การศึกษาเปรียบเทียบค่าเฉลี่ยของระดับฮอร์โมนโปรเจสเตอโรนในซีรั่มและระยะลูเตียลภายหลังการฝังจีเอ็นอาร์เอชอะโกนิศต์เดสโลเรลินระยะสั้นหรือระยะยาวในสุนัขระยะแอนเอสตรัส

นาย ธิติ ลือพงศ์ลัคณา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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COMPARISON OF MEAN SERUM PROGESTERONE LEVELS AND LUTEAL PERIOD FOLLOWING ANESTROUS BITCHES IMPLANTED WITH GNRH AGONIST, DESLORELIN, SHORT OR LONG-TERM

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A Thesis Submitted in Partial Fulfillment of the Requirements
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การศึกษานี้มีวัตถุประสงค์เพื่อสังเกตผลของการฝังจีเอ็นอาร์เอชอะโกนิสต์ชนิดเดสโลเรลินระยะสั้นหรือระยะยาวต่อระยะลูเตียลและระดับฮอร์โมนโปรเจสเตอโรนในกระแสเลือดในสุนัขเพศเมีย โดยใช้สุนัขเพศเมียที่มีสุขภาพแข็งแรงซึ่งอยู่ในระยะแอนเอสตรัสจำนวน 18 ตัว และสุนัขเพศผู้ที่มีความสมบูรณ์พันธุ์จำนวน 2 ตัว ทำการแบ่งสุ่มสุนัขเพศเมียออกเป็น 4 กลุ่ม กลุ่มที่ 1 จำนวน 4 ตัวได้รับการฝังเดสโลเรลินในขนาด 4.7 มิลลิกรัมและทำการถอนออกในวันแรกของระยะเอสตรัส กลุ่มที่ 2 จำนวน 6 ตัวได้รับการฝังเดสโลเรลินขนาดเดียวกันตลอดการทดลอง สุนัขทั้ง 2 กลุ่มนี้ได้รับการผสมพันธุ์ตามธรรมชาติและการผสมเทียมในระยะเอสตรัส และทำการตรวจการตั้งท้องโดยวิธีอัลตร้าซาวน์ กลุ่มที่ 3 จำนวน 4 ตัว มีวิธีการทดลองเหมือนกับกลุ่มที่ 2 แต่ไม่ได้รับการผสมพันธุ์ และกลุ่มที่ 4 จำนวน 4 ตัว เป็นกลุ่มควบคุมโดยมีวงรอบการเป็นสัดตามธรรมชาติ การเปลี่ยนแปลงระยะต่างๆของวงรอบการเป็นสัดประเมินโดยการเปลี่ยนแปลงทางกายภาพและพฤติกรรม การตรวจเซลล์เยื่อบุช่องคลอด และการวัดระดับฮอร์โมนโปรเจสเตอโรนในกระแสเลือดตามช่วงเวลาที่กำหนด หลังสิ้นสุดการทดลองทำการเปรียบเทียบค่าเฉลี่ยของระยะไดเอสตรัสในแต่ละกลุ่มในทางสถิติโดยใช้การวิเคราะห์ความแปรปรวน และระดับฮอร์โมนโปรเจสเตอโรนในกระแสเลือดโดยใช้สถิติเชิงพรรณนา ผลการทดลองพบว่าสุนัขทุกตัวแสดงอาการเป็นสัดและมีการตกไข่ยกเว้น 1 ตัวในกลุ่มที่ 3 ค่าเฉลี่ยของระยะไดเอสตรัสในกลุ่ม 3 (31.7±2.7 วัน) สั้นกว่ากลุ่มที่ 1 2 และ 4 อย่างมีนัยสำคัญ ($p<0.05$) (56.3±3.1 วัน 45.3±5.0 วัน และ 54.8±1.7 วัน ตามลำดับ) การเปลี่ยนแปลงของระดับฮอร์โมนโปรเจสเตอโรนในกลุ่มที่ 1 และ 2 คล้ายคลึงกับสุนัขที่มีวงรอบการเป็นสัดตามธรรมชาติ ค่าเฉลี่ยสูงสุดของระดับฮอร์โมนโปรเจสเตอโรนในกระแสเลือดของกลุ่มที่ 3 มีค่าต่ำกว่ากลุ่มอื่นๆและมีการลดระดับเร็วกว่ากลุ่มอื่นๆอีกด้วย สุนัข 3 ตัวจากกลุ่มที่ 1 และ 2 ตรวจพบว่ามีการตั้งท้อง สุนัขทุกตัวที่ตั้งท้องในกลุ่มที่ 1 สามารถตั้งท้องและคลอดได้ตามปกติ ในทางกลับกันสุนัข 2 จาก 3 ตัวที่ตั้งท้องในกลุ่มที่ 2 แท้งในวันที่ 51 และ 53 ตามลำดับ และเกิดการแท้งอย่างสมบูรณ์ภายใน 3 วัน สรุปผลการทดลองได้ว่าการฝังเดสโลเรลินขนาด 4.7 มิลลิกรัมในสุนัขเพศเมียที่อยู่ในระยะแอนเอสตรัสสามารถเหนี่ยวนำการเป็นสัดได้อย่างมีประสิทธิภาพและมีวงรอบการเป็นสัดคล้ายคลึงกับสุนัขที่มีวงรอบการเป็นสัดตามธรรมชาติในกรณีที่ถอนเดสโลเรลินออกในวันแรกของระยะเอสตรัส ในทางตรงกันข้ามการฝังฮอร์โมนแบบระยะยาวเพื่อคุมกำเนิดจะทำให้ระยะไดเอสตรัสสั้นลงหรือเกิดการแท้งขึ้นแต่การแท้งอาจไม่เกิดขึ้นกับสุนัขทุกตัว ดังนั้นควรหลีกเลี่ยงการผสมพันธุ์ในช่วงที่เป็นสัดหลังจากการเหนี่ยวนำโดยเดสโลเรลิน

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THITI LUEPONGLUKANA : COMPARISON OF MEAN SERUM PROGESTERONE LEVELS AND LUTEAL PERIOD FOLLOWING ANESTROUS BITCHES IMPLANTED WITH GNRH AGONIST, DESLORELIN, SHORT OR LONG-TERM. ADVISOR : ASSOC. PROF. SUDSON SIRIVAIDYAPONG, D.V.M., Ph.D., 43 pp.

