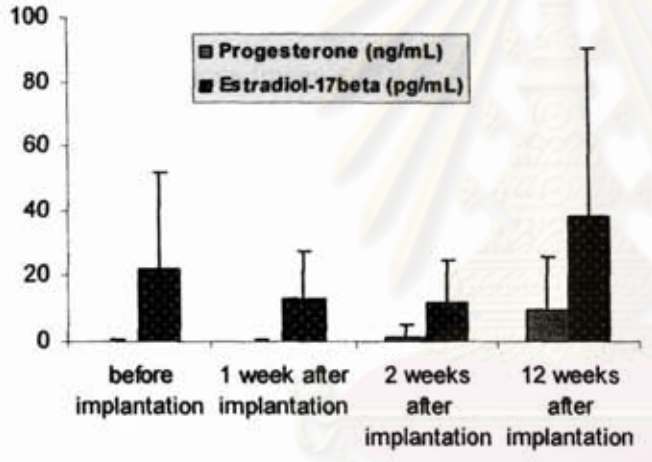

Fig. 6B The levels of serum estradiol-17β and progesterone in bitches during different stages of placebo implantation, mean ± SD

4.4.2 Immunohistochemistry

The positive immunolocalization of the ERα and PR were found in the nuclei of alveolar and tubular epithelium of canine mammary tissues in both groups (Fig. 7 and Fig. 8 respectively). The ERα and PR immunostaining scores (mean ± SD) before implantation and 1 week, 2 weeks and 12 weeks after implantation, in both deslorelin and placebo groups were shown in table 6 and 7 respectively.

Table 6. ERα score of the canine mammary tissues, at different stages of deslorelin and placebo implantations (mean ± SD)

Stage of implantation	Deslorelin implantation	Placebo implantation
Before implantation	$35.14 \pm 7.33^{A, a}$	$38.56 \pm 8.11^{A, a}$
1 week after implantation	$31.00 \pm 11.98^{A, a}$	$39.28 \pm 15.78^{A, a}$
2 weeks after implantation	$59.33 \pm 19.27^{A, b}$	$31.49 \pm 11.11^{B, a}$
12 weeks after implantation	$32.67 \pm 10.84^{A, a}$	$32.78 \pm 11.46^{A, a}$

Mean (± SD) within the same column followed by the different superscript small letters, and within the same row followed by the different superscript capital letters are significantly different ($P \leq 0.05$)

Table 7. PR score of the canine mammary tissues, at different stages of deslorelin and placebo implantations (mean ± SD)

Stage of implantation	Deslorelin implantation	Placebo implantation
Before implantation	$35.59 \pm 9.50^{A, a}$	$68.22 \pm 27.69^{B, ab}$
1 week after implantation	$38.57 \pm 12.87^{A, ac}$	$74.11 \pm 28.30^{B, b}$
2 weeks after implantation	$60.04 \pm 10.36^{A, bc}$	$72.44 \pm 20.61^{A, ab}$
12 weeks after implantation	$53.36 \pm 11.21^{A, ac}$	$49.18 \pm 19.07^{A, a}$

Mean (± SD) within the same column followed by the different superscript small letters, and within the same row followed by the different superscript capital letters are significantly different ($P \leq 0.05$)

