CHAPTER 3

Materials and methods

3.1 Animals

Eighteen clinically healthy prepubertal female dogs aged 4 months old from 6 litters of full-sib (3 dogs in each litter) were used in this study. The dogs were housed in pairs in indoor-outdoor runs and were fed with a standard commercial dog food twice daily until the dogs reached 8 months old and once a day afterwards. Water was available *ad libitum*. All dogs were vaccinated against rabies and DA_2LCPV vaccine (Distemper, Adenovirus type 2, Leptospirosis and Canine Parvovirus vaccine) twice started at 4 months old. To prevent heart worm and round worm infestation including ectoparasites, Ivermectin (300 microgram/kg body weight, Merck®) was subcutaneously injected every month for the whole period of the study.

Vulvar appearance and sexual behavioral changes were monitored, and samples taken for vaginal cytology and serum progesterone concentrations were investigated twice a week to confirm their anoestrous stage. Animals were anoestrus if serum progesterone was <1ng/ml (Feldman and Nelson, 1996). The animals in each litter were randomly divided into 3 groups, and implanted subcutaneously with a deslorelin containing implant (Peptech Animal Health, Sydney Australia) (9.4 mg/implant) at 4 months old (n = 6), at 7 months old (n = 6), and with placebo at 4 months old (n = 6). The implantation site was the interscapular region. Prior to implanting, aseptic preparation was performed by clipping and applying Betadine and alcolhol. A sterile deslorelin-containing or placebo implant was inserted subcutaneously via a single use disposable syringes. After implantation the implantation sites were observed daily for 10 days, for signs of inflammatory or allergic reactions.

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

3.2 The drug

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 5 mg of active ingredient deslorelin. Two implants (10 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. In a real time *in vitro* dissolution system, these implants released doses of > 1 µg / day for periods of > 1 year (Dr TE Trigg, personal communication).

3.3 Research design

The study was conducted as an experimental study (Randomized, placebo-controlled trial).

The observation period continued until the female dogs reached puberty, and all dogs were observed until they exhibited oestrus. At 13 months old, an ovariohysterectomy was performed on each dog; the ovaries and uteri were collected and examined for histological differences between control and experimental dogs.

3.4 Reproductive performance :

Stage of oestrus was evaluated by the following parameters

The signs of oestrus including physical signs (vulvar swelling, serosanguineous vaginal discharge), vaginal cytology and serum progesterone concentration were evaluated 2 times a week after implantation for the first 4 week period, and every 2 weeks thereafter until the dogs reached 13 months old).

3.4.1 Physical signs of oestrus

Changes in external genital appearance were examined as vulvar swelling and serosanguineous vaginal discharge.

3.4.2 Vaginal cytology

Vaginal exfoliative cytology samples were obtained by using "Cotton Swab Technique" (Feldman and Nelson, 1996). The cotton swab (a sterile, 5- to 7-inch-long cotton tipped applicator) was moistened with saline solution. The lips of the vulva were gently separated with one hand. The cotton-tipped end of this swab was passed into the dorsal commissure of the vulva. The cotton tip initially was gently pressed against the caudodorsal surface of the vaginal vault and then advanced in a craniodorsal direction, toward the vertebral column, until it passed over the ischial arch. The swab was inserted at least the distance needed to reach the pelvic canal. The applicator was then rotated one complete revolution in each direction and withdrawn. Once the cotton swab was withdrawn, the cotton tip was rolled gently from one end of 2 glass microscope slides to the other. The slides with the exfoliated cells were air dried and then dipped once in absolute methanol. Vaginal smear glasses were stained with Modified Wright-Giemsa stain (Diff-quik, Dada Diagnostics PR, Auburn, MI), and cellular identified parabasal or intermediate or superficial cell was based on criteria reported by Concannon and Digregorio (1986) and Holst and Phemister (1974). Vaginal cytology was evaluated by the percentage of vaginal epithelial cells in a smear that were polygonal in outline as determined by the examination of 200 epithelial cells (x 100 magnification) (Wright and Parry, 1989) as described in Chapter 1.

3.4.3 Serum progesterone concentrations

Blood samples (2 ml) were collected from cephalic vein into sterile tubes and centrifuged, then serum was assayed for progesterone (on the day of collection). Serum progesterone concentrations (ng/ml) were measured by Electro-chemiluminescence immunoassay using Elecsys®Systems

1010/2010/ Modular Analytics E170. The measurement range was 0.03-60 ng/ml. The sensitivity of the assay was 0.2 ng/ml progesterone.

3.5 Reproductive performance at the end of study

Dogs were ovariohysterictomized at the end of experiment under total anaesthesia. After premedication with 0.04 mg/kg atropine and 2 mg/kg xylazine (Rompun; Bayer®) administered intramuscularly, anaesthesia was induced with sodium thiopental. After endotracheal intubation, anaesthesia was maintained using halothane and oxygen, which was delivered in an opened system. All animals received an electrolyte solution (Acetar) intravenously during surgery. OVH was performed via ventral midline incision and routine technique, using 2/0 polyglactin suture material and placement of subcuticular sutures for closure of the skin.

3.5.1 Vulvar measurement

Vulva were measured in width and height (centimeter; cm) prior to OVH

3.5.2 Histology of ovary and uterus

Ovary and uterine tissues were fixed in 10 % Para-formaldehyde for conventional histological preparation. The sections were stained with haematoxylin and eosin.

3.6 Statistical analysis

Chi-Square analysis was used for analyzing the proportion of dogs in the treatment and control groups showing oestrous signs after GnRH agonists implantation. Paired-t-test analysis was used for analyzing the relationship of reproductive performance between experiment and control dogs in the same litters. A value of $p<0.05$ was considered statistically significant. All tests were accessed by using SAS Version 6.12 Cary NC. USA 1996.