

The Effect of a Long-acting GnRH Agonist (Deslorelin) on Estrogen Receptor Alpha (ER α) and Progesterone Receptor (PR) Expressions in Canine Mammary Tissue

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Abstract

The objective of this study was to evaluate the expression of estrogen receptor alpha (ER α) and progesterone receptor (PR) in normal mammary tissues of bitches before and after the bitch was implanted with the GnRH agonist, deslorelin. Fifteen mature bitches with no pathological condition of the mammary gland, and during anestrus, were selected and divided into two groups: five bitches were implanted with placebo (placebo group); and the ten bitches were implanted with 9.4 mg deslorelin (deslorelin group). Mammary tissues were collected from all bitches before implantation and at 2 and 12 weeks after implantation. The expressions of ER α and PR were investigated by using immunohistochemistry. The highest scores of both ER α and PR were found at 2 weeks after deslorelin implantation which differed significantly from those of other stages. At 12 weeks after implantation, ER α score decreased to the same level as before implantation while PR score tended to decrease, though not significantly differed from at 2 weeks after implantation. In placebo group, neither difference of ER α nor PR scores was observed at any time of the implantation. This finding indicated that deslorelin may effect the expression of ER α and PR in the bitch mammary tissue, in which it may stimulate the expression of ER α and PR at 2 weeks after deslorelin implantation. On the other hand, at 12 weeks after deslorelin implantation, ER α returned to the same level as before deslorelin implantation while PR decreased but not significantly different. This may indicate that ER α was more sensitive to deslorelin implantation than PR. In addition, the similar level of PR expression between the deslorelin and placebo group at 2 weeks after the implantation also suggests that some other factors besides deslorelin, or together with deslorelin, may have the effects on PR expression in the bitch mammary tissue.

Keywords : canine, deslorelin, ER α , mammary, PR

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บทคัดย่อ

ผลของจีเอ็นอาร์เอช อะโกนิสต์ ชนิดออกฤทธิ์นาน (เดสโรรีลิน) ที่มีต่อการแสดงออกของตัวรับฮอร์โมนเอสโตรเจน ชนิดอัลฟา และตัวรับฮอร์โมนโปรเจสเตอโรน บนเนื้อเยื่อเต้านมของสุนัข