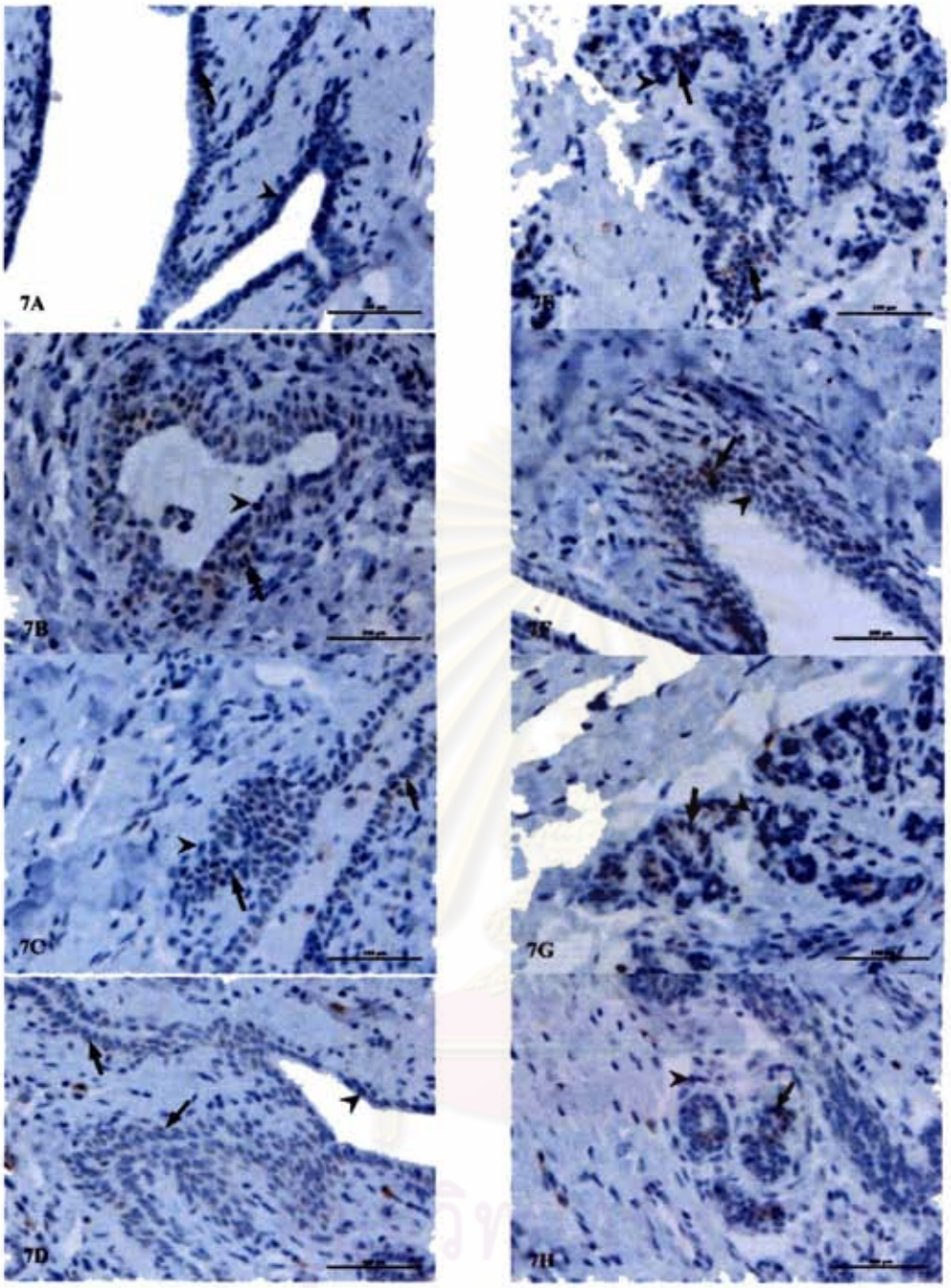

Fig. 7 ERα immunostaining in normal canine mammary tissue during different stages of deslorelin and placebo implantations, 7A, 7B, 7C and 7D represent ERα immunostaining at before, after 1 week, after 2 weeks and after 12 weeks of deslorelin implantation respectively.7E, 7F, 7G and 7H represent ERα immunostaining at before, after 1 week, after 2 weeks and after 12 weeks of placebo implantation respectively. Arrow head and arrow show, respectively, negative and positive immunostaining cells.

