ผลต่อสมรรถนะทางระบบสืบพันธุ์ภายหลังการฝังฮอร์โมน GnRH-agonist (Deslorelin)
ในแมวเพศผู้ และเพศเมียก่อนวัยเจริญพันธุ์

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EFFECT OF GnRH-AGONIST (DESLORELIN) IMPLANTATION ON REPRODUCTIVE PERFORMANCES IN EARLY PREPUBERTAL MALE AND FEMALE CATS

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ณิโคล ศิริโศภิษฐ์ เมห์ล : ผลต่อสมรรถนะทางระบบสืบพันธุ์ภายหลังการฝังฮอร์โมน GnRH-agonist (Deslorelin) ในแมวเพศผู้ และเพศเมียก่อนวัยเจริญพันธุ์ (EFFECT OF GnRH-AGONIST (DESLORELIN) IMPLANTATION ON REPRODUCTIVE PERFORMANCES IN EARLY PREPUBERTAL MALE AND FEMALE CATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร. สุดสรร ศิริไวทยพงศ์, 92 หน้า.

การทดลองดังกล่าวมีวัตถุประสงค์เพื่อศึกษาผลของ จีเอนอาร์เอช อะโกนิสต์ (เดสโรลีลิน) ต่อพฤติกรรมทางเพศ สมรรถนะของระบบสืบพันธุ์ และการแสดงออกของโปรตีนและmRNA ของตัวรับฮอร์โมน LH และ FSH ในแมวเพศผู้และเพศเมียก่อนวัยเจริญพันธุ์

การศึกษาในส่วนแรก เป็นการทดลองเพื่อศึกษาถึงผลของการฝังฮอร์โมน จีเอนอาร์เอช อะโกนิสต์ ในแมวเพศผู้ก่อนถึงวัยเจริญพันธุ์ เพื่อศึกษาถึงผลของการฝังฮอร์โมนต่อพฤติกรรมทางเพศ สมรรถนะทางระบบสืบพันธุ์ การแสดงออกของตัวรับของลูทีไนซิงฮอร์โมน (LH) และฟอลิคูลาร์สติมูเลติงฮอร์โมน (FSH) และเปรียบเทียบลักษณะทางกายภาพของอัณฑะ และเนื้อเยื่ออัณฑะ ระหว่างแมวเพศผู้ก่อนถึงวัยเจริญพันธุ์ และแมวเพศผู้ที่อยู่ในวัยเจริญพันธุ์แล้ว ในส่วนแรกของการทดลองดังกล่าว ใช้แมวเพศผู้อายุ 3 เดือน 2 กลุ่ม โดยมีแมวจำนวนกลุ่มละ 6 ตัว แมวกลุ่มแรกจะได้รับการฝังฮอร์โมน เดสโลรีลิน ขนาด 4.7 มิลลิกรัม และกลุ่มที่สองเป็นกลุ่มควบคุมที่ไม่ได้รับการฝังฮอร์โมน หลังจากการฝังฮอร์โมน 48 สัปดาห์แมวทั้งสองกลุ่มถูกทำการรีดน้ำเชื้อ และตรวจคุณภาพน้ำเชื้อ ก่อนที่จะทำหมันเพศผู้โดยการผ่าตัดต่อมา เนื้อเยื่ออัณฑะของแมวทั้งสองกลุ่มได้ถูกนำมาตรวจหาการแสดงออกของเอ็มอาร์เอ็นเอ (mRNA) และโปรตีนของตัวรับฮอร์โมน LH และFSH ในเนื้อเยื่ออัณฑะ น้ำหนักตัว และปริมาตรของอัณฑะของแมวทั้งสองกลุ่ม ถูกนำมาคำนวณเปรียบเทียบทางสถิติด้วยทีเทสต์ (T-Test) การแสดงออกของโปรตีน และ mRNA ของตัวรับฮอร์โมน LH และ FSH และ น้ำหนักของอีพิดิไดมิส ถูกนำมาคำนวณเปรียบเทียบทางสถิติด้วยเจนเนอรัล ลีเนียร์ โมเดล (GLM) ส่วนน้ำหนักของอัณฑะ เส้นผ่านศูนย์กลางของเซมินิฟเฟอรัสทิวบูล และคะแนนความสมบูรณ์ของเนื่อเยื่ออัณฑะ ถูกนำมาคำนวณปรียบเทียบทางสถิติด้วย วิลคอกซัน แรงค์ซัมเทสต์ (Wilcoxon rank sum test) จากการทดลองดังกล่าวสามารถเก็บน้ำเชื้อได้จากแมวกลุ่มควบคุมที่ไม่ได้รับการฝังฮอร์โมนเท่านั้น ส่วนแมวที่ได้รับการฝังฮอร์โมนนั้น ไม่สามารถทำการรีดเก็บน้ำเชื้อได้ และยังไม่พบการแสดงออกของพฤติกรรมทางเพศในแมวกลุ่มดังกล่าวตลอดระยะเวลาการทดลอง แต่แมวกลุ่มควบคุมนั้น มีการแสดงออกของพฤติกกรมทางเพศตั้งแต่ อาทิตย์ที่ 28 ของการทดลอง นอกจากนี้ยังพบว่าตั้งแต่สัปดาห์ที่ 30 หลังการทดลองนั้นปริมาตรของลูกอัณฑะของแมวกลุ่มที่ได้รับการฝังฮอร์โมนนั้น มีขนาดเล็กกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ นอกจากนี้ยังพบว่า คะแนนการความสมบูรณ์พันธุ์ของเนื้อเยื่ออัณฑะ ขนาดเส้นผ่านศูนย์กลางของเซมินิเฟอรัสทิวบูล และการแสดงออกของโปรตีนของตัวรับฮอร์โมน LH นั้นต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ แต่อย่างไรก็ตามจากการทดลองดังกล่าวนั้น ไม่พบความแตกต่างของการแสดงออกของ mRNA ของตัวรับฮอร์โมน LH และการแสดงออกของโปรตีนของตัวรับฮอร์โมน FSH ระหว่างกลุ่มทดลอง และกลุ่มควบคุม แต่พบว่าการแสดงออกของ mRNA ของตัวรับฮอร์โมน FSH ในกลุ่มควบคุมนั้นสูงกว่ากลุ่มที่ได้รับการฝังฮอร์โมนอย่างมีนัยสำคัญ ในส่วนที่สองของการทดลอง ได้ทำการเก็บตัวอย่างอัณฑะ จากแมวเพศผู้ก่อนวัยเจริญพันธุ์จำนวน 6 ตัว และในวัยเจริญพันธุ์จำนวน 6 ตัว หลังจากได้รับการผ่าตัดทำหมันเพศผู้ เพื่อนำมาเนื้อเยื่ออัณฑะของแมวทั้งสองกลุ่มได้ถูกนำมาตรวจหา การแสดงออก mRNA และโปรตีน ของตัวรับฮอร์โมน LH และFSH ในเนื้อเยื่ออัณฑะ โดยไม่พบความแตกต่างระหว่างกลุ่มตัวอย่างของการแสดงออกของ โปรตีนของตัวรับฮอร์โมนทั้ง 2 ชนิด แต่พบว่า การแสดงออกของ mRNA ของตัวรับฮอร์โมน FSH ในเนื้อเยื่ออัณฑะของแมวก่อนวัยเจริญพันธุ์นั้นสูงกว่าอย่างมีนัยสำคัญ นอกจากนี้ยังพบว่าน้ำหนักของอัณฑะ และอิพิดิไดมิส ขนาดเส้นผ่านศูนย์กลางของเซมินิฟเฟอรัสทิวบูล และคะแนนความสมบูรณ์พันธุ์ของเนื้อเยื่ออัณฑะในแมววัยเจริญพันธุ์นั้น สูงกว่าแมวก่อนวัยเจริญพันธุ์อย่างมีนัยยะสำคัญ