The objectives of the study were to investigate the effect of short or long-term GnRH agonist deslorelin implantation on duration of luteal period and serum progesterone concentrations in bitches. Eighteen healthy anestrous bitches and two fertile male dogs were used in this study. All bitches were randomly divided into 4 groups. Group 1 (n=4), bitches received 4.7 mg deslorelin which was removed at the first day of estrus. Group 2 (n=6), received 4.7 mg deslorelin which was removed at the end of experiment. Group 1 and 2 were mated and inseminated during estrous period and detected pregnant by ultrasonography. The same protocol as group 2 was used for group 3 but without insemination. And group 4 (n=4), bitches process a spontaneous estrous cycle which were used as control. All bitches were examined for the stage of estrous cycle by physical and behavioral signs, vaginal cytology and serum progesterone concentrations on required dates. Statistic analytical process using ANOVA for evaluated a mean interval of day in luteal period (mean±SEM) in each group. Mean concentrations of progesterone were analyzed using descriptive statistic. The results showed that all bitches came into estrous stage and ovulated except 1 bitch in group 3. Mean diestrous period in group 3 (31.7±2.7 days) was significantly shorter than group 1, 2 and 4 (56.3±3.1 days, 45.3±5.0 days and 54.8±1.7 days, respectively) ($P<0.05$). Progesterone profiles in group 1 and 2 resemble natural estrous dogs. Mean serum progesterone concentrations in group 3 exhibited a low peak and premature declined compared with other groups. Three bitches in group 1 and 2 were found to be a pregnant. All pregnant bitches in group 1 had a normal pregnancy and whelping. In group 2, 2/3 pregnant bitches started aborting on day 51 and 53, respectively and aborting was completed within 3 days. In conclusion, the results of this present study clearly revealed that short-term implantation of 4.7 mg deslorelin in anestrous bitches is effective and practical method for estrous induction. Bitches processed normally estrous cycle as natural cyclic dogs if the implants were removed at the first day of estrus. In contrast, long-term implantation to generate contraception produced premature luteal failure, resulting shortened diestrus or interrupted pregnancy but this effect may be incomplete. Therefore, a mating in flare-up period should be avoided.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
CL	corpus luteum
CMIA	chemiluminescent microparticle immunoassay
FSH	follicular stimulating hormone
GnRH	gonadotropin releasing hormone
GPCRs	G-protein coupled receptors
LHRH	luteinizing-hormone releasing hormone
LH	luteinizing hormone
MHz	mega hertz
n/a	not available
PRL	prolactin
SEM	standard error of the mean
µg	microgram

CHAPTER I

INTRODUCTION

Important and Rationale

The diestrous period in bitches is unique compare to other domestic animals. Serum progesterone level increases for as long as 2 months either cyclic diestrus or pregnancy. Progesterone profiles are indistinguishable until late in diestrus or gestation (Concannon et al., 1975; Özyurtlu et al., 2005). But mean fecal progesterone concentration in pregnant bitches is higher than non-pregnant bitches (Gudermuth et al., 1998). The corpus luteum (CL) is the unique source of circulating progesterone during diestrus, which plays a critical role in the maintenance of pregnancy (Kiso and Yamauchi, 1984). Both prolactin (PRL) and luteinizing hormone (LH) have been proposed to be a luteotrophic factor around the second half of diestrus and pregnancy (Concannon et al., 1987; Okkens et al., 1990). Progesterone level in the diestrous period can be suppressed by many synthetic compounds, following diestrous or pregnancy interruption, such as antiprogestin, prolactin-lowering drugs, LH-antiserum and GnRH analogues (Gobello, 2007).

Gonadotrophin releasing hormone (GnRH) is the key hormone of reproductive function in mammals. GnRH is secreted by the hypothalamus, stimulating the release of LH and FSH (follicular-stimulating hormone) from the anterior pituitary gland to the blood stream. These hormones regulate the gonads to produce sex steroid hormones (Schneider et al., 2006). GnRH agonists have been widely developed and information about their applications are abundant in the literature (Tilbrook et al., 1992; Herbert et al., 2006; Trigg et al., 2006; Fontaine and Fontboone, 2011). GnRH agonists are now produced in a sustained-release form which is an implant administered to subcutaneous

tissue (Trigg et al., 2001). GnRH agonists have 2 major effects; stimulation and inhibition of estrous cycle, which result numerous clinical applications.

Long-term GnRH agonists administration produce an inhibition effect; these are used for contraception. However, estrous induction after the beginning of implantation is the considerable problem. Because induced bitches could mate and become pregnant. Trigg et al. (2001) noted that if progesterone level is more than 5 ng/ml at the time of implantation, they did not show an induced estrus but this technique is not practical for stray dogs because most of their estrous cycle is anestrus (Jöchle and Andersen, 1976). Following the advice of Trigg et al. (2001), Wright et al. (2001) treated with progestin before implantation to prevent the initial stimulating effect but the results obtained are equivocal (Corrada et al., 2006; Sung et al., 2006).

Wright et al. (2001) and Romagnoli et al. (2009) reported that a formulation delivering 6 or 4.7 mg deslorelin over about 6 months in anestrous bitches, produced pregnancy failure at approximately day 40 of gestation followed by initial estrous induction. Also, estrus was induced in 7 bitches by 2.1 mg deslorelin, two out of three conceived bitches started aborting around 6-7 week after pre-ovulatory LH surge and only one remained pregnant to term (Kutzler et al., 2001). These studies showed that even if bitches were mismated in the initial stimulating period with GnRH agonist (flare-up effect), pregnant dogs aborted. However, the effect of down-regulation in deslorelin-induced bitches may complicate (Volkmann et al., 2006b) and should be investigated.

In contrast, the stimulating effects of GnRH agonist have been presented in many studies. Kutzler et al. (2009) demonstrated that high pregnancy rates were achieved after removal of the 2.1 mg deslorelin implants from the vestibular mucosa when progesterone concentration first exceeded 1.5 ng/ml. In same study, implants were inserted in the subcutaneous tissue between the shoulder blades during the luteal period, 37.5% treated bitches produced premature luteal failure. The result agrees with

Walter et al. (2011) who removed the 4.7 mg deslorelin implants when the first day of bloody vaginal discharge was detected. Pregnancy rate did not differ from untreated dogs. Unfortunately, early ovariohysterectomy was performed before investigations of the corpus luteum entire luteal phase.

Deslorelin implants offer many benefits for management and control of estrus in bitches. All treated animals seem to respond in the same way, but many factors related to the mechanism of hormone action is not yet clear, especially in luteal period.

Hypothesis

Mean serum progesterone levels and duration of diestrous period after short-term implantation with 4.7 mg GnRH agonist deslorelin in anestrous bitches is greater than long-term implantation

Objectives

1. To compare mean serum progesterone levels and duration of the luteal period in anestrous bitches implanted subcutaneously with 4.7 mg of the GnRH agonist, deslorelin
2. To investigate the effect of long-term implantation of GnRH agonist, deslorelin, in anestrus to pregnant and non-pregnant bitches
3. To determine the effect of estrous induction by short-term GnRH agonist, deslorelin, on the entire luteal period

Definition of words

Desensitization: a process to reduce or eliminate a sensitivity of receptor cells following prolongs exposure to a substance or stimulus.