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาการแสดงออกของตัวรับฮอร์โมนเอสโตรเจน ชนิดอัลฟา และตัวรับฮอร์โมนโปรเจสเตอโรน บนเนื้อเยื่อเต้านมปกติของสุนัขเพศเมียก่อนและหลังการฝังจีเอ็นอาร์เอช อะโกนิสต์ (เดสโรรีลิน) ทำการแบ่งสุนัขเพศเมียที่อยู่ในวัยเจริญพันธุ์ และไม่มีพยาธิสภาพของเต้านม จำนวน 15 ตัว ออกเป็นสองกลุ่ม คือ กลุ่มยาหลอกโดยฝังยาหลอกให้แก่สุนัข จำนวน 5 ตัว และกลุ่มเดสโรรีลินโดยฝังเดสโรรีลิน 9.4 มก.ให้แก่สุนัข จำนวน 10 ตัว ทำการเก็บตัวอย่างเต้านมจากสุนัขทั้งสองกลุ่มที่ก่อนและหลังการฝังที่ 2 และ 12 สัปดาห์ ตรวจหาการแสดงออกของตัวรับฮอร์โมนเอสโตรเจน ชนิดอัลฟา และตัวรับฮอร์โมนโปรเจสเตอโรน ด้วยขบวนการทางอิมมูโนฮิสโตเคมี พบคะแนนของตัวรับฮอร์โมนทั้งสองชนิดมีค่าสูงสุดหลังจากการฝังเดสโรรีลิน 2 สัปดาห์ หลังจากฝังเดสโรรีลิน 12 สัปดาห์ คะแนนของตัวรับฮอร์โมนเอสโตรเจน ชนิดอัลฟา ลดลงเท่ากับก่อนทำการฝังเดสโรรีลิน ในขณะที่คะแนนของตัวรับฮอร์โมนโปรเจสเตอโรนมีแนวโน้มว่าจะลดลง แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มที่เก็บตัวอย่างหลังจากฝังเดสโรรีลิน 2 สัปดาห์ ในกลุ่มสุนัขที่ฝังยาหลอกไม่พบความแตกต่างของคะแนนของตัวรับฮอร์โมนทั้งสองชนิดตลอดการศึกษา ผลการศึกษาแสดงให้เห็นว่า เดสโรรีลินอาจมีผลต่อการแสดงออกของตัวรับฮอร์โมนทั้งสองชนิดบนเนื้อเยื่อเต้านมในสุนัข โดยมีการกระตุ้นการแสดงออกของตัวรับฮอร์โมนทั้งสองชนิดให้เพิ่มขึ้นหลังจากการฝังเดสโรรีลินไปแล้วที่ 2 สัปดาห์ ในทางตรงกันข้ามที่ 12 สัปดาห์หลังจากฝังเดสโรรีลิน คะแนนของตัวรับฮอร์โมนเอสโตรเจน ชนิดอัลฟา ลดลงจนอยู่ในระดับเดียวกับก่อนฝังเดสโรรีลิน ในขณะที่คะแนนของตัวรับฮอร์โมนโปรเจสเตอโรนมีแนวโน้มลดลงแต่ไม่พบความแตกต่างกับที่ 2 สัปดาห์หลังจากฝังเดสโรรีลิน อาจกล่าวได้ว่าแสดงออกของตัวรับฮอร์โมนเอสโตรเจนชนิดอัลฟามีความไวต่อการฝังเดสโรรีลิน มากกว่าการแสดงของตัวรับฮอร์โมนโปรเจสเตอโรน นอกจากนี้ระดับของตัวรับฮอร์โมนโปรเจสเตอโรนที่ไม่แตกต่างกันระหว่างกลุ่มที่ฝังเดสโรรีลินและกลุ่มยาหลอกที่ 2 สัปดาห์หลังการฝังอาจบ่งชี้ว่าน่าจะมีปัจจัยอื่นนอกเหนือจากเดสโรรีลินหรือปัจจัยอื่นร่วมกับเดสโรรีลินมีอิทธิพลในการแสดงออกของตัวรับฮอร์โมนโปรเจสเตอโรนในเนื้อเยื่อเต้านมสุนัข

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Introduction

In all species, the mammary glands are composed of various cell types, such as the epithelia that are embedded in a fat pad, and they are the major target for cell proliferation of the mammary tissue (Anderson and Clarke, 2004). Estrogen and progesterone are major mitogens for the normal mammary epithelium. The ova-

rian 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). The study in the bitches showed that steroid hormone receptors have been detected in normal mammary tissue and mammary tumor (Hamilton et al.,

1977; MacEwen et al., 1982; Donnay et al., 1995; Geraldles et al., 2000; Nieto et al., 2000).

The GnRH agonists have been reported to control fertility in dogs and also in hormone-dependent human breast cancer and canine mammary tumor. 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 gonadotropins secretion and consequent loss of ovarian steroid production (Emens and Davidson, 2003). In an earlier study, treatment with 60 µg/kg of GnRH-agonist, goserelin every 21 days for 12 months showed an inhibitory effect on the growth of hormone-dependent canine mammary tumors after 3 months (Lombardi et al., 1999). GnRH agonists initially stimulate gonadotropins secretion, but subsequently, upon continuous exposure cause a down-regulation of GnRH receptors resulting in the inhibition of luteinizing hormone production. 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 decreases ovarian estradiol production indirectly by blocking the hypothalamic-pituitary-ovarian axis. The GnRH agonists have been reported to control fertility in dogs (Herbert and Trigg, 2005; Kutzler and Wood, 2006; Trigg et al., 2006; Trigg et al., 2001) as well as in different species (Bertschinger et al., 2001; Farquhar et al., 2001; Kutzler and Wood, 2006; Munson et al., 2001).

Deslorelin is a synthetic GnRH agonist in a biocompatible, slow release subcutaneous implant. Following subcutaneous implantation to dogs with 12 mg, average plasma level of deslorelin was >1 µg/day for period of more than 1 year (Trigg et al., 2001). Some 9.4 mg deslorelin-implanted bitches showed estrus within 1-2 weeks after implantation (Trigg et al., 2006).