การศึกษาในส่วนที่สองนั้น ได้แบ่งแมวเพศเมียเป็น 3 กลุ่ม โดยกลุ่มแรกนั้น เป็นแมวเพศเมียก่อนวัยเจริญพันธุ์จำนวน 6 ตัว ที่ได้รับการฝังฮอร์โมนเดสโลรีลินขนาด 4.7 มิลลิกรัม เมื่ออายุ 3 เดือน กลุ่มที่สอง (จำนวน 18 ตัว) และกลุ่มที่สาม (จำนวน 6 ตัว) เป็นแมวที่ไม่ได้รับการฝังฮอร์โมน แมวกลุ่มที่ 1 และ2 ถูกเลี้ยงในสภาพแวดล้อมเดียวกัน และถูกบันทึก น้ำหนัก ความเข้มข้นของฮอร์โมรเอสตราไดออล ในอุจจาระ และการแสดงออกของพฤติกรรมทางเพศ และการเป็นสัด ตลอดระยะเวลาการทดลอง (48 สัปดาห์) หลังจาก 48 สัปดาห์ของการทดลอง แมวกลุ่มแรก และกลุ่มที่สอง จะได้รับการผ่าตัดทำหมันเพศเมีย เพื่อเก็บรังไข่และมดลูกไปศึกษา โดยแมวในกลุ่มที่สองนั้น ได้รับการผ่าตัดเมื่ออยู่ในระยะฟอลลิคูลาร์ (6 ตัว) ระยะลูเตียล (6 ตัว) และระยะที่รังไข่ไม่มีการทำงาน (6 ตัว) ส่วนแมวในกลุ่มที่สามนั้น ได้รับการผ่าตัดทำหมันเพศเมีย เพื่อเก็บตัวอย่างรังไข่และมดลูกตั้งแต่ก่อนถึงวัยเจริญพันธุ์ (อายุ 3 เดือน) รังไข่ และมดลูกของแมวทั้งหมดถูกทำไปวิเคราะห์ลักษณะทางกายภาพโดยละเอียด นอกจากนี้ยังทำการตรวจหาการแสดงออกของ mRNA และโปรตีนของตัวรับฮอร์โมน LH และ FSH ในเนื้อเยื่อรังไข่ ในแต่ละกลุ่มการทดลอง ความแตกต่างของน้ำหนักตัวของแมวกลุ่มที่ 1 และ2 ถูกนำมาคำนวนเปรียบเทียบทางสถิติด้วย t-Test น้ำหนักของรังไข่ ขนาดเส้นผ่านศูนย์กลางของต่อมของผนังมดลูก ความหนาของผนังมดลูกในแต่ละชั้น จำนวนของฟอลิเคิล ชนิด ไพรไมเดียล ปฐมภูมิ ทุติยภูมิ และฟอลิเคิลขนาดใหญ่ และการแสดงออกของ mRNA และโปรตีนของตัวรับฮอร์โมน LH และ FSH ในเนื้อเยื่อรังไข่ ของแมวทั้ง 3 กลุ่มการทดลอง ถูกนำมาคำนวนเพื่อเปรียบเทียบทางสถิติด้วย GLM จากการทดลองดังกล่าวพบว่า แมวกลุ่มควบคุม (กลุ่มที่2) มีน้ำหนักตัวสูงกว่ากลุ่มที่ได้รับการฝังฮอร์โมนอย่างมีนัยสำคัญ ในช่วงสัปดาห์ที่ 22 ถึง 26 ของการทดลอง นอกจากนี้ยังพบว่าแมวในกลุ่มควบคุมที่ไม่ได้รับการฝังฮอร์โมนนั้น มีการแสดงออกของพฤติกรรมทางเพศ และพบการเปลี่ยนแปลงของความเข้มข้นของเอสตราไดอัลตามวงรอบการเป็นสัด ในขณะที่แมวในกลุ่มที่ถูกฝังฮอร์โมนนั้นไม่พบพฤติกรรม และการเปลี่ยนแปลงของเอสตราไดอัลดังกล่าว และจากการทดลองดังกล่าวยังพบว่า การฝังฮอร์โมนเดสโรรีลินนั้น ทำให้ น้ำหนักของรังไข่ และจำนวนของฟอลิเคิลขนาดใหญ่ ลดลงอย่างมีนัยสำคัญ แต่ไม่มีผลต่อความหนาของมดลูกในชั้นเอนโดมีเทรียม และเส้นผ่านศูนย์กลางของต่อมของผนังมดลูก ความหนาของชั้นกล้ามเนื้อของมดลูกในแมวที่ได้รับการฝังฮอร์โมนนั้นลดลง เมื่อเทียบกับแมวกลุ่มควบคุมในระยะ ฟอลลิคูลาร์ และลูเตียล การทดลองดังกล่าวไม่พบการแตกต่างของการแสดงออกของ mRNA ของตัวรับฮอร์โมน FSH และโปรตีนของตัวรับฮอร์โมน LH ระหว่างกลุ่มการทดลอง แต่พบว่าการแสดงออก

โดยสรุปแล้ว การฝังฮอร์โมนจีเอนอาร์เอช-อะโกนิสต์ ในแมวก่อนวัยเจริญพันธุ์เพศผู้ และเพศเมียนั้น ส่งผลต่อการระงับการแสดงออกของพฤติกรรมทางเพศ และสมรรถนะของระบบสืบพันธุ์ โดยไม่ส่งผลกระทบข้างเคียงต่อตัวสัตว์เป็นระยะเวลาอย่างน้อย 48 สัปดาห์ ในแมวเพศเมียนั้น น้ำหนักของรังไข่ การเจริญของฟอลลิเคิล การผลิตฮอร์โมนเอสตราไดออล และความหนาของชั้นกล้ามเนื้อของผนังมดลูก จะลดลงในแมวที่ได้รับการฝังฮอร์โมนจีเอนอาร์เอช-อะโกนิสต์ ซึ่งอาจเป็นผลจากการเปลี่ยนแปลงของการแสดงออกของ mRNA ของตัวรับฮอร์โมน LH ในเนื้อเยื่อรังไข่ ส่วนในแมวเพศผู้ที่ได้รับการฝังฮอร์โมน จีเอนอาร์เอช-อะโกนิสต์ นั้นพบว่ามีผลต่อการยับยั้งสมรรถนะการทำงานของระบบสืบพันธุ์ กดการแสดงออกของโปรตีนของตัวรับฮอร์โมน LH และส่งผลกระตุ้นการแสดงออกของ mRNA ของตัวรับฮอร์โมน FSH

ภาควิชา สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

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NICOLE SIRISOPIT MEHL: EFFECT OF GnRH-AGONIST (DESLORELIN) IMPLANTATION ON REPRODUCTIVE PERFORMANCES IN EARLY PREPUBERTAL MALE AND FEMALE CATS. ADVISOR: ASSOC. PROF. SUDSON SIRIVAIDYAPONG, D.V.M., Ph.D., 92 pp.

The main objective of this study was to investigate the effect of GnRH-agonist (Deslorelin) implantation on the sexual behavior, reproductive function and gonadal Luteinizing hormone receptor (LHR) and Follicular stimulating hormone receptor (FSHR) expression in pre-pubertal male and female cats. The study had two main parts.

In the *first part* of the study two experiments were performed. The 1st experiment was conducted to investigate the effect of GnRH-agonist implantation on sexual behavior, reproductive performance and expression of testicular LHR and FSHR in pre-pubertal tomcats, and in the 2nd experiment testicular characteristics, and LuteinLHR and FSHR expression were compared between pre-pubertal and adult tomcats (n = 6 / group).

In Expt.1, 3 months-old tomcats (n=6/group) were either implanted with or left without 4.7 mg deslorelin implants for a period of 48 weeks. Semen collection and evaluation were performed just before castration after 48 weeks of treatment; removed testes were analyzed for mRNA and protein expression of LHR and FSHR. Body weight and testicular volume were compared between groups using Independent t-test. General linear model (GLM) was performed to compare the protein and mRNA expression of LHR and FSHR and the epididymal weight. Wilcoxon rank sum test was performed to compare the testicular weight, the mean diameter of seminiferous tubules and the grade of seminiferous tubules between groups. It was possible to collect semen from all the control cats, whereas semen could not be collected in implanted cats. Sexual behavior was absent in the deslorelin-implanted cats throughout the study period whereas it was present in the control cats from 28th week of the experiment onwards. Testicular volume started to decrease from 30th week of the implantation onwards compared to the controls ($P < 0.05$). Testicular tissue score, seminiferous tubule diameter and LHR protein expression were found to be significantly lower in the implanted cats ($P < 0.05$) but no differences were observed in mRNA expression of LHR and protein expression of FSHR between groups. The mRNA expression of FSHR was higher in the implanted ($P < 0.05$) compared to control cats.

In Expt. 2, testes from pre-pubertal (n=6) and adult (n=6) male cats were collected after castration and analyzed for mRNA and protein expression of LHR and FSHR. No difference were observed in the protein expression of LHR and FSHR between the two groups, while mRNA expression of FSHR was higher in pre-pubertal cats ($P < 0.05$) compared to adult ones. Testicular and epididymal weight, diameter of seminiferous tubules and the testicular grade were higher in the adult compared to pre-pubertal cats ($P < 0.05$).

The *second part* of the study was performed in prepubertal female cats; they were either implanted with 4.7 mg deslorelin (Group 1: n=6) or left without implants (Group 2: n =18; Group 3: n =6). Body weights, fecal estradiol and sexual behavior of cats in Groups 1 and 2 were monitored for 48 weeks followed by collection of their ovaries and uteri. Ovaries and uteri were collected from the control cats (Group 2) at their follicular, luteal and inter-estrus stage (n = 6/group) of the estrous cycle. Ovaries and uteri were collected from cats in Group 3 while they were still pre-pubertal. Both ovaries and uteri were analyzed for anatomical and histological characteristics. Ovaries were also analyzed for LHR and FSHR protein and mRNA expression. Data were statistically analyzed; body weights were compared between Groups 1 and 2 by Independent T-test and GLM was used to compare the ovarian weight, endometrial gland diameter, thickness of endometrium and myometrium, number of primordial, primary, secondary and antral follicles, and the mRNA and protein expression of LHR and FSHR among the experimental groups. The control cats had significantly higher ($P < 0.05$) body weight compared to implanted cats only during the 22nd to 26th week of the experimental period. Unlike the control cats, neither fecal estradiol peak nor estrus behavior was observed in the implanted cats. Deslorelin significantly ($P < 0.05$) reduced the ovarian weight and the number of antral follicles. Endometrial thickness and gland diameter were not affected by deslorelin. However, myometrial thickness of the implanted cats was significantly ($P < 0.05$) lower than the control cats at the follicular and luteal stage. Ovarian LHR mRNA expression was significantly ($P < 0.05$) lower in the implanted compared to the control cats at follicular stage of estrous cycle. FSHR mRNA and LHR protein expression did not differ among the 3 groups. FSHR protein expression was, however, significantly ($P < 0.05$) lower in pre-pubertal cats but was not affected by deslorelin-implantation.

In summary GnRH-agonist implantation of pre-pubertal male and female cats suppressed their sexual behavior and reproductive function without any adverse effects for at least 48 weeks. In male cats, the GnRH-agonist implants suppressed the protein expression of LHR and enhanced mRNA expression of FSHR along with suppression of reproductive function. In conclusion, GnRH-agonist implantation can be effectively used in pre-pubertal male and female cats to suppress their reproductive function and delay puberty without any adverse effects for at least 48 weeks. In female cats the ovarian weight, follicle development, estradiol production and myometrial thickness were suppressed by GnRH-agonist implantation possibly through a change in the ovarian mRNA expression of LHR.

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Student's Signature .....
Advisor's Signature .....