Down-regulation: a process which decrease the number of receptors on the surface of target cells, creating the cells less sensitive to hormone or another agent.

Flare-up effect: the outcome of greatly increase in LH and FSH after initial stimulation of GnRH agonists following exhibit estrous signs.

Premature luteal failure: an early decrease in a serum progesterone concentration during diestrous period after continuous exposure of GnRH agonists.

Up-regulation: a process which increase the number of receptors on the surface of target cells, creating the cells more sensitive to a hormone or another agent.

Expected output

1. To apply the protocols of implantation for reproduction control in female dogs
2. To increase the alternative choices for estrous induction and non-surgical contraception in female dogs

CHAPTER II

LITERATURE REVIEW

The estrous cycle

The estrous or reproductive cycle in the domestic dog (*Canis familiaris*) is generally described as non-seasonal monoestrus, because bitches may exhibit estrus at any time of the year and show only one estrus per estrous cycle. Pubertal estrus occurs variably at 6-14 months in most breed. The estrous cycle has been divided into 4 stages: proestrus, estrus, diestrus and anestrus (Jöchle and Andersen, 1976).

Proestrus is the first stage of the reproductive cycle of the bitches. It lasts on average 9 days (from 0-27 days). It is recognized by bloody or serosanguineous vaginal discharge, swelling and edema of vulva. In addition it is not receptive to mating. Vaginal cytology presents mixture of epithelial cells type and erythrocyte, bacteria and neutrophil in early to mid proestrus. Serum concentrations of estradiol increase throughout proestrus correlated with follicular development, while serum concentrations of progesterone remain at basal level (< 1.0 ng/ml) until the LH surge (Johnston et al., 2001).

Estrus, when the bitch accepts the male lasts about 9 days. The vulva remains enlarged but is usually softer than it was during proestrus. Vaginal cytology in the first day of estrus presents more than 90 percent of superficial cells. Ovulation occurs approximately 2-3 days after a pre-ovulatory LH surge. Canine oocytes ovulate as primary oocyte and maturated into secondary oocyte in 2-3 days (Tsutsui, 1989; Johnston et al., 2001). Serum progesterone concentrations rapidly increase after the LH surge and attain maximum levels at 25-30 days after the onset of estrus (Jöchle and

Andersen, 1976) and progesterone assay are a reliable technique for the detection of ovulation in bitches (Lévy and Fontbonne, 2007).

During diestrus, bitches become refractory to breeding, with diminished attraction of male dogs and vulval edema is slowly resolved. This period begins with a sharp decrease in the percentage of superficial cells by at least 20 percent, and increase in the percentage of intermediate and parabasal cells. Neutrophilic leukocytes are found in variable numbers in vaginal smears (Holst and Phemister, 1974). During diestrus, CLs are fully functional and serum concentrations of progesterone rise to a peak of 15-90 ng/ml between 15-30 days after LH surge and progressively decline in late diestrus. Diestrus lasts an average of 60 days (Feldman and Nelson, 1996a; Johnston et al., 2001).

Anestrus is the period of transition between one cycle and the next. Bitches in anestrus are not attracted to or receptive to male dogs. The ovaries are inactive, uterine involution and endometrial repair occurs. The vulva is small with no or minimal discharge. Parabasal and intermediate cells are the predominant cell types in the vaginal smears at this time. The onset of anestrus occurs when serum progesterone decline to levels persistently below 1-2 ng/ml (Feldman and Nelson, 1996b).

Luteal physiology

The diestrous period or luteal period is defined as the phase of progesterone dominance following estrus, but if breeding occurs during estrus, diestrus may be replaced by pregnancy. In bitches, the CL is the unique source of circulating progesterone during both the estrous cycle and pregnancy (Kiso and Yamauchi, 1984). Serum concentrations of progesterone rise above basal level at the end of proestrus to a level greater than 1-2 ng/ml at the onset of estrus. The maximal rate of secretion occurs approximately 2-3 weeks after the beginning of diestrus, usually in the range around 15-

90 ng/ml. After this time, serum concentrations of progesterone begin a gradual decline that continues for 5-6 weeks, and less than 1-2 ng/ml at the end of diestrus or prior to whelping (Johnston et al., 2001; Verstegen-Onclin and Verstegen, 2008). Progesterone plays a critical role in the maintenance of pregnancy, including endometrial growth, secretion of uterine fluid, maintenance of placental integrity and inhibition of uterine contraction (Feldman and Nelson, 1996b; Chu et al., 2001). Concannon and Hansel (1977) and Vickery and McRae (1980) noted that pregnancy was terminated if a premature drop of progesterone to lower than 2 ng/ml for more than 48 hours, occurs. Also, hypoluteoidism a disease of the CL during which insufficient progesterone is produced, can interrupt pregnancy in bitches (Görlinger et al., 2005).

There does not appear to be much difference between pregnant and non-pregnant bitches, in the duration of the luteal phase or the mean maximum progesterone level. However, pregnant bitches reach baseline progesterone concentrations earlier than non-pregnant (Concannon et al., 1975; Özyurtlu et al., 2005). Moreover, between days 26-45 after ovulation, mean fecal progesterone concentration is higher in the pregnant than the non-pregnant bitch, but this difference is usually not found in plasma or serum concentrations because of an increase in hemodilution, metabolism and clearance of progesterone in pregnant bitch (Gudermuth et al., 1998).

PRL a peptide hormone primarily associated with lactation and mammary gland development, is synthesized and secreted by pituitary gland. PRL increases during the second half of diestrus, the peak levels of PRL occur around parturition and remain high throughout lactation. This is maintained by suckling of the pups (Jöchle, 1997; Concannon et al., 1978). Increasing of plasma PRL is often involved in the etiology of pseudopregnancy in bitches (Gobello et al., 2001; Tsutsui et al, 2007). Plasma PRL levels in pregnant bitches are much higher than in non-pregnant bitches during the last

part of luteal phase, but PRL concentrations are unreliable for pregnancy diagnosis because of individual variation (De Coster et al., 1983; Kutritz, 2005).

The source of estradiol in the luteal phase originates from CL (Nishiyama et al., 1999). Its role in the regulation of the canine CL has not been clearly identified, but in other species such as rats, rabbit and pig, estradiol plays a role in luteal regulation (Hilliard, 1973; Garverick et al., 1982). Plasma estradiol increases rapidly reaching peak during late proestrus, and falls rapidly during estrus. Estradiol increases again during late estrus or early diestrus and remains elevated throughout luteal period, with a similar pattern in pregnant and non-pregnant bitches (Jones et al., 1973; Onclin et al., 2002). However, Concannon et al. (1975) reported that estradiol levels in pregnant bitches are higher than in non-pregnant bitches during the last part of luteal period. Increases in fecal estradiol concentrations are pregnancy specific (Gudermuth, et al., 1998).