It is widely accepted that deslorelin-induced changes in circulating ovarian steroid hormones levels, can alter the expression of the ovarian steroids receptors. However, there were no data collected on the effects of deslorelin on normal mammary tissue. Therefore, to assist understanding the alteration of the ovarian steroid receptors expression on canine mammary tissue, the immunopresence of ERα and PR on normal canine mammary tissue in various stages of deslorelin implantation requires investigation.

In the present study, the investigation of the expression of ERα and PR in normal canine mammary glands before and after deslorelin implantation was performed by using immunohistochemical technique.

Materials and Methods

Experimental animals and management: The experimental plan was approved by the Ethical Committee for Experimentation with Animals at Faculty of Veterinary Science, Chulalongkorn University. Fifteen healthy early anestrus bitches (3 months after estrous signs were shown), aged 1-3 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 given a placebo implant, (placebo or control group), group 2 given 9.4 mg deslorelin (deslorelin group). Blood samples were taken before the experiment from both groups to assess hematology (complete blood count and blood parasite) and blood chemistry (creatinine, BUN, SGPT and ALP) values, as well as progesterone circulating levels. Stage of estrous cycle at this time was also confirmed by cytological examination of vaginal cytology and serum progesterone level (Feldman and Nelson, 1996). In order to reduce the variation among bitches before deslorelin implantation, only bitches with normal range of progesterone levels (<0.5 ng/mL) at anestrus and showed normal expression of steroid receptors in the mammary

gland were included in the study, therefore 10 bitches and 5 bitches were used in deslorelin group and placebo group respectively.

Drug administration and sample 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 (Suprelorin®). Implants were manufactured by a proprietary method that involved extrusion of deslorelin with a matrix consisting principally of low-melting point lipids and a biological surfactant. Each implant contained 4.7 mg of the 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°C until use. Sterile deslorelin-containing or placebo implants were inserted subcutaneous via single-use syringes. In a real time *in vitro* dissolution system, 6 mg implants of similar composition released more than 1µg/day for period of more than 1 year (Trigg et al., 2001).

Blood samples were taken from all dogs at 2 and 12 weeks after implantation, for evaluation of the hematology and blood chemistry values, as well as circulating levels of progesterone.

Normal mammary tissues were obtained before implantation and at 2 and 12 weeks after implantation in order to investigate the stimulatory effect (week 2) and suppression effect of deslorelin (week 12) by incisional biopsy under general anesthesia (premedicated with 1 mg/kg of xylazine hydrochloride and 0.04 mg/kg atropine sulfate, inducted and maintained with 10 mg/kg thiopental sodium). No samples were collected in the period between week 2 and week 12 since the level of serum progesterone after deslorelin implantation may still be high at that period (T. Swangchan-uthai, unpublished observations). Therefore, the suppression effect of hypothalamic-pituitary-ovarian axis from deslorelin should occur already at week 12 (Trigg et al., 2001) when the

tissues were sampled in order to investigate the suppression effect of deslorelin. Immediately after surgical excision, they were fixed in 4% (w/v) 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.

Progesterone assays: Serum progesterone levels 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. Sensitivity of this assay is 0.02 ng/ml. The within-assay coefficients of variation ranged from 7 to 9 %. All samples were determined at the same time.

Immunohistochemistry of estrogen receptor alpha and progesterone receptor: Before immunohistochemistry, the specimens were deparaffinized in xylene and rehydrated in graded alcohol. The immunohistochemical protocol was modified from Sukjumlong et al. (2003). Antigen retrieval technique was in effect by means of heating in the microwave oven with 0.01 M citrate buffer, pH 6.0 at high power (750 W) for 15 min (5 min. x 3). A standard avidin-biotin immunoperoxidase technique (Vectastain® ABC kit, Vector Laboratories, Inc., USA) was applied to detect ERα and PR proteins. The primary antibodies used were mouse monoclonal antibody to ERα (DAKO, clone 1D5, dilution 1:50) and PR (Immunotech, clone 10A9, dilution 1:100) for 2 hours in a humidity chamber at room temperature.

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

In the final step, 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.

Evaluation of immunohistochemical data: The immunostaining for ER α and PR 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 ER α and PR. The evaluations of positively stained cells were carried out as blinded preparation by the same person. The human score system was used for evaluate the immunohistochemical results. In brief, ER α 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).