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LIST OF ABBREVIATIONS

%	percent
°C	degree celcius
Arg	Arginine
bp	base pair
cAMP	Cyclic adenosine monophosphate
CL	corpus luteum
cm^3	cubic centimeter
COOH-	Carboxyl group
DAB	3,3'-diaminobenzidine
DNA	Deoxyriboneucleic acid
EIA	Enzyme immunoassay
Expt	Experiment
fg/µg	femtogram per microgram
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
fZP	felines' zona pellucida
G	grams
GLM	General Linear Model
Gly	Glycine
GnRH	Gonadotropin releasing hormone
H	hours
H&E	hematoxillin and eosin
His	Histidine
HPG	Hypothalamo-pituitary-gonadal
IgG	immunoglobulin G
IHC	immunohistochemistry
Kg	kilogram
Leu	Leucine

LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
M	molar
mg	milligram
min	minutes
mm^2	millimeter
MPA	medroxyprogesterone acetate
mRNA	messenger ribonucleic acid
ng/g	nanogram per gram
ng/ml	nanogram per milliliter
ng/µl	nanogram per microliter
NH^2-	amino group
NTC	Non-template control
OVH	ovariohysterectomy
PBS	phosphate buffer solution
PCR	polymerase chain reaction
pg/mg	picogram per microgram
pg/ml	picogram per milliliter
POA	preoptic area
Pro	Proline
pyroGlu	pyrophosphate glutamic acid
pZP	zona pellucida protein
qPCR	quantitative real-time polymerase chain recation
RIA	radioimmunoassay
RNA	ribonucleic acid
RPM	round per minute
s	second
SEM	standard error of the mean
Ser	Serine
sperms/ml	sperms per milliliter
SQ	Starting Quantity

TNR	Trap neuter return
Trp	Trypsin
Try	Tyrosine
UV	ultraviolet
v/v	volume by volume
w/v	weight by volume
wk	week
ZP	zona pellucida
µg/kg/day	microgram per kilogram per day
µl	microliter
µm	micrometer
π	Pi

CHAPTER I

INTRODUCTION

1.1 Importance and rationale

Across the world, more than 25 percent of households in big cities own a dog or a cat. Despite the fact that more health care for pets is provided nowadays, there are still a massive number of pets which are left out of health care. Cats that are left out of health care could develop diseases and therefore may become unhealthy and suffer to death. On the other hand, overpopulation that could occur from the abundant of health care and/or birth control, could lead to a number of serious issues such as environmental problems, zoonotic diseases, nuisance to people in the community and disruption of the ecosystem mainly to the wild life of the area/region. Although many educational programs (including help in management) like knowledge of responsible pet ownership, 'trap neuter return' (TNR) program, aggressive encouragement of adoption or putting feral cats into shelters (Joshua and Pamela, 2007; Robertson, 2008) are provided to the owners and the workers alike who are responsible of cats, there are still a large amount of cats that are left out and remain unattended of these management programs. In some situations, death is the ultimate conclusion for such animals, which is basically against the welfare and/or ethics. Birth control could offer an acceptable option to this problem of overpopulation.

Methods to control the overpopulation of cats are being developed but yet, none of the recent methods available are compatible and could completely help manage and/or reduce the overpopulation of cats. The novel contraceptive method is supposed to be the one which 1) is reliable and effective, 2) has minimal or no toxicity or harmful side effects on the animal, 3) is of low cost, and 4) is easier to perform/conduct (Munson, 2006; Robertson, 2008). The development of such a method will certainly be able to contribute to an effective tool for population control.

Limitations to the success of cat overpopulation control programs are mainly the lack of responsibility by the owners and/or communities, the natural physiology of

reproduction and/or the natural behavior of cats. Owners should be responsible in controlling unwanted litters of their own pet. Free roaming of intact pets should be strictly limited. The community should be aware of the problem, contraception programs such as TNR should be supported by the community. Adoption of feral animals in the community should be supported intensively. Unawareness of the owners and people in the community has been reported to be one of the major limitations for pet overpopulation control (Finkler and Terkel, 2012). The next limitation in the cat population control is the natural behavior of cats. Roaming behavior especially of the intact male cats and female cats during their follicular phase of the estrous cycle is out of control for many owners. Roaming of these pet cats could lead to unexpected mating with other roaming or feral cats in the area. Such mating at the most appropriate timings can be highly successful and could result in a high number of offspring per litter. Cats are the animals that can adapt into many diverse situations, they can survive either with or without depending on humans. Adaptability of cats contributes to the higher survival rate of feral cats observed throughout many countries. Success rate of breeding has been reported in most feral cats especially when they are in areas with unlimited resources, they could provide up to 5 offspring for several times in one year. Presence of feral cat colonies in the remote areas with abundant prey and the intact females during their follicular period pose a great difficulty to the population control programs in each community (Fisher et al., 2014). Another limitation to cat population control is the reproductive physiology of cats, especially female cats which are seasonally polyestrous and induced ovulatory. Estrus in female cats lasts for about 5 – 7 days and if fertilization does not occur, they return to estrus again in every 2 – 3 weeks and this may continue for several months until fertilization and pregnancy (Malandain et al., 2011). Breeding season depends on the day length. Tropical countries such as Thailand has almost 12 hours day length for more than 8 months a year, that cats being long day breeders would mean that cats in countries like Thailand will have a long breeding season extending to almost whole year. The long duration of breeding season leads to an increase in the population and the associated problem of unwanted litters and feral cats. Puberty in cats may occur at a very young age (4 – 12 months) (Johnston et al., 2001a), and as soon as puberty

is achieved, unexpected mating and unwanted litters could occur due to unawareness of most of the owners.

Contraception is one of the most successful methods for population control in many animal species. Contraception is presently achieved by ovariohysterectomy (OVH) and/or castration but the procedure is invasive with risk of animals undergoing general anesthesia especially in juvenile, senile and unhealthy cats. Progestins are widely used to control reproduction in cats, however, its undesirable effects such as cystic endometrial hyperplasia, mammary gland hypertrophy, mammary gland neoplasia and diabetes mellitus, warrants its use (Johnston et al., 2001a).

Many methods have been launched to help in managing the overpopulation problem in cats. Non-surgical contraception seems to be an ideal method for population control, but as long as scientists are developing the ideal non-surgical contraception method, the surgical contraception program like TNR remains the most proper method for population control (Robertson, 2008).

An earlier age of puberty in cats requires the contraception programs to be intensive and applied since early age. Neutering of cats in their early age is called “prepubertal contraception”. The main objective of prepubertal contraception is to eliminate any chance of mating and to eliminate any unwanted behavior from the effect of sex steroid hormones. Due to its advantages, prepubertal neutering is an appropriate method to assist overpopulation control in cats (Joyce and Yates, 2011). Even though surgical contraception could be easily done by an experienced veterinarian with a high rate of success, performing surgery in young animals could lead to a higher risk from the anesthesia protocols, such as the higher sensitivity to many drugs, an impaired hepatic function which may lead to prolonged effects of drugs and lower capacity of compensation by the cardiovascular system (Joyce and Yates, 2011). Nonsurgical contraception will be an ideal alternative to surgical method in the future (Spain et al., 2004). However, so far it has not been met with a complete success and further research studies are required in this respect and so is the main objective of the work reported in this thesis.

1.2 Literature review

1.2.1 Puberty in cats

The definition of puberty in male and female mammals is implied as the onset of normal reproductive function, complete of spermatogenesis or the onset of first ability to ejaculate in male and complete cyclicity of estrous cycle in female animals (Evans and Ganjam, 2001; Johnston et al., 2001a). Onset of puberty in domestic cats could occur from 4 – 12 months of age in queens and from 7 – 10 months of age in tomcats (Johnston et al., 2001a). Many factors such as the photoperiod, body weight or the environment could influence the onset of puberty in cats (Root et al., 1997). At puberty, many physical and behavioral changes are observed in both male and female domestic cats, most the behavioral changes are mainly due to the changes in the concentrations of hormones in the circulation.

1.2.1.1 Hormonal changes

At puberty, development of the hypothalamo-pituitary-gonadal (HPG) axis is complete and it becomes fully functional to control the reproductive system in both males and females. During the prepubertal period, the levels of reproductive steroid hormones such as estrogen and progesterone in female cats and testosterone in male cats will be found at basal level, but at puberty the concentrations of these hormones increase as to initiate and maintain normal reproductive function. A study on prepubertal domestic cats found that the levels of sex steroid hormones (testosterone in males and estrogen in females) will increase in the earlier weeks postnatal, then gradually decrease to a basal/low levels and will increase again at the age of puberty (Faya et al., 2013). The testosterone levels in male adult cats range from non-detectable to 20 ng/ml plasma and the peak serum estrogen level during estrus stage is more than 70 pg/ml (Johnston et al., 2001a). Apart from the increase in sex steroid hormone levels, the levels of other hormones in the HPG-axis such as luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland and gonadotropin releasing hormone (GnRH) from the hypothalamus, also increase around

the time of onset of puberty. The serum LH levels in male adult cats are reported to have a wide range (from 2.1 to 29.2 ng/mg) but serum LH levels in female cats remain low/basal until the time of coital or spontaneous ovulation (Johnston et al., 2001a).

1.2.1.2 Behavioral and physical changes

Estrus behavior is one of the obvious behaviors that could be detected as a queen reaches puberty. Stages of estrous cycle in pubertal female cats include follicular, luteal and interestrus. During estrus female will show behaviors such as crouching with their hind limbs pressed to the ground, hyperextension of the back, lordosis, rubbing and rolling, perineal grooming and presentation of their vulva. Apart from the behavioral symptoms, estrus in female cats could also be confirmed by the changes in vaginal cytology which are mainly due to the effect of reproductive steroidal hormones.

In male cats, when they reach puberty, their testes could be obviously seen in their scrotal sacs with spherical to ovoid shape, penile spines at the shaft of penis increase in number because of an increase in testosterone concentrations. Other than the phenotypic changing there are also reproductive behaviors that will express at the onset of puberty such as copulatory behavior, erection and ejaculation (Johnston et al., 2001a).