LH and FSH are produced by anterior pituitary gland, regulate gonadal function. During the mid to late luteal phase, mean LH and FSH level elevated throughout in pregnant relative to in non-pregnant bitches. Their concentrations decrease around parturition and lactation (Onclin et al., 2001; Onclin et al., 2002). The role of LH and FSH during luteal phase of the dog is still controversial.

Relaxin is the only plasma hormone that can be used for pregnancy diagnosis, because it appears to be entirely of placental origin (Tsutsui and Stewart, 1991). It rises approximately 21-24 days after the LH sure, peak around 2-3 weeks before whelping and remains detected after parturition for 4-60 days. The roles of relaxin is not yet clear; but it is thought to promotes PRL secretion (Steinetz et al., 1987; Concannon et al., 2001).

Luteal regulation

Luteal regulation in the diestrous or pregnant bitch is complex and is influenced by several hormones, the most important being progesterone, PRL and LH. In bitches, the CL is the unique source of progesterone during diestrus or pregnancy (Tsutsui, 1983; Kiso and Yamauchi, 1984). The ovaries and pituitary seem to be required for maintenance of a normal progesterone secretion or pregnancy (Concannon, 1980; Tsutsui, 1983; Eilts et al., 1993). Luteal progesterone secretions in both pregnant and non-pregnant bitches appear to be dependent on both LH and PRL.

Progesterone secretion was inhibited by hypophysectomy (Concannon, 1980) or an injection of equine LH antiserum (Concannon et al, 1987), this significantly reduces progesterone concentration during the second part of the luteal phase (Okkens et al., 1986). Also, LH suppression by the GnRH antagonist was sufficiently luteolytic to terminate pregnancy when used at low and high dose in mid-pregnancy (Vickery et al., 1989; Valiente et al., 2009a). These indicated that LH is necessary for maintenance of progesterone levels during the last part of luteal period. However, another study reported that PRL was the main luteotrophic factor in the cyclic dog during luteal period (Okkens et al., 1990; Hori et al., 2004).

The luteotrophic effect of PRL was confirmed by using dopamine agonists which lower prolactin at the mid-luteal phase or later, resulting in luteal suppression or pregnancy termination (Post et al., 1988; Onclin and Verstegen, 1997). The role of PRL supports the longevity of the CL and its function rather than directly stimulating progesterone secretion (Onclin and Verstegen, 1997). In dogs and others species, PRL may regulates LH receptors on the CL (Grinwich et al., 1976; Onclin and Verstegen, 1997). Onclin et al (2000) conclude that LH administration does not stimulate progesterone secretion, but may stimulate PRL secretion in the last third of pregnancy.

The role of estradiol and relaxin for luteal regulation in bitches is not clear, but appears different from other species such as rat, rabbit and pig (Hilliard, 1973; Garverick et al., 1982; Huang et al., 1993). Prostaglandin $F_2\alpha$ which originates from the endometrium, and which is a causative factor for luteolysis in ruminants (Silvia et al., 1991), is not present during the luteal phase of the cyclic dogs (Okkens et al., 1985; Hoffman et al., 1992).

In conclusion, during the first half of pregnancy the CL functions independently of pituitary support. This differs from the second half where the pituitary gland is essential to maintain luteal function. However, luteotrophic factors are not well understood and require further investigation.

GnRH and GnRH receptors

The decapeptide hormone gonadotropin releasing hormone (GnRH) (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2), also known as luteinizing-hormone releasing hormone (LHRH) is produced by neurosecretory cells, called GnRH neurons. They are found in the preoptic area of the hypothalamus (Guyton and Hall, 1996). In response to neural signals, GnRH is released from hypothalamic nerve ending in a pulsatile manner in every 30-120 minutes and delivered to the anterior pituitary gland by the hypophyseal portal veins (England, 1998; Millar, 2005). In the anterior pituitary gland, GnRH binds to the GnRH receptors on gonadotropic cells to stimulate the releasing of gonadotropins, FSH and LH to the circulation. They act on the gonads to regulate gametogenesis and hormonogenesis (Gobello, 2007). At present, there are 3 isoforms of GnRH have been identified, GnRH-I, -II and –III. GnRH-I or type one mammalian GnRH (mGnRH) is the hypothalamic form of GnRH. It regulates reproduction across wide variety of organisms. The second form of GnRH-II first identified from chicken brain is ubiquitous in vertebrates from bony fish to humans and the third form, type three GnRH or GnRH-III. It has been

discovered in lamprey fish (Millar et al., 2004). In mammals, there are 2 forms, GnRH-I and –II but GnRH type I is the only one that plays a key role in the regulation of the reproductive function (Millar et al, 2004; Millar, 2005).

The GnRH receptor is a member of the seven-transmembrane, G-protein coupled receptors (GPCRs) family. It is expressed on the surface of pituitary gonadotrope cells as well as other tissues (Rispoli and Nett, 2005). Normally, receptor molecules are degraded and replaced on a regular basis, and the number of GnRH receptor is dependant on the stage of estrus (Clayton et al., 1980; Savoy-Moore et al, 1980). In the follicular phase, gonadotropes, increase in their GnRH receptor number and receptor affinity is increased above normal, called up-regulation of GnRH receptors. It is important for complete expression of the pre-ovulatory LH surge (Lozach et al., 1998; Stanislaus et al., 1998; Rispoli and Nett, 2005). In contrast, a down-regulation mechanism decreases the number of GnRH receptors during the luteal phase when the corpus luteum actively secretes progesterone (Rispoli and Nett, 2005). Desensitization of gonadotropins release by pituitary gland when GnRH is administered or secreted in a continuous mode normally divided into 2 categories; short-term desensitization which impairs the GPCRs function due to receptor phosphorylation enhances the binding of ß-arrestin, which leads to G-protein uncoupling and also serves to target the desensitized receptors for internalization and long-term desensitization associated with a decrease in receptor numbers (down-regulation) (Shacham et al., 2001). A unique feature of mammalian GnRH receptor is the absence of the carboxyl-terminal tail (C-terminal tail) (Millar, 2005). This receptor does not exhibit short-term rapid desensitization because the C-terminal tail is necessary for its phosphorylation and internalization. (Davidson et al., 1994; McArdle et al., 1999; Willars et al., 1999; Pawson and McNeilly, 2005). However, the mechanisms relating gonadotrope desensitization have not been clearly elucidated.