Statistical analysis: Data were processed and statistically analyzed using the SAS statistical package (version 8, SAS Institute, Inc., 2002, Cary, NC, USA). Normal distribution of residuals from the statistical models was tested using UNIVARIATE procedure option NORMAL to test normality of data by using Kolmogoroff-Smirnoff test. Differences in mean numbers of ER α score, PR score and progesterone levels were tested using analysis of variance. The statistical model included the fixed effects of stage (four groups: before 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 ER α and PR scores and serum level of progesterone were evaluated using Pearson correlation coefficients. A p-value < 0.05 was considered statistically significant except for mean scores of ER α and PR in which $p < 0.01$ was considered significantly different.

Results

In all bitches, there was no allergic or inflammatory reaction observed at the site of implantation at any time during the study. The hematology and serum chemistry parameters tested every 2 weeks, were in normal range according to Plumb (1995) (data not shown).

Before implantation, there was no incidence of estrus in any bitch of either group. In the deslorelin group, all animals showed bloody vaginal discharge after 1 week of implantation and signs of estrus were observed up to 2 to 3 weeks later.

Serum hormonal levels: Before implantation, all 15 bitches in both deslorelin and placebo groups were in anestrus as classified by vaginal cytology and serum progesterone level. The average serum progesterone levels (mean $\pm$ SEM) in the deslorelin group and placebo group were 0.21 $\pm$ 0.06 ng/ml and 0.16 $\pm$ 0.05 ng/ml, respectively.

The serum progesterone levels (mean $\pm$ SEM) in both experimental groups are shown in Table 1. In deslorelin group, the serum progesterone level significantly increased at 2 weeks after implantation (4.96 $\pm$ 2.72 ng/ml) ($p < 0.05$), which was in estrous levels and subsequently decreased to anestrus levels (0.25 $\pm$ 0.16 ng/ml) at 12 weeks after implantation. In the placebo group, the serum progesterone levels at different stages of the implantation were not significantly different, which were in anestrus throughout this investigation.

Immunohistochemistry: Positive immunolocalization of the ER α and PR was found in the nuclei of alveolar and tubular epithelium of canine mammary tissues in both groups (Fig. 1). The ER α and PR immunostaining scores (mean $\pm$ SEM) before implantation, 2 weeks and 12 weeks after implantation, in deslorelin and placebo groups are shown in Table 2 and 3 respectively.

A significantly higher ER α score in the deslorelin group was shown at 2 weeks after implantation, while the ER α immunostaining score at other stages was not significantly different. When compared between groups,

Table 1 Serum progesterone levels of bitches, at different times following deslorelin and placebo implantation (mean±SEM)

Stage of implantation	Deslorelin implantation	Placebo implantation
Before implantation	0.21 ± 0.06 ^a	0.16 ± 0.05 ^a
2 weeks after implantation	4.96 ± 2.72 ^b	0.31 ± 0.13 ^a
12 weeks after implantation	0.25 ± 0.16 ^a	0.50 ± 0.21 ^a

Mean (±SEM) within the same column followed by the different superscript letters is significantly different ($p < 0.05$)

Table 2 ERα score of the canine mammary tissues, at different times following deslorelin and placebo implantation (mean±SEM)

Stage of implantation	Deslorelin implantation	Placebo implantation
Before implantation	34.26 ± 2.05 ^{A,a}	32.99 ± 2.27 ^{A,a}
2 weeks after implantation	55.83 ± 5.93 ^{B,b}	23.05 ± 3.70 ^{A,a}
12 weeks after implantation	33.51 ± 3.62 ^{A,a}	24.01 ± 1.95 ^{A,a}

Mean (± SEM) 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 ($p < 0.01$)

Table 3 PR score of the canine mammary tissues, at different times following deslorelin and placebo implantation (mean±SEM)

Stage of implantation	Deslorelin implantation	Placebo implantation
Before implantation	35.31 ± 2.87 ^{A,a}	47.77 ± 1.26 ^{A,a}
2 weeks after implantation	60.09 ± 3.44 ^{B,b}	58.76 ± 4.97 ^{B,a}
12 weeks after implantation	54.13 ± 3.23 ^{B,b}	56.49 ± 6.56 ^{B,a}

Mean (±SEM) within the same row followed by the different superscript capital letters, and within the same column followed by the different superscript small letters are significantly different ($p < 0.01$)

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. While in the placebo group, the PR score was not significantly different in all stages.