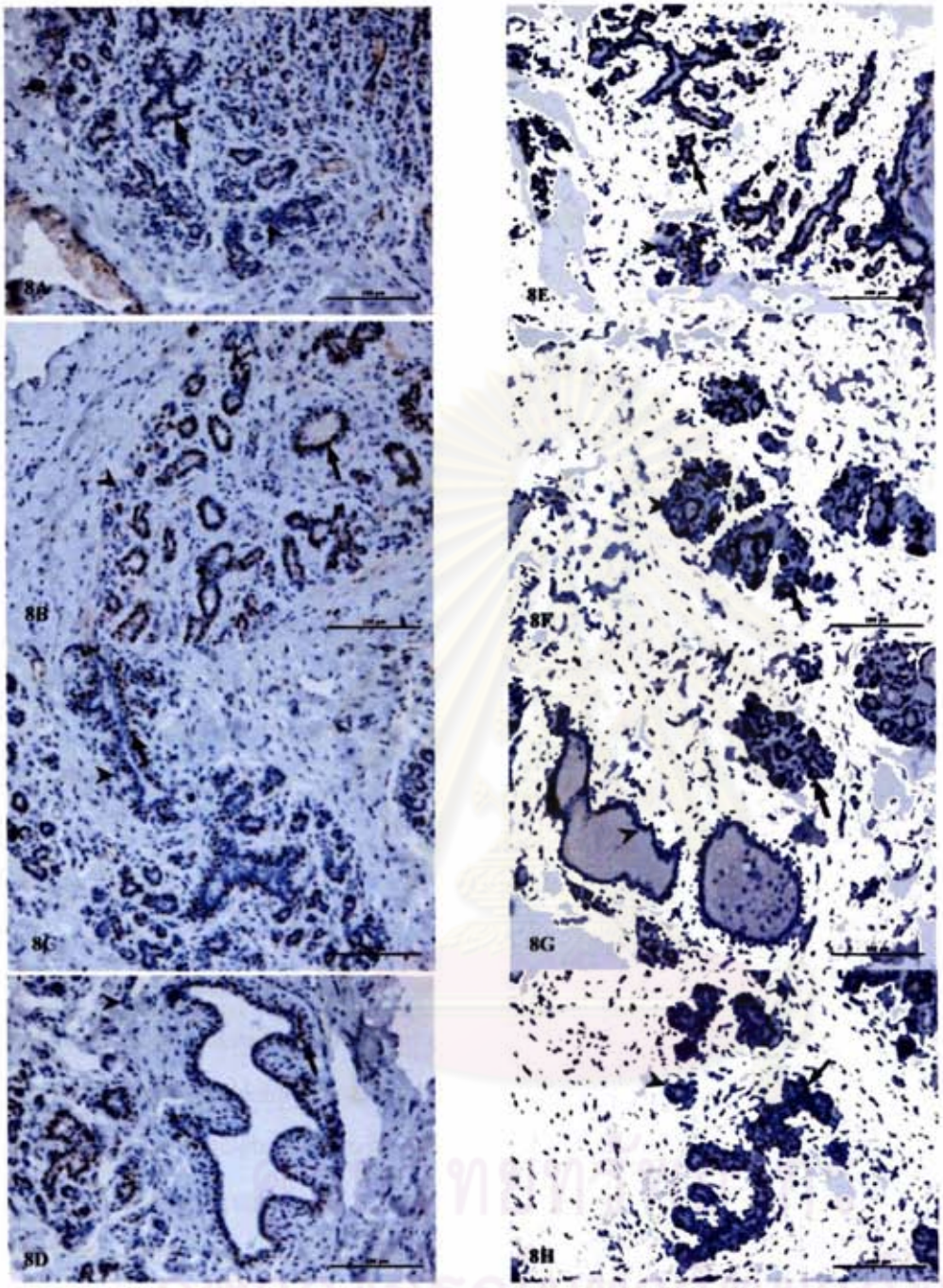

Fig. 8 PR immunostaining in normal canine mammary tissue during different stages of deslorelin and placebo implantations, 8A, 8B, 8C and 8D represent PR immunostaining at before, after 1 week, after 2 weeks and after 12 weeks of deslorelin implantation respectively, 8E, 8F, 8G and 8H represent PR immunostaining at before, after 1 week, after 2 weeks and after 12 weeks of placebo implantation respectively. Arrow head and arrow show, respectively, negative and positive immunostaining cells.