1.2.2 Hypothalamo-pituitary-gonadal axis (HPG axis)

1.2.2.1 Physiology and function of the HPG axis

Vertebrates' reproductive tracts are controlled by a neuroendocrine system (Sower et al., 2009). The center of this system starts at the hypothalamus by its action on controlling the release of gonadotropins from the anterior pituitary. The hypothalamic neurons secrete GnRH which is responsible for the release of the gonadotropins [luteinizing hormone (LH) and follicle stimutating hormone (FSH)] from the anterior pituitary (Clarke and Pompolo, 2005; Noakes et al., 2009). During the fetal development the hypothalamic GnRH is activated, and has lower level of activity during the postnatal/prepubertal period, and finally its activity is increased again at puberty (Conn and Crowley, 1994). GnRH acts on the hypophyseal gonadotropes to

control the release of LH in a pulsatile pattern and maintain FSH synthesis (Clarke and Pompolo, 2005).

Ovulation in queens and other domestic species which are induced ovulators is induced by the stimulation of the sensory receptor in the vagina and cervix to activate the GnRH neurons in the surge center to release GnRH, followed by the release of LH surge (Noakes et al., 2009) which induces ovulation of the preovulatory follicle(s) in female cats (Davidson, 2005). During the feline estrous cycle, in the beginning of proestrus, follicle development is induced by FSH. After initial follicular development, estradiol secreted from the dominant follicle stimulates the signs and physical changes of estrus, mating at this stage induces the secretion of LH surge and the ovulation of dominate follicles. Corpus luteum is developed after ovulation and secretes progesterone to help maintaining pregnancy. The high concentrations of serum estrogen and progesterone have negative feed back on GnRH secretion from the hypothalamus. (Bristol-Gould and Woodruff, 2006). LH in male cats is secreted in a pulsatile manner by the episodic stimulation from the GnRH neurons and controls testosterone synthesis from the Leydic cells in the testes. FSH stimulates the growth of follicles and the production of estrogen in female cats and stimulates spermatogenesis in male cats (Gilbert, 2005).

The control of the HPG axis by reproductive steroid hormones

Gonadal steroids have a negative effect on the secretion of GnRH and/or gonadotropins. Inhibin and androgen plays an important role in the regulation of gonadotropins in male mammals. Testosterone and its metabolite (estradiol 17-β) are known for their negative feedback effect on GnRH. Inhibin which is produced by the sertoli cells in the testes also plays an important role to control the regulation of the HPG axis especially the regulation of FSH by suppressing the release of FSH from the pituitary (Tilbrook and Clarke, 2001). In female mammals, estradiol and progesterone are responsible for the feedback effects on GnRH and gonadotropins. In other mammals estradiol also controls the positive feedback to LH but in felid species, LH surge is induced by the stimulation during the copulation of male felids. Estradiol also acts as negative feedback on FSH and LH by regulating the mRNA encoding the

gonadotropins. Moreover, hypothalamus and pituitary are the sites of action of the negative feedback by estradiol and the action of estradiol depends on its dosage. Progesterone in the luteal phase acts as a negative feedback to LH by reducing the pulse frequency through the hypothalamic GnRH and LH respectively (Couzinet and Schaison, 1993).

The control of reproductive steroid hormones on the HPG axis is explained by a brief model of the HPG axis in Figure 1.

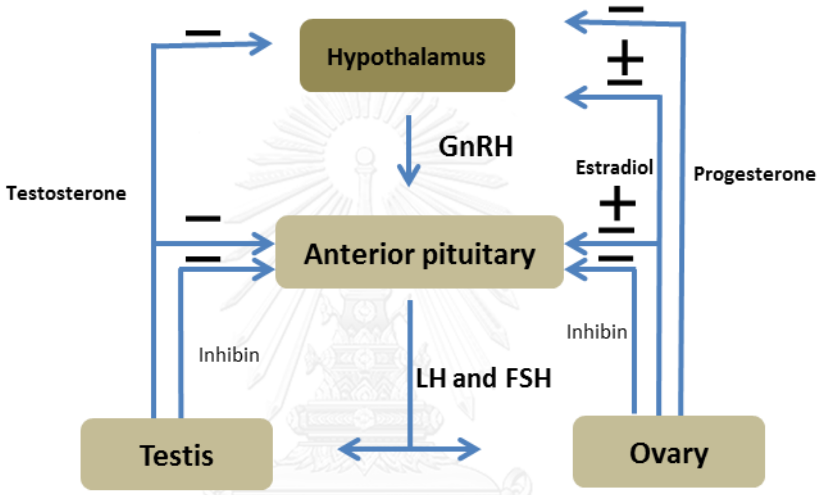

Figure 1 *A model of hypothalamic pituitary gonadal axis in mammals*

1.2.2.2 Gonadotropin releasing hormone (GnRH)

The HPG axis regulates the gonadal function of mammals (Aparicio, 2005) and the key hormone in this axis is Gonadotropin releasing hormone (GnRH) which is synthesized by the hypothalamic GnRH neurons and is released in a pulsatile manner (Conn and Crowley, 1994). GnRH is a decapeptide (Figure 2) and each of the amino acid residues have their own functional properties as shown in Table 1.

The GnRH neurons which secrete the GnRH are located at the basal forebrain around the preoptic area (POA) and the mediobasal hypothalamus (Clarke and Pompolo, 2005). GnRH cells could be influenced by many intrinsic and extrinsic factors, which may include the gonadal feedback, stress, nutrition and season (Clarke and Pompolo, 2005). More than 20 decapeptide forms of GnRH have been identified but only 2 forms are identified in mammals which are redundant to GnRH I and GnRH II.

GnRH I is known to regulate the gonadotropins and GnRH II acts as a neuromodulator and stimulates sexual behavior. Type II receptor is present in the nervous system where GnRH II plays a role as a neuromodulator as well as in non-neural reproductive tissues (ex. prostate tissue). The finding of GnRH II in sensitive neurons and brain areas in some species has leaded to a speculation of its role in eliciting reproductive behaviors in those species (Millar, 2005). Among the 20 forms the terminal sequences are conserved as NH2- and COOH- terminal. These end terminals are the binding site for GnRH decapeptides to their receptors (Millar, 2005). Figure 2, shows the model of GnRH decapeptide and the function of each peptides are shown in Table 1.

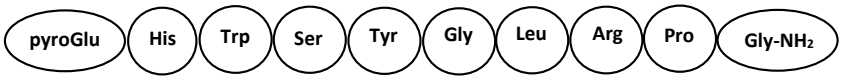

Figure 2 *The model of mammalian GnRH decapeptide hormone*

Table 1 *The individual functions of each amino acid residues from the GnRH decapeptide.*

Aminoacid residues	Function
pyroGlu	Uncharged – CONHCHO-N-terminus required
His	Aromaticity required
Trp	Aromaticity required
Ser	Any small chain acceptable
Tyr	Aromaticity required
Gly	Essential to assume bent conformation and D-aminoacid substitution enhances binding
Leu	Not essential but must be uncharged
Arg	Essential for high affinty binding but not if D-aminoacid is in position six
Pro	Essential conserved residue
Gly-NH_2	Small uncharged C-terminus required

Data modified from Padula (2005)

GnRH stimulates the gonadotrophs in the anterior pituitary to release gonadotropins such as LH and FSH. The pulsatile secretion of GnRH is reflexed in the the gonadotropins release (Krsmanovic et al., 2009), the gonadotropins then stimulate the gonads. The pituitary GnRH receptors mediate the reproductive function as well as determine the ability of pituitary gland to respond to GnRH (Rispoli and Nett, 2005). Other roles of GnRH action include neuroendocrine, paracrine, autocrine and neurotransmitter roles. The regulation of the hypothalamic pituitary gonadal axis depends on the positive and negative feedback of sex steroid hormones to the reproductive axis. Aside from the central nervous system, GnRH and its receptors are also localized in various tissues such as the placenta, the gonads, peripheral nervous system, immune cells, breast cancer cells and prostatic cancer cells. The GnRH and its receptor in the gonads suggest their neuroendocrine role by direct regulation before the evolutionary development of the pituitary regulation (Millar, 2005).

1.2.2.3 Luteinizing hormone, Follicular stimulating hormone and their receptors

Gonadotropin hormones regulate normal function and development of the gonads (O'Shaughnessy et al., 1997) by binding to their receptors present in the gonads of mammals (Howell-Skalla et al., 2000; Ahda and Soeharso, 2003; Saint-Dizier et al., 2007; Nogueira et al., 2010). LH and FSH receptors expression has been reported since the fetal and neonatal phase, and their level of expression were found to be related to the oocytes' development.

Although many studies reported the presence of LH and FSH receptors in both gonadal and other tissues, the physiological function and the regulation of these receptors are not fully understood. Gonadotropins-suppressed animal may be an ideal model to get further information on the regulation and functions of these receptors. Morever, such studies might also explain the effects of GnRH-agonist implantation more thoroughly.

1.2.3 Contraception in felids

1.2.3.1 Methods of contraception

Contraception is used to control the animal's reproductive status and the methods used in cats could be classified into categories of surgical and non-surgical. Surgical contraception in cats are known as ovariohysterectomy in females, vasectomy and castration in males. Non-surgical contraceptive methods include the use of pharmacological or chemical barriers or area restriction to block or control fertilization at different levels of reproductive function with the main aim to arrest the reproductive system of male and/or female animals (Munson, 2006). Nowadays, ethics has become a major issue in many countries, and surgical contraceptive techniques are criticized for ethical reasons. For this reason non-surgical contraceptive methods have been introduced since many years in domestic animals and wild captive animals. Below is a simple description of such methods.