Correlation between ERα and PR scores: A 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).

Discussion

In the present study, all bitches in the deslorelin group showed estrus within 1-2 weeks after implantation and subsequently became quiescent after 4 weeks. This finding demonstrated the stimulatory effect of deslorelin on the hypothalamic-pituitary-ovarian axis, which induced pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) production (Trigg et al., 2001). Subsequently, ovarian steroid secretion was induced but it was suppressed after sustained stimulation caused by desensitization of GnRH receptors in the anterior pituitary gland and brain (Corbin, 1982; Herbert and Trigg, 2005).

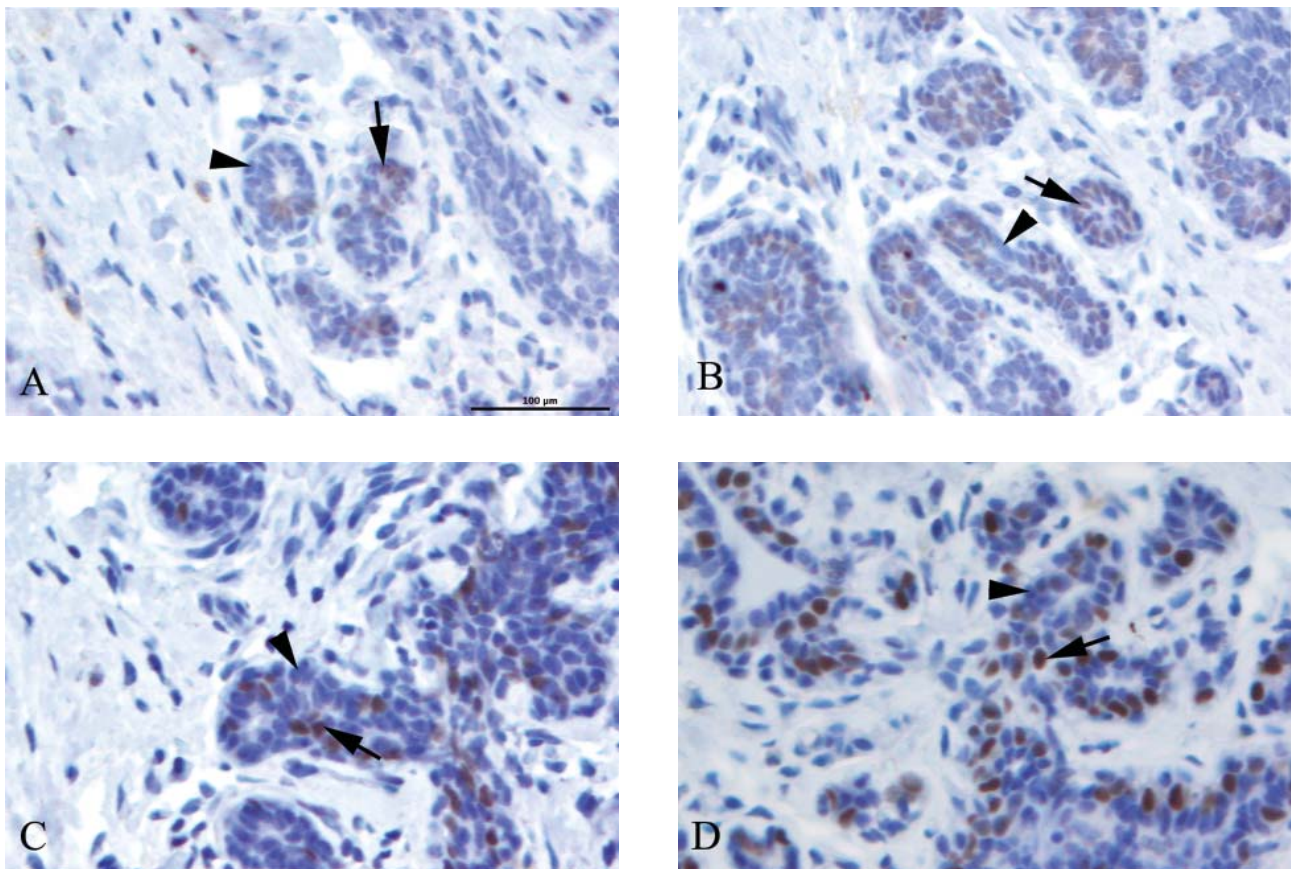


Figure 1 The immunohistochemical staining of ER α and PR in normal canine mammary tissue. A and B represent ER α immunostaining before and after 2 weeks following deslorelin implantation. C and D represent PR immunostaining before and after 2 weeks following deslorelin implantation. Arrow head and arrow show negative and positive immunostaining cells respectively.