The significantly higher ERα score in the deslorelin group were shown at 2 weeks after implantation, while the ERα immunostaining score at other stages was not significantly different. When compared between groups, at 2 weeks after implantation the ERα score in the deslorelin group was significantly higher than the placebo group.

The score of the PR positive cells in the deslorelin group was highest at 2 weeks after implantation, which was significantly different to before implantation. In the placebo group, the lowest score was observed at 12 week after implantation which was significantly different to 1 week after implantation. When compared between deslorelin group and placebo group, the significantly higher of the PR scores were observed in the placebo group before implantation and 1 week after implantation.

4.4.3 Correlation between serum estradiol-17β and progesterone, and ERα and PR scores

The positive correlation was found between the ERα score and PR score in both deslorelin and placebo groups ($r = 0.48$, $P \leq 0.01$ and $r = 0.39$, $P \leq 0.01$ respectively).

The positive correlation was found between the levels of serum progesterone and ERα score in deslorelin group ($r = 0.53$, $P \leq 0.01$).

4.5 Discussion

In the present study, all bitches in deslorelin group showed estrous signs after implantation and subsequently turned to quiescent stage. This finding demonstrated the stimulating effect of deslorelin on the hypothalamic-pituitary-ovarian axis, which induced pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH). Subsequently, ovarian steroid secretion was induced but it would be suppressed after sustained stimulation which caused by desensitization of GnRH receptor in the anterior pituitary and brain (Corbin, 1982).

The highest ERα score at 2 weeks after implantation were observed in deslorelin group. At this stage, all 12 bitches showed the estrous sign, detected by vaginal cytology and hormonal pattern in which estradiol-17β concentrations being decreased while progesterone concentration increased. This finding indicated the stimulating effect

of this GnRH agonist; deslorelin, at early stage after implantation. The ERα score was low at 12 weeks after implantation in deslorelin group, which may due to the suppression effect of GnRH agonist through the ovarian function. This finding supported the study in human uterine leiomyomas which demonstrated the decreasing of ERα expression after long term GnRH agonist therapy (Vu et al., 1998). In placebo group, the ERα score was constantly low in all stages of implantation due to anestrous stage of bitches, in which serum progesterone concentration was not changed throughout the stage. When compared between deslorelin and placebo groups, the ERα score in deslorelin group was higher than in placebo group at 2 weeks after implantation, this may be caused by the stimulating effect of deslorelin at early stage of implantation.

As for ERα, PR score was significantly high at 2 weeks after implantation in deslorelin group. This similar pattern of the expression of both receptors may be due to the stimulating effect of deslorelin on the ovarian activity as previously described for ERα. In placebo group, the constant score of PR was observed at before, 1 and 2 weeks after implantation, whereas lower score was found at 12 weeks after implantation. This may be caused by the increasing serum progesterone concentrations at 12 weeks after implantation resulting in the down-regulation of PR at that stage for placebo group. When compared between groups before the implantation, the mean PR scores in placebo group were higher than in deslorelin group, which may cause by the variation of receptor expression in the individual bitch rather than from the effect of deslorelin. However, the PR scores in placebo group were similar before implantation until 2 weeks after implantation. At 12 weeks after placebo implantation, two bitches in this group were in estrus which made the serum estradiol-17β increased. Moreover, two bitches in this group turned to the stage of diestrus which made the serum progesterone increased as well, and may result in the down-regulation of PR at that stage. However, the rest of the bitches were still in anestrus.