Non-surgical contraception methods in cats

a.) Confinement

Confinement is one of the traditional methods used especially by the owners who want more litters at the right time with the right tom. Owners could restrict their cat in estrus period in a certain area to avoid contact with other intact male cats. Although this method is easy to conduct but it is not permanent. Moreover, vocalizing and behavior of cats under confinement is highly unappreciated (Jackson, 1984).

b.) Hormonal contraception methods

Hormones of the HPG aixs could be used for the purpose of contraception and/or to arrest at least one pathway of the functions of the reproductive tract. Hormones or synthetic agonist/antagonists used for cat contraception had a wide range and include progestins, GnRH-agonists and antagonist and androgens. Progestins that are commercially available are medroxyprogesterone acetate (MPA), megestrol acetate, levonogestrol and melengestrol acetate. Progestin administration inhibits the estrous cycle in female cats by a not quite clearly understood method but there are suggestions that progestin administration might act either inducing a negative feedback

effect on the GnRH and gonadotropins or cause oocyte transportation and fertilization failure or embryonic transplantation failure by affecting the tubular movement and the endometrial function. The progestins have been used by implantation, injection or even oral administration. Although progestins therapy is affordable with high variety of application and with highly reliable effects but significant adverse effect from progestins are needed to be aware of (Munson, 2006).

c.) Chemical contraception methods

Chemicals are used to arrest spermatogenesis by injecting chemicals directly into the male cats' testicular parenchyma. Zinc gluconate has been used for this purpose. Although this method has great potential as was reviewed earlier (Oliveira et al., 2013; Fagundes et al., 2014), it is presently unacceptable because it is found to be toxic, causes irritation and trauma to cats (Munson, 2006).

d.) Pharmacological contraception methods

Certain medicines used for other medical reasons are found to also have effect on reproductive function. Bisdiamine compounds are an example of such drugs which are normally used for their amoebicidal properties but they also cause spermatogenesis arrest in male cats. This suppression of spermatogenesis is, however, reversible after the termination of drug administration with no adverse effect in male cats but administration in pregnant female cats could cause teratogenic effects (Munson, 2006).

e.) Immunological contraception method

Immunizing against reproductive antigens is the key of this method. Antigens which are recently studied and used to control the reproductive system include zona pellucida (ZP) proteins in female cats, LH receptor protein in female cats and GnRH receptor protein in both male and female cats.

Immunizing against ZP antigen will lead to a disruption in sperm binding to the oocyte. In female cats, immunizing against ZP was firstly induced by porcine zona pellucida protein (pZP), unfortunately the success rate was quite low because the pZP could not completely cross react with felines' ZP (fZP). ZP protein of other species also has the same trend in cats, and after immunization cats remained cyclic and were able to be pregnant. Later on, in some other studies immunization against fZP has been

reported to result in lower litter size (Ringleb et al., 2004; Levy, 2011) but the success of the immunization against fZP still remains in doubt.

GnRH is another reproductive antigen which is a target of immunization. GonaCon™ is a synthetic vaccine against GnRH antigen. A part of both male and female cats vaccinated with GonaCon™ did not develop a high titer of antibody against GnRH and were without suppression of their reproductive function. However, some animals was able to develop high titer with the suppression of their reproductive function. Male cats that developed high titer against GnRH were monitored with the suppression of testosterone, the decline of semen production and the change in phenotype of male characteristics but their reproductive function recovered in 3 years after vaccination. While female cats with high titer was reported to be infertile for up to 5 years after a single vaccination (Kutzler and Wood, 2006). Vaccine against LH receptor is another recent immunocontraceptive technique. Estrus behavior suppression was found in a study of female cats implanted with a subcutaneous implantation against LH receptor protein (Saxena et al., 2003).

1.2.4 GnRH antagonist

GnRH antagonist is another application used for contraception. The antagonist binds with the GnRH receptor in the pituitary. The GnRH antagonist occupies the pituitary GnRH receptors and therefore stops the binding of endogenous GnRH to its receptors. After the administration of GnRH antagonist, the release of gonadotropins will be suppressed followed by the suppression of the reproductive function (Herbert and Trigg, 2005). Although the development of GnRH antagonist and its application is quite limited but there are some studies show that GnRH antagonist may be an application for contraception in mammals in the future (Padula, 2005; Garcia Romero et al., 2009).

1.2.5 GnRH-agonist and its application

GnRH-agonist is a type of GnRH analog which acts after binding to its highly potent G-protein coupled GnRH receptors (Conn and Crowley, 1994). The high affinity for the GnRH receptor and the stability of analogs are the two major points of concern for GnRH analog development (Padula, 2005). The modification of the decapeptide at the

6th position (glycine) with a D-amino acid and the replacement of an ethylamide group instead of the C-terminal glycinamide residue helps enhance the affinity of the GnRH molecules (Herbert and Trigg, 2005). New GnRH-agonist applications such as deslorelin are developed to be easily used and could induce GnRH actions for a long period of time for long-term fertility suppression (Herbert and Trigg, 2005). Deslorelin is a GnRH-agonist known as a superagonist. The potency of deslorelin was found to be 144 times greater than natural GnRH and was found to have good thermal stability (Padula, 2005). The applications of GnRH and its analogs in clinical use are seen as two different models. The first application is to restore fertility in GnRH deficient patients, either to imitate the normal frequency of endogenous GnRH secretion or by its action during the up regulation effect of the analog. The second application is the suppression of the pituitary gonadal axis by the down-regulation effect of the analog (Conn and Crowley, 1994). The GnRH treatment is shown to have a flare up effect in the initial phase of treatment, which results in the increase of plasma LH and FSH concentration, but as the treatment continues and the exposure remains, a down-regulation effect results with the decreasing amount of plasma LH and FSH concentration (Herbert and Trigg, 2005). The suppression by GnRH-agonist analogs normally is a selective and reversible suppression to the reproductive system and are mainly used in the parenteral route because of the degradation of peptides from gastrointestinal peptidases (Padula, 2005). Intramuscular and subcutaneous are proven sites of administration that show the effect on reproductive tract suppression in mammals. The duration of action on fertility suppression is proven to have a positive correlation with the dosage of GnRH-agonist given, but the mechanism and actions still remain unclear (Herbert and Trigg, 2005).

Studies of GnRH-agonist in felids are done mostly in wild felid species, mainly for the reversible action and convenient procedure of the hormonal castration by GnRH-agonist implantation (Bertschinger et al., 2007; Asa et al., 2012). Recently there are more studies on domestic cats due to the increasing problems of over population of domestic cats. Goericke-Pesch et al. (2011) demonstrated a castration related effect in pubertal tomcats after the treatment with subcutaneous GnRH-agonist implantation containing 4.7 mg deslorelin in a study lasting for 36 weeks (9 months). Munson et al.

(2001) found a suppression of estrous cycle in cats from the treatment of subcutaneous GnRH-agonist implantation containing 6 mg deslorelin in a study lasting for 56 weeks (14 months). Toydemir et al. (2012) also demonstrated a suppression of ovarian cycle in pubertal female domestic cats after the treatment with subcutaneous GnRH-agonist implantation containing 4.7 mg deslorelin in a study lasting for 74 weeks (18.5 months). An administration of subcutaneous GnRH-agonist implantation containing 4.7 mg deslorelin in pubertal tomcats found that the application could suppress the function of the reproductive tract, but the onset of effects were varied among individual cats. They also found a complete reversible reproductive function in tomcats after implantation removal. This study also recommended a need for more comprehensive examination of the cats to monitor the suppressive and/or other effects (Novotny et al., 2012).

There are still only a few studies that report usage of GnRH-agonists in prepubertal animals. For GnRH therapy a number of species have been tried as a model for humans. Prepubertal rats were the first one in the list; they were given low dose (20 μg/kg/day) and high dose (200 μg/kg/day) of leuprolide acetate [D-Leu^{6}, des-Gly-$NH_2{}^{10}$, Pro-ethylamide^{9}], a synthetic GnRH analog. Lower weight and atrophic changes in the reproductive organs were found in the treatment groups compared to the control group. Histology of reproductive organs returned to normal in both males and females after 140 days of recovery period, reproductive function was recovered back to normal in almost every group except the females treated with high dose of leuprolide (Lehrer et al., 1992). The long-term use of GnRH-agonist applications results as an anti-fertility effect. Lacoste et al. (1989) used subcutaneous daily administration of GnRH Ethylamine [D-Trp^{6}, des-Gly-$NH_2{}^{10}$] in prepubertal dogs and investigated the effects of long term or chronic GnRH-agonist administration as a model for humans. The administration of GnRH-agonist took place for 23 months (the same duration as the usage in treatment of precocious puberty in boys and girls); the pituitary gland, the reproductive tract and the accessory gland (prostate gland) of the treated dogs were found to be suppressed, but were returned back to normal after a recovery period of 14 months. Trigg et al. (2006) performed a study on both 4 and 7 months old female dogs with either 9.4 mg deslorelin subcutaneous implantation for 38 weeks. No signs

of estrus were found in the group implanted at 4 months old, while estrus signs were observed 1 – 2 weeks after implantation in the 7 months old dogs. However, no estrus signs were detected in this group between 12 – 28 weeks after implantation. This leads to a conclusion that the reproductive tract of 4 months old prepubertal dogs could be suppressed without a flare up effect by the implantation of 9.4 mg deslorelin GnRH-agonist. In another study 4-months old prepubertal bitches were given Gonazon®, a GnRH-agonist device containing azagly-naferelin and were compared with a control group without the treatment at the age of 8 – 16 months old. One year delay in puberty in the young was observed in prepubertal bitches without up-regulation of the reproductive system, while the control group which were treated at 8 – 16 months old shown signs of up regulation effect after the treatment then following by the down-regulation effect. The absence of an up regulation effect in 4 months dogs was explained on the basis of immaturity of the hypothalamo-pituitary axis and/or immaturity of the ovaries (Rubion et al., 2006). A study of the subcutaneous GnRH-agonist implantation containing 4.7 mg deslorelin in female domestic cats at the age of 114.4 ± 12.7 days old to postpone puberty. Puberty was detected at 180 – 428 days old in deslorelin implantation group, while in the control group, puberty was detected at 134 – 286 days old. This showed that the implantation of 4.7 mg deslorelin at the age of 114.4 ± 12.7 days old was postponed to only 180 – 428 days old (6 – 14.2 months old). Whereas, the control group without treatment presented pubertal sign at 134 – 286 days old (4.46 – 9.53 months old). Although they reported very low side effects in the deslorelin administration group, one female cat showed clinical signs of pyometra at 92 days after administration and another cat administrated with deslorelin agonist showed signs of estrus after 13 days of GnRH-agonist implantation (Risso et al., 2012). Another study was conducted in postnatal cats implanted with 1.6 mg deslorelin, puberty was postponed until the age of 16 months old (Carranza et al., 2014). From the previous studies, we could conclude that the GnRH-agonist affects the reproductive tract by an up-regulation effect during the first period of treatment, but in a long lasting treatment, it has a down-regulation effect. However, in studies done on prepubertal animals with an immature HPG axis, the up-regulation effect does not

occur and the period of down-regulation effect seems to last longer than the treatment in pubertal animals.