GnRH agonists

GnRH is necessary for reproductive function but endogenous GnRH has a short half-life of 2-4 min due to its rapid cleavage by endopeptidases in the hypothalamus and pituitary gland. Moreover, the binding affinity of endogenous GnRH to its pituitary receptor is quite low (Florio et al., 2002). Many GnRH agonists, which are generally longer acting and more potent than native GnRH have been developed. GnRH agonists are peptides that are similar to native GnRH but are modified at site of enzymatic degradation of GnRH. Substitution of amino acid in position 6 and 9 increase receptor affinity and decrease enzyme degradation (Padula, 2005).

In the past, use of native GnRH or short-acting formulations of agonists required frequent injections or constant infusion. Daily product administrations were necessary to obtain a clinical effect. However these protocols are not practically use (Cain et al., 1988; Cain et al., 1989). Sustained-release forms of GnRH agonist such as leuprolide, nafarelin, azagyl-nafarelin, buserelin, goselerin and deslorelin have been developed. The mode of delivery includes delivery from osmotic mini-pumps, sylastic polymer containing and microencapsulate forms of the agonists. Advanced developments aim for convenience of application and long-term fertility control in domestic animals (Inaba et al., 1998; Herbert and Trigg, 2005; Rubion et al., 2006).

GnRH agonist is mainly divided into 2 categories; that effect stimulation and inhibition of fertility. In male dogs, the contraceptive effect of GnRH agonist is predominant. Its use is for chemical sterilization (Trigg et al., 2001; Trigg et al., 2006) and sex hormone-related conditions treatment include benign prostate-related conditions (Ponglowapan et al., 2002; Smith, 2008), and behavioral disorders (Goericke-Pesch et al., 2010). Meanwhile, both effects are necessary for bitches. Their properties are used for estrous induction, contraception (Gobello, 2007), treatment of hormone-dependent mammary tumors (Lombari et al., 1999; Pagnini et al., 2002) and post-

spaying disorders such as urinary incontinence (Reichler et al., 2003, Reichler et al., 2006) and hair coat changes (Reichler et al., 2008). Up to now, only 2 products, Gonazon® (18.5 mg azagyl-nafarelin, Intervet/Schering-Plough, France) and Suprelorin® (4.7 and 9.4 mg deslorelin acetate, Peptech Animal Health, Australia), that have been licensed to use in canine reproduction (Concannon, 2006; Driancourt et al., 2006).

GnRH agonist deslorelin

Deslorelin is a potent and long-acting of GnRH agonist. Its potency is 110-150 times than native GnRH with following structure p-Glu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-NHEt. It has been used for induction and inhibition of reproductive function in female canids (Herbert and Trigg, 2005; Schneider et al., 2006). GnRH agonist, deslorelin, treatment initially induces up-regulation of the receptors and increases of LH and FSH concentrations (flare-up effect). Continued exposure results in down-regulation of GnRH receptors on gonadotrope cells, suppressed pulsatile secretion of LH and decreased progesterone secretion (premature luteal failure) (Herbert and Trigg, 2005). The flare-up effect was not observed when bitches with progesterone levels in excess of 5 ng/ml were treated (Trigg et al., 2001) and in prepubertal bitches (less than 5 months) (Trigg et al., 2006). Wright et al. (2001) noted that oral megestrol acetate (dose 2.0 mg/kg) 7 days before deslorelin implanted was able to prevent the induced estrus. However, in trial by Sung et al. (2006) using the same protocol, 4 of the 5 bitches expressed estrus. Moreover, the potential of unfavorable side effects of progestins could be of concern (Cox, 1970). Some authors have recommended administering GnRH agonists during diestrus (Romagnoli et al., 2009), but this should not be effective in all cases. Estrous induction was reported in some bitches with progesterone levels greater than 60 ng/ml (Fontaine and Fontbonne., 2010).

Many studies have investigated the effect of GnRH agonists and adapted the knowledge for clinical applications. GnRH analogue (Ovuplant®, Peptech Animal Health, Australia), is licensed for use in equine practice, and contains 2.1 mg deslorelin in a 2 day release format has been used for estrus induction in 7 anestrous beagle bitches. Estrus was induced in all bitches, 3/7 became pregnant, but 2/3 subsequently lost their pregnancies around 39 and 51 days after the LH surge, perhaps due to failure of pregnancy caused by down-regulation of GnRH receptor (Kutzler et al., 2001). The results agree with Kutzler et al. (2009) but if the implants were removed when serum progesterone concentrations first exceeded 1.5 ng/ml, rate of premature luteal regression were lower than bitches were received deslorelin throughout the study (Kutzler et al., 2009). Deslorelin implant provides an effective and practical method of estrous induction in anestrous bitches and the pregnancy rate is not different from dogs with a natural estrus (Volkmann et al., 2006a). However, the effect of 2.1 mg deslorelin to suppress reproductive cycle is not complete because of its rapid release (MaCue et al., 2000; Volkmann et al., 2006b).

A new deslorelin implant (Suprelorin®) is now available which contains 4.7 and 9.4 mg deslorelin acetate and is licensed for suppression of male sexual function (Trigg et al., 2006). It is applied for using in bitches (Trigg et al., 2006) and other species (Eymann et al., 2007; Bertschinger et al., 2008; Wagner et al., 2009; Goericke-Pesch et al., 2011). In the studies by Wright et al. (2001) which using 6 mg deslorelin implants in anestrous bitches, they found that treated bitches lost their pregnancy approximately day 40 of gestation with low serum progesterone concentrations after initial flare-up period. The results resemble those of Romagnoli et al. (2009), where pregnancy failed around day 35 following a 4.7 mg deslorelin implantation in anestrus. These results most likely present complete premature luteal failure but the luteal phase was not investigated (Romagnoli et al., 2009). Walter et al. (2011) who removed the 4.7 mg deslorelin implants

when the first day of bloody vaginal discharge was detected showed pregnancy rate did not differ from those of dogs having a natural estrus. Unfortunately, early ovariohysterectomy was performed before further investigations of the CL could be made.

The aims of this study are (i) to compare mean serum progesterone levels and duration of the luteal period following subcutaneously implanted with GnRH agonist, deslorelin, in 2 patterns, (ii) to investigate the effect of long-term implantation of GnRH agonist, deslorelin, in anestrus to pregnant and non-pregnant bitches, and (iii) to determine the effect of estrous induction by short-term GnRH agonist, deslorelin, on the entire luteal period. This study, uses 4.7 mg deslorelin (Suprelorin®), implanted into anestrous bitches to investigate luteal periods and serum progesterone levels.