The present study demonstrated the highest estradiol-17β concentration before implantation in deslorelin group. This finding may be due to the fluctuation of the estradiol-17β concentration in individual bitch at anestrus which are assumed to derive from waves of follicle development (Feldman and Nelson, 1996) or the stage of estrous cycle may be in late anestrus. However, the highest estradiol-17β concentration from

the bitches of this group might be shown during first and second week (at mid-estrous stage) was in between the time of data collection of the study. In placebo group, the highest estradiol-17β concentration was found at 12 weeks after implantation, which caused by the estrous bitches at that time. For the results of correlations, the deslorelin group demonstrated that the ERα score was positive correlated with the serum progesterone level. This finding was not in agreement with the previous studies in normal mammary tissues in other species (Battersby et al., 1992; Shyamala et al., 2002) and canine uterine tissue (Vermeirsch et al., 2000; Vermeirsch et al., 1999), which were demonstrated the negative correlation. This can be explained that at the stimulation stage of deslorelin (at 2 weeks after implantation), the serum progesterone level was increasing but were not high enough to suppress the expression of the ERα. This explanation revealed the specific physiological estrous steroid hormones in dog which is different from other species. Moreover, the positive correlation between ERα score and PR score that found in the present study supported the hypothesis that the presence of PR is indicated that ER was function. Since PR was identified as an ER-regulated gene product (Graham and Clarke, 1997; Osborne et al., 2005).

In conclusion, this is the first report on the effect of deslorelin on ovarian steroid receptors in normal mammary tissues. The results from the present study showed the similar pattern on the expression of both ERα and PR, which was up-regulated in the stimulation stage (at 2 weeks after deslorelin implantation) and down-regulated in the quiescent stage (12 weeks after deslorelin implantation). In addition, further study on the longer-period effect of deslorelin implantation in hormone-dependent canine mammary tumor should be performed to investigate the alteration of mammary tumor tissue in relation to the ovarian steroid hormone receptors and to make the alternative method for canine mammary tumor therapy.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

The growth and development of the canine mammary gland and etiology of the mammary gland tumors were regulated by numerous factors. The ovarian steroid hormones; estrogen and progesterone also played an important role in the development of the normal and tumoral mammary tissue. In order to better understand the regulation and role of ovarian steroid receptors on the various conditions of canine mammary tissues, the immunohistochemical detection of estrogen receptor alpha (ERα) and progesterone receptor (PR) was investigated.

5.1. Methodological considerations

5.1.1. Animals

The bitches in experiment 1 and 3 had a reproductive history with no complications from any reproductive disease. All of these bitches showed normal estrous cycle with no any pathological finding on the mammary glands. In experiment 2, the difference of the steroid receptors localization between normal and mammary tumor tissues in the same bitch was compared. Normal mammary tissues from the same bitch were collected from contra-lateral mammary gland and confirmed with the histological examination.

5.1.2. Immunohistochemical assay

The advantages of immunohistochemical assay with respect to others biochemical assays are the ability to observe the precise histological identification of tissue structure with ovarian steroid receptors protein. Moreover, this technique can use the routine histological methods for tissue fixation and processing. However, the variation in sensitivity and specificity of the antibodies used i.e. different antibodies to the same protein as well as different protocols may give different results. The scoring method used in this study followed the human scoring system, which counted the different levels of the intensity, calculated and presented as continuous data.

5.2. Immunohistochemistry

The steroid receptors ERα and PR are discussed separately for each condition of mammary tissues. In general, the immunostaining of the ERα and PR was localized in the nuclei of alveolar and tubular epithelial cells.

5.2.1. Normal mammary tissues in cyclic bitches

The same pattern of the ERα and PR scores in both cross-sectional group and longitudinal group was observed. The high ERα score at proestrus, estrus and the lowest ERα score at diestrus were observed in both groups. The variation in ER score during different stages of the estrous cycle may be related to the serum estradiol-17β and progesterone levels, it was high when serum level of estradiol-17β was high, whereas low score was observed when serum progesterone level was low. Also for PR, The lowest PR score was observed during diestrus and mid diestrus in cross-sectional group and longitudinal group respectively may due to the increasing progesterone levels at that stage.