1.2.6 Cats' behavior characteristics

Gonadal hormones play an important role in the behavior of dogs and cats. The typical male cat characteristics are roaming, fighting, and spraying which are affected by surgical castration in most of the animals with some variation in the magnitude of these effects (Hart and Eckstein, 1997). Behavioral characteristics of some animals do not change after castration. On the other hand, castration in male cats may evoke some male sex behavior in male cats more than older ages. As no behavioral problems were found in a study where castration was done at a very early age (Hart and Eckstein, 1997), prepubertal castration is suggested to be the best way to avoid the evocation of adverse behavior observed after castration and the best way to manage behavior problems in male cats. The behavioral characteristics of female cats are usually shown during the estrous cycle, which are vocalization and calls to the male, rubbing and rolling, head rubbing, lordosis, tail deviation, crouching with four quarters pressed to the ground and hyperextension of the back (Johnston et al., 2001a). The behaviors are presented normally at the first estrus. Some of these behaviors may be unwanted by some owners, moreover mating may occur after the first estrus although cats still be very young. To eliminate these behaviors, the suppression of the reproductive tract such as ovariohysterectomy is needed to be performed. Moreover, if unwanted mating is out of control, prepubertal spaying could be an alternative method to control this problem. Reproductive behaviors in mammals are controlled mainly by the gonadal steroid hormones. Therefore, the management of such unwanted behaviors which are caused from gonadal steroid hormones is by suppressing the production of these hormones. Alternatively, one can think of any methods which could prevent these problems from occurring and if such methods are also safe and highly effective, it could be highly useful. A suitable method of prepubertal contraception could be one of the answers to solve these problems.

It is clear from the literature reviewed above that GnRH-agonist implantation has been used to try to delay the puberty and/or suppress the reproductive function

in different species including small and large animals. However, most of these studies were not comprehensive enough and very few were performed in prepubertal animals, particularly prepubertal cats. Moreover, none of these studies have tried to investigate the mechanism by which reproductive function could be suppressed, so this project was planned to test the following hypothesis.

1.3 Research Outline

This research was performed on a total of 48 cats (18 males, 30 females) from June 2012 to October 2013. These cats were randomly subjected to the treatment with GnRH-agonist or control group for the study of the hormone implantation in male and female prepuberty cats.

The first part of research (Chapter III), experiment 1; 12 captive domestic male cats were subjected to two groups, GnRH implantation at the age of 3 months old and without. Sexual behaviors, male characteristics, fecal testosterone level and body weight of all cats were monitored. At the end of the study period, semen collection by electro-ejaculation technique was performed following by surgical castration. Testes and epididymis were weighted and were morphological investigated, testes were collected for the inspection of histological morphology, immunohistochemical localization and the mRNA of LHR and FSHR in the testes. In experiment 2; testes and epididymis from puberty and prepuberty cats collected from the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University were weighted and were morphological investigated, testes were collected for the inspection of histological morphology, immunohistochemical localization and the mRNA of LHR and FSHR in the testes as well.

The second part of research (Chapter IV) captive domestic female cats were subjected to two groups, GnRH implantation at the age of 3 months old, without implantation (controls) and the last group were ovariohysterectomized at the age of 3 months old. Estrus behaviors, fecal estrogen level, and body weight of all cats were monitored. At the end of the study period, ovariohysterectomy was performed. The cats in the control group was ovariohysterectomized when they were found to be in

different stages of estrous cycle (follicular, luteal and inactive stage) (n=6 per each stages). Ovaries and uterus were morphological invested, ovaries were weighted, both ovaries and uterus were collected for the inspection of histological morphology. Immunohistochemical localization and the mRNA of LHR and FSHR were observed in the ovaries.

1.4 Research Hypothesis

1.4.1 The GnRH-agonist implantation of prepubertal male and female cats delays puberty in both male and female cats by suppressing the development and/or function of reproductive tract including the protein and mRNA expressions of gonadal LH and FSH receptors in prepubertal male and female cats.

1.4.2 There will be difference of development and function of the reproductive tract including the protein and mRNA expressions of LH and FSH receptors between prepubertal male and female cats with pubertal male cats and female cats in different stages of estrus cycle.

1.5 Research Objectives

1.5.1 To investigate the effects of GnRH-agonist implantation at prepubertal age on the different parameters necessary for the initiation of puberty in both male and female cats.

1.5.2 To determine the effects of GnRH-agonist implantation at prepubertal age on the development and function of reproductive tract including the gonadal LH and FSH receptors in both prepubertal male and female cats.

1.5.3 To investigate the differences in the development and function of the reproductive tract including the gonadal LH and FSH receptors between prepubertal male and female cats with pubertal/adult male cats and female cats at different stages of estrous cycle.

1.6 Research Benefits

The main benefit of this study will be that it will demonstrate the usage and effectiveness of GnRH-agonist implantation in prepubertal cats regarding the suppression of the reproductive tract development and/or function. So the results obtained will help to achieve the ultimate goal to achieve population control. From the scientific point of view, apart for this main benefit, this study will also provide useful information on the morphology of reproductive tract of cats implanted with GnRH-agonist at early prepubertal age. Moreover, this study will also assess the effects of the GnRH-agonist treatment on the reproductive organs of the cats and may also reveal part of the mechanism by which GnRH-agonist treatment affects the reproductive tract development and/or function.

1.7 Keywords

Cat, Contraception, Deslorelin, GnRH-agonist, Reproductive behavior

CHAPTER II

GENERAL MATERIALS AND METHODS

2.1 Animals and hormonal implantation

2.1.1 Animals

Three months old tomcats and female cats that were proven to be clinically healthy and attended a complete vaccination program were housed in an open-air room with natural daylight at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Thailand. They were fed with a commercial diet twice daily and water was always available ad libitum.

Each cat was housed separately in its' own cage and was set free in the open area in the room for each behavior monitoring period.

2.1.2 Hormonal implantation

In deslorelin-Implanted groups both male and female cats, were subcutaneously implanted with 4.7 mg deslorelin GnRH-agonist (Suprelorin® 4.7mg, Virbac Animal Health, France) in the interscapular area using aseptic technique.

2.2 Monitoring of adverse effects

Implanted animals were monitored for any potential adverse effects like tissue reaction at the implantation site and/or infection for a period of one week and any rashes, oedema, erythema of implantation area, and other lesions were recorded. Body temperature was measured daily for one week after the hormonal implantation to monitor any infection and if found, blood was collected for blood profile monitoring.

Body weight of all the cats in both groups was recorded fortnightly until the end of the experiment (48 weeks).

2.3 Tissue collection and tissue processing

Reproductive organs were collected from both the control and implanted cats by castration or ovariohysterectomy which was performed after 48 weeks of the start of the experiment by scrotal sac incision immediately after semen collection and by left flank incision, respectively. Both male and female reproductive organs were investigated for their morphology; shape, size and consistency of the testes and epididymis were recorded. Consistency was categorized as soft, firm or hard. The weight of each testis, epididymis and ovaries was recorded in grams. This was followed by fixing part of both testes ovaries and uterine horn in 4% (w/v) paraformaldehyde (Merck, MA, USA) in PBS for 48–72 hours and then storage in 70% ethanol until processing. The second part of the testes and ovaries were snap frozen with liquid nitrogen and kept in -80° C until RNA extraction.

2.4 Immunohistochemical staining technique and the evaluation by image analysis

2.4.1 Immunohistochemicl staining of LHR and FSHR

After deparaffinization with graded alcohol, immunohistochemical staining was performed as described previously (Ponglowhapan et al., 2007). Briefly, the tissue sections were placed in boiling 0.01 M sodium citrate then cooled down to room temperature for 35 min to demask epitopes. Slides were then rinsed three times in phosphate buffer solution (PBS). Endogenous peroxidase activity was inactivated by immersing slides in 1% (v/v) hydrogen peroxide in methanol for 10 min, then rinsed again three times in PBS. Sections were subsequently blocked for 60 min in a humidified chamber using a blocking solution, comprising 1% normal horse serum (Vector Laboratories, CA, USA) diluted in PBS, 20% (v/v). After washing slides three times in PBS, the slides were incubated overnight at 4ºC in a humidified chamber with LHR (H – 50) polyclonal antibody (Santa Cruz biotechnology, Inc., USA) at a dilution of 1:50 or with FSHR (N – 20) polyclonal antibody (Santa Cruz Biotechnology, Inc., USA) at a dilution of 1:50. The negative control sections were treated in the same manner with PBS and biotin mixture in the absence of primary antibodies. After incubation, sections were washed with PBS three times. Then, secondary antibody (Biotinylated

anti-mouse anti-rabbit IgG, Vector Laboratories, Inc., USA for LHR localization and Biotinylated anti-goat IgG, Vector Laboratories, Inc., USA for FSHR localization) was applied to the sections and incubated for 30 min. Sections were washed again three times in PBS, then tissue sections incubated at room temperature with 20% (v/v) avidin-biotin complex solution (VECTASTAIN® Vector Laboratories, Inc., USA) for 30 min. Tissue sections were then incubated with DAB peroxidase substrate (Vector Laboratories, Inc., USA) until color development. All slides were counterstained with Mayer's hematoxylin. Brown staining was observed on tissue sections with positive staining for both LHR and FSHR and no staining was observed for negative controls for either receptor (Figure 3).