CHAPTER III

MATERIALS AND METHODS

Animals

Female dogs

Eighteen healthy bitches, 2-5 years old and weighing between 8.5-13 kg were used. All bitches had no history of hormonal treatments and reproductive diseases and had a record of being fertile. Bitches were considered anestrus when serum progesterone concentration less than 1.0 ng/ml and vaginal cytology contained more than 90% parabasal and intermediate cells (Feldman and Nelson, 1996a). All bitches were randomly divided into 4 groups. Group 1 (n=4), bitches received 4.7 mg deslorelin which was removed at the first day of estrus (Concannon, 2009; Johnston et al., 2001). This day was designated day 0. Group 2 (n=6), received 4.7 mg deslorelin which was removed at the end of experiment. Group 1 and 2 were mated and inseminated during estrous period and detected pregnant by ultrasonography. The same protocol as group 2 was used for group 3 (n=4) but without mating and insemination. And group 4 (n=4), bitches process a spontaneous estrous cycle which were used as control. All bitches were examined for stage of estrus cycle by physical and behavioral signs, vaginal cytology and serum progesterone concentration on required dates.

Male dogs

Two healthy and fertile male dogs, 1-3 years old and weighing between 13-15 kg were used for natural mating and artificial insemination. They were determined healthy by physical examination, complete blood count and serum chemical profiles. Fertility confirmed by reproductive history, reproductive examination and semen evaluation. Normal semen parameters issued by Kustritz (2007).

The animals were house in indoor-outdoor runs and were fed with standard commercial dog food once daily. Water was available *ad libitum*.

The studies were approved by the Ethical Committee of the Faculty of Veterinary Science, Chulalongkorn University

GnRH agonist deslorelin

The potent long acting GnRH agonist deslorelin prepared as a biocompatible cylindrical implant (12.5 mm long × 2.3 mm in diameter) was developed, manufactured and supplied by Peptech Animal Health Pty Limited, NSW, Australia. Implants were manufactured by proprietary method that involved extrusion of deslorelin with a matrix consisting principally of low melting point lipid and a biological surfactant. Each implant contained 4.7 mg of active ingredient deslorelin. Implant was 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 ºC until use. In a real time *in vitro* dissolution system, these implants release doses of > 1 µg/day for periods of > 6 months

The site of implantation

The implantation site was the concave site of the left pinna. Aseptic techniques were used to implant the animals. This required clipping the site and applying betadine and alcohol. Bleeding was stopped after implantation by putting pressure with gauze. For implant removal, bitches were sedated with xylazine (0.4 mg/kg, intravenous, once), then a scalpel blade (# 19) was used to incise the skin over the implant and the implant out carefully excised. The implantation site was intensively observed daily for 10 days after those methods, for signs of inflammatory and allergic reaction.

Determination of the stages of estrous cycle

The stage of estrous cycle was determined by physical and behavioral signs, vaginal cytology and serum progesterone concentration. Starting on the day of deslorelin implantation, physical and behavioral signs and vaginal cytology were evaluated everyday until the first day of diestrus. Serum progesterone concentration was measured in day 0 and 4 to confirm the first day of estrus and fertilization period, respectively. In the first day of diestrus, serum progesterone concentration was measured 1-2 times per week until the level less than 1.0 ng/ml

Physical and behavioral signs

The stage of the estrous cycle was determined by the receptiveness of bitches to male dog and change in the appearance of the external genitalia such as vulva swelling and serosanguineous vaginal discharge (Johnston et al., 2001).

Vaginal cytology

Exfoliated cells were obtained by passing a cotton-tipped swab into the cranial vagina. Care was taken to avoid obtaining sample from the clitoral fossa. The cotton swab was moistened with saline solution and inserted craniodorsal direction to the cranial vagina. Then it was rotated to obtain sample and subsequently withdrawn. The cotton tip was rolled gently on a glass microscopic slide. Air-dried smears were sequentially immersed in methanol and the two solutions that constitute the Dip-Quick stain (modified Wright-Giemsa stain) (Johnston et al., 2001). Evaluation of the types and number of vaginal cells was based on criteria reported by Holst and Pheminister (1974) and Post (1985).

Serum progesterone concentration

Blood sample (2 ml) was collected from cephalic vein into a sterile tube, and serum separated by stored at 4°C. The serum was assayed for progesterone (on the day of collection) by routine approved laboratory for canine progesterone measurement

using chemiluminescent microparticle immunoassay (CMIA) (ARCHITECT® Progesterone, Abbott laboratories, USA). The sensitivity of the assay was 0.1 ng/ml. The within-assay coefficient of variation ranged from 0.2 to 3.9%.

Stage of estrous cycle

The first day of proestrus

The onset of proestrus was determined as the first day of bloody vaginal discharge and enlargement or swelling of the vulva. Vaginal smears presented increase in the percentage of intermediate cells and decrease in the percentage of parabasal cells (Johnston et al., 2001).

The first day of estrus

The onset of estrus was defined when the proactive receptivity to mounting by male, vaginal cytology contained more than 90% superficial cells (Johnston et al., 2001; Concannon, 2009).

Diestrous period

The first day of diestrus was identified by a sharp decrease in the percentage of superficial cells by at least 20% and increase in the percentage of intermediate and parabasal cells (Holst and Phemister, 1974). The end of diestrous period was identified by the day of serum progesterone concentration declined below 1.0 ng/ml (Feldman and Nelson, 1996b).

Semen collection and evaluations

The sperm-rich fraction of the semen was collected by digital manipulation into a glass funnel and 15 ml centrifuge tube. Collection procedure was adapted from Kutzler (2005). Semen was examined for physical appearance, motility and total number of spermatozoa before used in the insemination procedure. The examination procedures and the normal range of semen parameters were issued by Kustriz. (2007).

Natural mating and artificial insemination

Natural mating

Male and female dogs (group 1 and 2) were housed together and let them mate naturally once to investigate the behavioral signs in the estrous stage.

Artificial insemination

All bitches in group 1 and 2 were inseminated intravaginally in day 2, 4 and 6 with fresh semen from the same dog used for natural mating (England and Concannon, 2002).

Ultrasonographic examination

Ultrasonographic examination was with transducers of 5.0 and 7.5 MHz. The animals were held in a dorsal recumbent position, the hair clipped from the area of ventral abdomen and conduction gel was applied to the skin. Ultrasonographic examinations began 17 days after day 0 and performed daily until day 25 (Yeager and Concannon, 1990b). If pregnancy was detected, examination was performed twice a week until the serum progesterone concentration fell to less than 1.0 ng/ml or parturition.

Statistical analysis

Statistic analytical process using one-way analysis of variance (ANOVA) for evaluated a mean interval of day in luteal period in each group. The values were presented as mean ± standard error of the mean (SEM). The level of significance was set at $P < 0.05$. Mean concentrations of progesterone were analyzed using descriptive statistic.