The ERα and PR scores were negative correlated with the serum progesterone level, while the level of estradiol-17β was not correlated with the receptors scores. In conclusion, the results showed that ERα and PR were regulated by ovarian steroid hormones, which may be down-regulated by progesterone. Estradiol-17β seems to have the up-regulation on the presence of ERα and PR.

5.2.2. Normal mammary tissues and tumoral mammary tissues

Immunostaining was recognized in normal mammary tissues as well as benign and malignant mammary tumors. The ERα and PR scores between normal and tumoral mammary tissues were not different in the same bitch. This finding suggested similar regulation of both steroid receptor expressions in those tissues within the same bitch. However, it seems that normal mammary glands have higher mean percentage of ERα as well as PR than that in mammary tumors from the same bitch though not significant.

A positive correlation was found between the number of ERα and PR in both normal and tumoral mammary tissues. The positive correlation between ERα and PR from the present study may indicate that either ERα or PR could be used as a prediction parameter of hormonal therapy in bitches. Moreover, it appears that half of the mammary tumors from the present study are hormone-independent tumors (ERα and PR negative).

This finding supports the multifactorial causes of mammary tumor in animal and human. Therefore, the treatment of canine mammary tumor by hormonal therapy should be carefully considered.

5.2.3. Mammary tissues of deslorelin implanted bitches

The immunostaining of ERα and PR were observed in both deslorelin implanted and placebo implanted groups. The ERα and PR score in deslorelin implanted group were highest at 2 weeks after implantation, in which deslorelin played the stimulatory effect on the hypothalamic-pituitary-ovarian axis. Moreover, the receptor scores were low at 12 weeks after implantation which showed the suppression of the hypothalamic-pituitary-ovarian axis after sustained stimulation. In placebo implanted groups, the receptor scores were not significantly different, this finding may be caused by the stage of the estrous cycle of the bitches in this group were mostly in anestrus.

The positive correlation between the level of progesterone and ERα score was observed in deslorelin implanted group. This finding was not in agreement with other previous studies in normal mammary tissues in other species (Battersby et al., 1992; Cheng et al., 2005; Ricketts et al., 1991; Soderqvist et al., 1993) and canine uterine tissue (Vermeirsch et al., 1999), which demonstrated the negative correlation. This can be explained that at the stimulation stage the serum progesterone level was increasing but were not high enough to suppress the expression of the ERα. In conclusion, deslorelin may have the effect on the expression of ovarian steroid receptors of canine mammary tissue by acting on the hypothalamic-pituitary-ovarian axis, in which stimulated the expression when initially implanted and subsequently suppressed after sustained stimulation.

5.3. Conclusions

The immunopresence of ERα and PR in various conditions of canine mammary tissues in the present study supported the hypothesis that the ovarian steroid hormones, estrogen and progesterone play an important role in the growth and development of normal canine mammary gland and also in pathogenesis of the canine mammary tumors. The estradiol-17β and progesterone levels had the effects on the expression of their receptors, which was up-regulated by estrogen and down-regulated by progesterone.

The status of the receptors can be used as a predictive marker for endocrine therapy response and can also be a useful tool to select mode of therapy in canine mammary tumors. The GnRH agonist (Deslorelin) had an effect on the expression of ERα and PR in normal canine mammary tissues by acting on hypothalamic-pituitary-ovarian axis, which suppressed the production of ovarian steroid hormones, therefore at 12 weeks after implantation the expression of steroid receptors, both ERα and PR was down-regulated.

Further study on receptors mRNA should be performed to investigate the functional state of the receptors. Moreover, deslorelin implantation should be performed on the bitches with hormone-dependent mammary tumor to investigate the effect of deslorelin on mammary tumor and to make the alternative method for the treatment of canine mammary tumor.

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