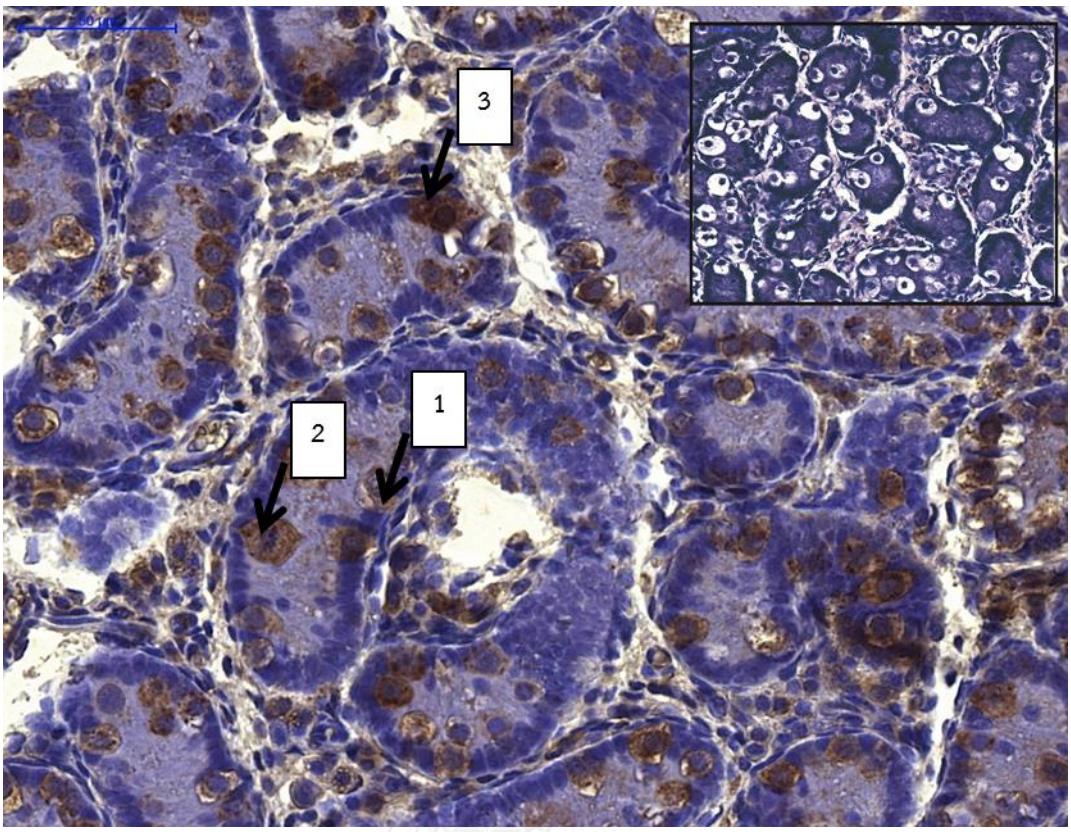

Figure 3 *Testicular tissue with positive staining of FSHR immunohistochemical localizing in the germ cells and sertoli cells of deslorelin-implanted male cats with the difference grade of staining (1, 2 and 3) and the negative control tissue (upper right frame).*

2.4.2 Quantification of immunohistochemical staining

The expression of LHR and FSHR staining was semi-quantified using a light microscope at 200X magnification. Ten fields per section of each tissue sample were assessed, tissue staining was semi-quantitatively scored with the Image-pro plus 7.0 program (Media Cybernetics, Inc., Singapore) as staining grade from 1 to 3, which represents weak, moderate and strong staining respectively (Ponglowhapan et al., 2007) (Figure 3). Expression index was calculated for statistical analysis from the percentage of each expression multiplied by the intensity score of staining, the score was ranged from 0 to 300 (Ishibashi et al., 2003; Ponglowhapan et al., 2008).

2.5 Quantitative real-time polymerase chain reaction (qPCR) for the LHR and FSHR mRNA in the testicular tissue

2.5.1 RNA extraction and reverse transcription of mRNA

Total RNA was extracted from the frozen testicular/ovarian tissues by the RNeasy mini kit (QIAGEN®, Alameda, CA, USA) following the manufacturer's instructions after intensely grinding the tissues with the homogenizer at 10,000 to 20,000 RPM for 10-20 s. Concentration and purity of the extracted RNA were accessed by spectrophotometer (ND-2000, NanoDrop,Wilmington, DE, USA).

2.5.2 Quantitative real-time PCR (qPCR)

Conventional PCR was performed to get the PCR product for the preparation of standards and analyzing the optimal melting and annealing temperature for each gene. The thermal cycler (G-Storm Thermal Cycler, Somerset, United Kingdom) was set at the condition of 15 min at 95ºC to activate Taq DNA polymerase, 30 cycles of 30 s at 94ºC for denaturing, 90 s at 57ºC for annealing, 30 s at 72ºC for extension and 10 min at 72º C for the final extension. Previously published sequences for both forward and reverse primers for feline LHR and FSHR (Sano et al., 2005; Tharasanit et al., 2014), and GAPDH (housekeeping gene) were used and are shown in Table 2. Each reaction was contained with the Qiagen Multiplex PCR Kit (QIAGEN®, Alameda, CA, USA). Amplified PCR products were run on 1.2% agarose gel (SIGMA-ALDRICH®, St, Louis, MO, USA) and visualized under UV gel document and analysis (SYNGENE® Cambridge,

United Kingdom) to confirm the presence of single products without dimers. Purification of the amplified products was performed with the QIAquik PCR purification kit (QIAGEN®, Alameda, CA, USA). Purified products were quantified by spectrophotometer (ND-2000, NanoDrop,Wilmington, DE, USA) and used to prepare standards for use in qPCR.

Real-time qPCR amplification was performed using the C1000™ Thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with the Bio-Rad CFX manager 3.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each reaction (20 µl) was contained with 10 µl of 2x qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd., London, United Kingdom), 0.8 µl of each forward and reverse primer, 5 µl of a DNA template (5 ng/µl), and RNase free water was added up to the amount of 20 µl. RNase free water was added instead of cDNA template in the Non-template control (NTC). Thermocycler was set for 38 cycles of denaturing at 95ºC for 5 s following with the optimum annealing temperature of 61.4ºC, 60ºC and 61.4ºC for 25 s and melting temperature of 82ºC, 80ºC and 76ºC for 10 s for GAPDH, FSHR and LHR, respectively with a gradient from 50 - 95ºC to investigate the gene expression recorded as Starting Quantity (SQ). Standards of each gene were used as the endogenous controls to determine the absolute value of mRNA in each reaction.

Table 2 *Sequence of forward and reverse primers for GAPDH as housekeeping gene, and feline LHR and FSHR as target genes.*

Gene	Primer sequence (5’ - 3’)	Length (bp)	Reference
GAPDH	F: GGAGAAAGCTGCCAAATATG	20	(Sano et al., 2005;
	R: AGGAAATGAGCTTGACAAAGTGG	23	Tharasanit et al., 2014)
LHR	F: CTAATGCCTTTGACAACCTAATA	23	(Tharasanit et al., 2014)
	R: CCCATTGAATGCATGACTTTGTA	23	
FSHR	F: CATGCTGCTAGGCTGGATCTT	21	(Tharasanit et al., 2014)
	R: CTTGGCGATCTTGGTGTCACT	21	

CHAPTER III

THE EFFECTS OF GnRH-AGONIST IMPLANTATION ON THE REPRODUCTIVE PERFORMANCE OF PREPUBERTAL MALE CATS

3.1 Abstract

Over population of cats is a global problem with welfare implications. It can be controlled by surgical contraception, which is invasive, problematic and not always acceptable. Alternatively, pharmacological methods are considered a favourable option and continuous administration of GnRH has been used for this purpose. However, the exact period and mechanism of long-term 4.7 mg GnRH-agonist implantaion in prepubertal cats is not known. This study had two objectives: (1) to compare the testicular charateristics, LHR and FSHR expression in prepubertal and adult tomcats and (2) to investigate the effect of GnRH-agonist implantation in prepubertal tomcats on the sexual behavior, reproductive performance and the testicular LHR and FSHR expression.

In Experiment 1, three months old tomcats were either treated with deslorelin (4.7mg) (deslorelin-implanted; n=6) or left without (Non-implanted; n=6), and their sexual behavior was monitored. Semen collection and evaluation were performed 48 wk after the start of the experiment which was then followed by castration. All removed testes were analyzed for LHR and FSHR protein and mRNA expression by IHC and qPCR, respectively. No adverse effects were observed after the implantation. Sexual behavior was absent in the implanted cats throughout the study period of 48 wk. Lower testicular volume was found from 30 wk after treatment in implanted cats ($P \leq 0.05$). Semen production was found only in the non-implanted control cats. Lower testicular tissue score and seminiferous tubule diameter along with lower LHR protein expression was found in the implanted cats. However, no differences were observed in LHR mRNA expression between the 2 experimental groups. Moreover, there were no differences in the FSHR protein expression between the two groups but higher FSHR mRNA expression was found in the implanted cats ($P \leq 0.05$).

In Experiment 2, testes from prepubertal (n=6) and adult (n=6) male cats were collected after castration and analyzed for protein and mRNA expression of LHR and FSHR by IHC and qPCR, respectively. No differences were observed in the LHR and FSHR protein expression between the two groups, while mRNA expression of FSHR was higher in prepubertal cats ($P \leq 0.05$). Moreover, testicular and epididymal weight, diameter of seminiferous tubules and the testicular grade were higher in the adult cats ($P \leq 0.05$). In conclusion, suppression of reproductive performance was continued in the deslorelin implanted animals for at least 48 wks along with a suppression of LHR protein expression and an increased FSHR mRNA expression without any adverse effects from implantation.

3.2 Introduction

Contraception is one of the most successful methods for population control in many animal species. Traditional way of contraception by castration is presently in practice in cats as well. However, castration is an invasive surgical procedure and can only be performed on anesthetized animals, whereas anesthesia poses serious problems in juvenile and senile cats and in cats with health problems. Cats reach puberty by the age of 4 month (Johnston et al., 2001c) with a possibility of mating soon after. However, surgical neutering in early age may pose risks like higher sensitivity to many drugs including the anesthetics (Joyce and Yates, 2011). Therefore, nonsurgical neutering could be a welfare-friendly and viable alternative to surgical methods of neutering (Spain et al., 2004).