The significant increasing level of progesterone at 2 weeks after deslorelin implantation showed the stimulatory effect of deslorelin on the ovarian activity as described above. This level of the serum progesterone at 2 weeks after implantation was in estrous range; therefore deslorelin implantation may increase progesterone level from inactive stage into follicular stage. After sustained stimulation, progesterone returned to basal level as shown at 12 weeks after implantation.

In deslorelin group, the highest ER α score was found at 2 weeks after implantation. At this stage, all 10 bitches had already shown estrus, detected by vaginal cytology and the hormonal pattern in which progesterone concentration increased. This result together with the higher ER α score in deslorelin group compared with placebo group suggested the stimulatory effect of this GnRH

agonist; deslorelin, on the presence of ER α in the bitch mammary tissue at 2 weeks after implantation. On the other hand, the low ER α score at 12 weeks following deslorelin implantation may due to the suppression effect of deslorelin on the ovarian function. This finding supports the study in human uterine leiomyomas where ER α expression decreased after long term GnRH agonist therapy (Vu et al., 1998). In the placebo group, the ER α score was constantly low at all times following the implantation due to the bitches remaining in anestrus throughout the placebo implanted period. Besides, the constant ER α score confirmed that placebo implantation had no effect on the expression of ER α in the bitch mammary gland from the present study.

For PR, at 2 weeks, the immunostaining score was significantly higher at 2 weeks after implantation

than at other times, which may due to the stimulatory effect of deslorelin on the ovarian activity as described above for ER α . In contrast to placebo group, the constant high presence of PR indicated no effect from placebo at any stages of implantation. However, when comparing between deslorelin and placebo group at 2 weeks after implantation, PR score was not different. This may imply that deslorelin implantation may not directly have the stimulatory effect on PR expression. Instead the high score at 2 weeks was also from some other regulatory mechanisms found in both groups of bitches or that PR expression in the mammary gland was not only under the influence of ovarian steroid hormone. Some other factors such as insulin-like growth factor may have an important role on the expression of PR in the mammary tissues (Cline, 2007; Cui et al., 2003). At 12 weeks after deslorelin implantation, PR score appeared to decrease, though it was not significantly different to that at 2 weeks after implantation where ER α score was already significantly lower compared to at 2 weeks. This may indicate that ER α immunopresence in the bitch mammary gland was more sensitive to the suppression effect of deslorelin than PR in this study.

For the results of correlation, a positive correlation found between ER α and PR may indicate the same regulatory effects under the influence of deslorelin implantation on these steroid receptors. Moreover, it supports the hypothesis that PR is an ER-regulated gene (Osborne et al., 2005) and its presence indicates a functioning ER pathway or that the presence of progesterone receptor is under the influence of estrogen acting through ER as described before in the study of human breast cancer tissue (Horwitz and McGuire, 1978).

In conclusion, this is the first report on the effect of deslorelin on ovarian steroid receptors in normal canine mammary tissues. The results from the present study showed a similar effect of deslorelin on the immunopresence of both ER α and PR, which was up-regulated in the stimulation stage (at 2 weeks after deslorelin implantation) and returned to basal levels in

the quiescent stage (12 weeks after deslorelin implantation). However, for PR, other factors together with deslorelin may have the effects on PR expression in the bitch mammary tissues as there is no difference between deslorelin and placebo group at 2 weeks after the implantation. In addition, due to its suppressing effect on the ovarian steroid hormones secretion as well as the expression of steroid receptors, the therapeutic use of deslorelin may be useful in a long-term therapy of hormone-dependent canine mammary tumors. Further studies on the effect of deslorelin implantation in hormone-dependent canine mammary tumor and its relation to the ovarian steroid hormone receptors are suggested to investigate which may result in the development of an alternative method for canine mammary tumor treatment.

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