CHAPTER IV

RESULTS

Bitches did not exhibit any pain, allergic or inflammatory reactions at the site of implantation during the 10-days observation period or at any time during the experiment. The implants were easy to palpate under the skin and removal of the intact implants was easy to perform without infection or pain.

All bitches receiving deslorelin came into proestrous and estrous stage. The intervals between implant insertion and the first day of proestrus and estrus varied from 2-6 days (average 3.71 days) and 8-18 days (average 10.71 days), respectively. Ovulation failure was observed in only one bitch of group 3. Mean diestrous period did not differ significantly between group 1, 2 and 4. Meanwhile, mean diestrous period in group 3 was significantly shorter than other groups. Only data of bitches that had ovulated were used in this statistical analysis. The diestrous period in each group is summarized in table 1.

Serum progesterone concentrations rose during estrus and starting declined around 2-4 weeks after the day of estimated LH surge or the first day of estrus. Progesterone profiles in group 1 and 2 resemble natural estrous dogs (group 4). However, mean serum progesterone concentrations in group 3 exhibit a low peak and premature declined lower than 1.0 ng/ml compared with other groups. Mean serum progesterone concentrations in all groups are presented in figure 1

Table 1 The comparison of the duration of implantation to proestrus, estrus and diestrus (mean±SEM) between groups

Group	Duration (days, mean±SEM)			
	Implant-proestrus	Proestrus	Estrus	Diestrus
1*(n=4)*	4.0 ± 0.4	6.3 ± 0.8	6.3 ± 0.5	56.3 ± 3.1 [a]
2*(n=6)*	3.5 ± 0.6	6.7 ± 0.5	6.2 ± 0.9	45.3 ± 5.0 [a]
3*(n=4)*	3.8 ± 0.3	8.3 ± 2.0	7.5 ± 1.9	31.7 ± 2.7 [b]
4*(n=4)*	n/a	8.0 ± 0.8	7.3 ± 1.5	54.8 ± 1.7 [a]

The different superscript letters within columns are significantly different ($P < 0.05$)

n/a: not available

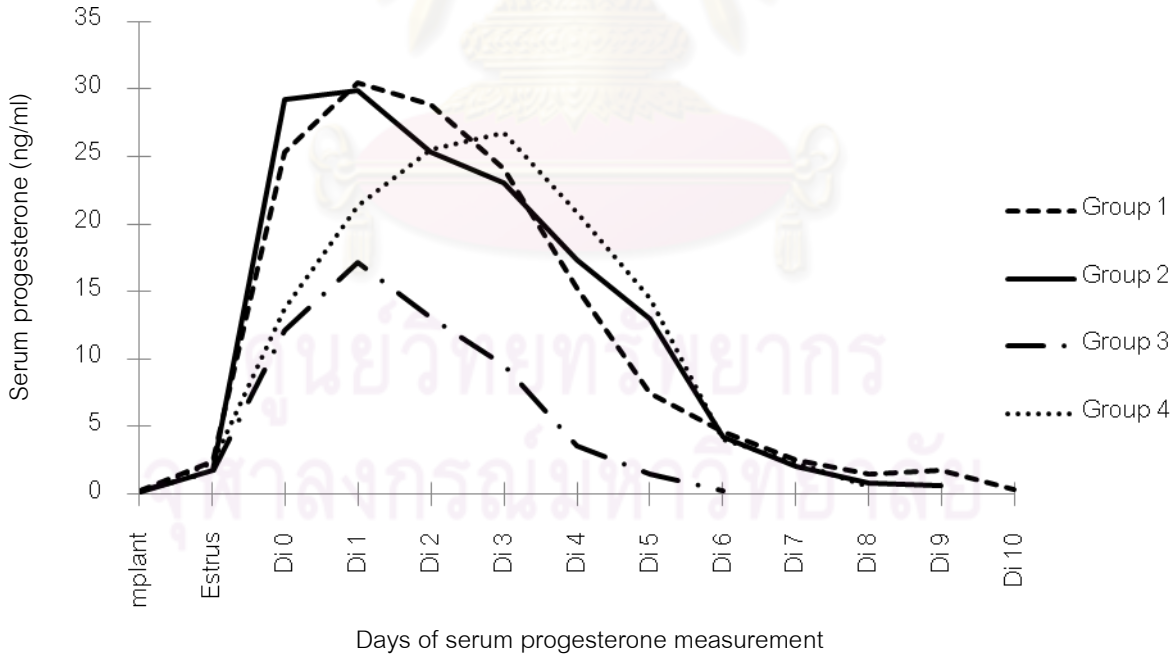

Figure1 Mean serum progesterone concentrations (ng/ml) from bitches in each group starting on the day of deslorelin implantation until the concentrations lower than 1.0 ng/ml (Di 0: the first day of diestrus, Di 1-Di 10: 1st – 10th week of diestrus)

Bitches in group 1 and 2 were mated (and inseminated) during the optimal period of estrus with normal range of semen parameters (table 2). Three bitches in group 1 (*n=4*) and group 2 (*n=6*) were found to be pregnant by ultrasonography. All pregnant bitches in group 1 had a normal pregnancy and whelping, they produced overall 13 live pups (average 4.3 pups). In group 2, two of three pregnant bitches detected 4 and 6 embryonic sacs. Then, they started aborting on day 51 and 53, respectively and aborting was completed within 3 days. Only one bitches in group 2 remained pregnant to term but she gave only one live pup.

Table 2 Minimum values of fresh semen quality used for artificial insemination

Group	Semen parameters		
	Physical appearance	Progressively motile sperm (%)	Total number of spermatozoa (x10^6)
1 (n=4)	Milky, 2.0 ml	80%	740
2 (n=6)	Milky, 2.3 ml	80%	713

CHAPTER V

DISCUSSION

The results of present study demonstrate the effects of shorted and prolonged deslorelin application in bitches on aspects of their reproductive.

GnRH agonists have been studied in male and female reproduction in many species. In female dogs, they have been used for many proposes such as estrous induction, contraception, mammary gland tumors treatment and post-spaying disorder treatment (Trigg et al., 2001; Pagnini et al., 2002; Reichler et al., 2006; Tsumagari et al., 2006; Gobello, 2007; Reichler et al., 2008). There are many licensed products used in canine practice. In this study, we used 4.7 mg deslorelin acetate (Suprelorin®), which provided temporary sterilization for at least 6 months in male dogs.

All bitches that received GnRH agonist, deslorelin, did not show any inflammatory and allergic reaction during 10 days intensive observation period or at any time of study. The body condition and general appearance of bitches in all groups were normal throughout the study, suggesting that deslorelin and its delivery system was safe for subcutaneous implantation (Trigg et al., 2001). Moreover, the site of implantation did not become infected or inflammated after removal, indicating that removal method was safe.

Many pharmacological compounds have been used to induce estrus in bitches over many years. However, some compounds are responsible for serious complication that prevents their use (Kutzler, 2007). The dopamine agonist cabergoline has been shown to be efficacious for estrous induction without side effects, but the wide variation of the interval from start of treatment until ovulation is its limitation (Verstegen et al., 1999; Gunay et al., 2004). In contrast, the time between treatment start and the onset of estrus

and ovulation is fairly constant for all protocols using GnRH agonists in anestrous bitches (Cain et al., 1989; Inaba et al., 1998; Volkmann et al., 2006a).

The present study demonstrated that 4.7 mg deslorelin implantation can reliability induce a rapid and synchronous estrus in anestrous bitches as all implanted bitches came into clinical proestrus-estrus and ovulated, except one bitch in group 3, as detected by serum progesterone levels greater than 5.0 ng/ml (Volkmann et al., 2006a). This supported the previous study, which used other doses of deslorelin (Trigg et al., 2001; Volkmann et al., 2006a; Kutzler et al., 2009). Up-regulation of GnRH receptors is the essential mechanism for this induction but prolonged administration of deslorelin may produce premature luteal failure (Herbert and Trigg, 2005). Lanna et al. (2010) attempted to avoid this negative effect by used the injectable formulation of 2 mg deslorelin. It proves to be effective in the induction of estrus in anestrous bitches but this requires multiple injections and some bitches did not exhibit estrous signs. In this study, we therefore removed the subcutaneous implants at the first day of estrus (bitches in group 1). The bitches presented normal estrous cycles and maintained pregnant to term. However, the day of removing the deslorelin implant is debatable question. In the study of Walter et al. (2011), who administed the same dosage of deslorelin implant subcutaneously on the medial side of the leg in 11 anestrous beagle bitches and the implants were removed when a bloody vaginal discharge was detected. All bitches showed estrous signs and ovulated. They were both bred and inseminated and the pregnancy rate did not differ from natural estrous dogs. The preliminary trials of Fontaine and Fontbonne. (2010) suggested that the implant should be removed after the ovulation took place because anovulation may occur when the implant was removed around the LH peak. Therefore, the optimal time for removing the implant requires investigation.

In the case of long-term 4.7 mg deslorelin implantation in anestrous bitches (group 2 and 3), the mean diestrous period was lower than group 1 and 4 and 2/3

pregnant bitches in group 2 aborted. In this case GnRH agonist down-regulated of GnRH receptors in the pituitary gland, causing a decrease in LH, at a time when CLs were still dependent on pituitary secretion of gonadotrophins, following luteolysis, decreasing serum concentrations of progesterone and a shortening of the diestrous period (Okkens et al., 1986; Trigg et al., 2001). The result resemble as Wright et al. (2001) and Romagnoli et al. (2009). Down-regulation of GnRH receptors may require continuous exposure of GnRH agonists for at least 4 weeks, this agrees with our result. Therefore treatment of GnRH agonists during the second half of pregnancy, treated bitches probably delivered live pups at term (Bertschinger et al., 2001). The pituitary desensitization presented not only in dogs but also in mare (Johnson et al., 2002). Johnson et al. (2000) reported that a 2.1 mg deslorelin prolonged the interovulatory interval by an average 6.2 days and suppressed serum LH and FSH concentrations until 19 days after the induced ovulation. However, incomplete premature luteal failure has been documented in anestrous beagle bitches after long-term implantation of 2.1 mg deslorelin (Volkmann et al., 2006b) because it is a shorter acting formulation with less dosage for complete suppression than 4.7 mg deslorelin.

In group 2, all bitches came into estrus but only 3 bitches became pregnant different from group 1. The reason for the poor fertility in this group is speculated to be due to induction in the early anestrous period and incomplete endometrial regeneration. The pregnancy rate of the bitches that were induced with leuprolide was higher in late anestrus than in early anestrus (Inaba et al., 1998). A period of uterine endometrial regeneration is necessary for both implantation and gestation to term. The authors suggested that endometrium in bitches is fully involuted around 12-15 weeks after the end of diestrus (Yeager and Concannon, 1990a; Orfanou et al., 2009). However, the duration of anestrus of all bitches used in this study was unknown. Haaften et al. (1993) reported that the responsiveness of the pituitary to GnRH was higher in late anestrus

than in early anestrus. The ovulation failure occurred in some early anestrous bitches that received 2.1 or 1.05 mg deslorelin after inducing a luteolysis with prostaglandin $F_2\alpha$ in diestrous period (Volkmann et al., 2006a). Fontaine and Fontbonne. (2010) reported that bitches that presented anovulatory cycle after estrous induction using deslorelin mainly implanted in early anestrus. These studies may help explain a bitch from this study failed to ovulate.

The mean diestrous period in group 2 was significantly greater than group 3, even though both groups had same implantation protocol. This suggests that pregnant bitches have a luteotrophic factor that more effectively supports the life span of CL than non-pregnant bitches. Relaxin, a pregnancy-specific in bitches, probably promoted progesterone and PRL secretion during pregnancy (Günzel-Apel et al., 2009). However, there is no known delicate information on this. Moreover, one pregnant bitch in group 2 maintained pregnant to term and gave only one live pup. Its gestation period is measured from the first day of cytologic diestrus as in normal range (51-60 days) (Holst and Phemister, 1974). This data showed a contraceptive failure after using a 4.7 mg deslorelin implantation in an anestrous bitches. The flare-up effect can be a problem of concern with the GnRH agonist. In the past, Trigg et al. (2001) using 52 bitches suggested that flare-up effect did not occur when serum progesterone levels more than 5 ng/ml at the time of implantation. The result disagrees with Fontaine and Fontbonne. (2010). Also, pre-treatment with progestin to prevent flare-up effect is not reliable (Sung et al., 2006). Valiente et al. (2009b) attempted to prevent this effect by combination of the GnRH antagonist acyline in bitches implanted with deslorelin during anestrus. Three quarters of treated bitches showed estrous signs and half of them ovulated. As yet, no reliable protocol has been designed to inhibit the stimulating effect and this is an area of research requiring more attention.

In conclusion, 4.7 mg GnRH agonist, deslorelin subcutaneous implantation in anestrous bitches is safe, effective and practical method for estrous induction. Bitches so implanted had a normal estrous cycle including the luteal period, if the implants were removed at the first day of the estrous stage or the time of the pre-ovulatory LH surge. In contrast, long-term implantation with GnRH to generate contraception produced premature luteal failure, resulting shortened diestrus and interrupted pregnancy. Unfortunately, the result of this study indicated that mating in flare-up period should be avoided because some implanted bitches maintained pregnant to term and whelped normally. The effective and safe method for preventing the flare-up effect should be further investigated.

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