Reproduction in mammals is controlled by the hypothalamic-pituitary-gonadal (HPG) axis and it has been shown that long-term continuous administration of GnRH desensitizes/downregulates the pituitary gland, profoundly suppresses the gonadotropins release and impairs the reproductive function (Goericke-Pesch et al., 2011). Accordingly, a contraceptive method has been developed; it is employed by GnRH-agonist implantation (Suprelorin®; Peptech Animal Health), and has been proven effective in pubertal tomcats (Munson, 2006; Goericke-Pesch et al., 2011) and female domestic cats (Munson et al., 2001; Munson, 2006; Toydemir et al., 2012). This method

results into long-term reversible contraception without any negative effects to the animals. The contraceptive effects of GnRH-agonist have also been reported in other species such as dogs, wild felids, gilts, flying fox and giraffes (Peltoniemi et al., 1995; Trigg et al., 2001; Wright et al., 2001; Herbert and Trigg, 2005; Patton et al., 2006; Wiebe and Howard, 2009; Melville et al., 2012). Moreover, Trigg et al. (2006) have reported that when 4 months old female pups were implanted with 9.4 mg of deslorelin, contraceptive effect was prolonged and lasted for at least 36 weeks while the contraceptive effect in pubertal dogs was varied from 24 to 48 wks. It is a possibility that this longevity effect might have been achieved by a delay in the age of puberty in these animals. Moreover, there are reports to suggest that early-age neutering could reduce undesirable behaviour of cats especially in adopted cats and could help reduce the unwanted litters in many pet shelters. Although GnRH implantation has been used in cats to suppress the reproductive function but the studies in prepubertal cats are rare and with variable results (Risso et al., 2012; Carranza et al., 2014).

The effects of GnRH implantation on the gonadotropins' release along with the suppression of reproductive function are well documented (Goericke-Pesch et al., 2011; Novotny et al., 2012). However, it is not known whether such effects are achieved through an alteration in the gonadal expression of receptors for LH and FSH and/or testosterone production. The present study was, therefore, designed to investigate the effects of long-term GnRH implantation [4.7 mg GnRH-agonist (Deslorelin)] on the reproductive performance, testicular morphology and expression of LHR and FSHR in prepubertal male cats. Testicular morphology and expression of LHR and FSHR were also compared between prepubertal and adult male cats.

3.3 Materials and methods

3.3.1 Animals, samples and data collection

Experiment I

Three months old tomcats from 3 litters that were proven to be clinically healthy and had attended a complete vaccination program, were randomly divided into 2 groups and were either implanted with 4.7 mg deslorelin, GnRH-agonist (Suprelorin® 4.7mg, Virbac Animal Health, France) in the interscapular area after aseptic technique (Treatment; n=6) or left without any implants (Control; n=6). The cats were housed together at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Thailand in an open-air room with natural daylight, fed with a commercial diet twice daily and water was available ad libitum.

Implanted animals were monitored for any potential adverse effects like tissue reaction or infection at the implantation site for a period of one week. Any rashes, oedema, erythema of implantation area, and other lesions were recorded. Body temperature was measured daily for one week after the hormonal implantation to monitor any systemic infection and if found, blood was collected for blood profile monitoring.

Body weight of all the cats in both groups was recorded fortnightly until the end of the experiment (48 weeks). Throughout the experimental period, functional evaluations of the reproductive organs such as the penile spines, testicular volume and consistency, and male sexual behavior characteristics were monitored at 2-weekly intervals in all the cats.

Experiment II

Testes were collected from prepubertal (4-6 months old, n = 6) or adult (1-3 years old, n=6) normal healthy male cats without any history of hormonal treatment after surgical castration with the consent from the owners at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Surgical castration was performed by scrotal sac incision after the injection of ten milligrams per kilogram of Zoletil100® (Virbac, Thailand) intramuscularly.

Testes were longitudinal sectioned into 2 parts immediately after castration. The first part was preserved in 4% (w/v) paraformaldehyde for 48 h and then transferred into 70% ethanol at room temperature and embedded in paraffin wax until preparation for histology and immunohistochemicalprocess. The second part of the testes was snap frozen with liquid nitrogen and kept in -80°C until RNA extraction.

3.3.1.1 Penile spine

Penile spines of male cats were visually observed as criteria of puberty since the day of implantation at 2-weekly intervals until the end of study period (48 weeks). Penile spines were evaluated as – or +, no penile spines observed or penile spines observed respectively (Goericke-Pesch et al., 2011).

3.3.1.2 Testicular volume and consistency

Testicular volume and consistency were observed at 2-weekly intervals after implantation until the end of study period. Vernier Calipers were used to measure the length, width and depth (including the scrotal sac) of the testes. Testicular volume was calculated with a modified spherical equation; volume (cm^3) = 4/3 x π x (1/2 length x 1/2 width x 1/2 depth) (Goericke-Pesch et al., 2011). Testicular consistency was determined by digital palpation at 2-weekly intervals by one observer and was noted as soft, firm or hard.

3.3.1.3 Male sexual behavior characteristics

Male sexual behavior such as marking behavior, mounting (with or without intromission), or fighting (Rosenblatt and Aronson, 1958) were observed for at least 30 minutes at 2-weekly intervals in all the cats.

3.3.1.4 Fecal collection for fecal testosterone assay

Feces were collected continuously every morning once every two weeks after implantation until the end of the study period and kept at -20°C until the enzyme immunoassay process is conducted at the Khao Kheaw Open Zoo Animal Hospital Laboratory.

3.3.1.5 Semen collection and evaluation

Semen collection was performed by electro-ejaculation technique after the injection of ten milligrams per kilogram of Zoletil50® or Zoletil100® (Virbac, Thailand) intramuscularly in treatment and control cats at 48 weeks after hormonal implantation (before surgical castration). Soon after collection, semen was evaluated for its volume, colour, motility, concentration, viability and sperm morphology). If semen ejaculation could not be accomplished, epididymal sperms were collected immediately after castration and evaluated

3.3.1.6 Castration

Surgical castration was performed in all cats at 48 weeks after implantation by scrotal sac incision immediately after semen collection. Both left and right testes, and epididymides were investigated for morphology. Shape, size and consistency of the testes and epididymis were observed. The consistency was categorized into soft, firm and hard consistency. The weight of each testis and epididymis was recorded in grams. This was followed by fixing both testes in 4% (w/v) paraformaldehyde for 48–72 hrs and then their storage in 70% ethanol until processing. The second part of the testes was snap frozen with liquid nitrogen and kept in -80°C until RNA extraction.

3.3.2 Morphology of the testes

Morphology of the testes was investigated in both the experiments. Tissue sections (5 µm of testicular tissue on non-coated glass slides) stained with hematoxylin and eosin were evaluated for seminiferous tubules, those with normal basement membrane were considered as normal and functional. Diameters of seminiferous tubules in each tissue section were measured by using occularmicrometer at 200X magnification. In each tissue section 200 seminiferous tubules were classified into st0, st1, st2, st3 and st4 using the criteria set up previously (Novotny et al., 2012); if they had only spermatogonia (st0), only spermatogonia and spermatocytes (st1), or with spermatids (st2), or with elongating spermatids present (st3) or with elongated spermatids present in the lumen (st4). Each tissue section was graded into 5 grades (0 – 4), based on the majority percent of seminiferous tubules found in the tissue section;

- Grade 0: Testicular tissue with the majority of st0 seminiferous tubules
- Grade 1: Testicular tissue with the majority of st1 seminiferous tubules
- Grade 2: Testicular tissue with the majority of st2 seminiferous tubules
- Grade 3: Testicular tissue with the majority of st3 seminiferous tubules
- Grade 4: Testicular tissue with the majority of st4 seminiferous tubules

Any pathological changes if present were investigated and recorded.

3.3.3 Localization and expression of testicular LHR and FSHR

The localization and expression of testicular LHR and FSHR was done by immunohistochemical staining as mentioned in chapter II, section 2.4.

3.3.4 mRNA expression of LHR and FSHR RNA in the testicular tissue

The method of quantitative real-time polymerase chain reaction (qPCR) was used to measure the mRNA expression of the testicular LHR and FSHR as mentioned in chapter II, section 2.5.

3.3.5 Statisticals analysis

Body weight and testicular volume were compared between the implanted and non-implanted (Expt 1) animals using Students'-test.

General linear model (GLM) was performed to compare the protein expression of LHR and FSHR, the concentration of LHR and FSHR mRNA and the epididymal weight between the prepubertal and adult (Expt 2) and implanted and non-implanted (Expt 1) cats. Wilcoxon rank sum test was performed to compare the testicular weight, the mean diameter of seminiferous tubules and the grade of seminiferous tubules between the prepubertal and adult cats (Expt 2) and between the implanted and non-implanted cats (Expt 1).

3.4 Results

No tissue reaction and/or infections were recorded after implantation.

Male sexual behaviour was absent in deslorelin-implanted cats but was fully present in the non-implanted controls after the study period of 28 weeks. A summary of the male sexual behavior is shown in Table 3. No difference in body weight of implanted and non-implanted cats was observed (Figure 4).

The implanted cat had significantly lower testicular volume from 30th week of the study onwards (Figure 5). Testicular consistency was firm in both the groups.

Penile spines in non-implanted cats were present from the 28th week of the study onwards and were absent in the implanted cats throughout the study period (Figure 6).

Significantly higher ($P \leq 0.05$) fecal testosterone levels were present in the non-implanted cats from 24th week of the study onwards (Figure 7). It was possible to collect semen by electro-ejaculation technique from the non-implanted cats and the data on semen evaluation are shown in Table 4. It was not possible to collect semen from the deslorelin-implanted cats and epididymal sperms were also uncollectable. Testicular morphology of all cats was normal, mean testes and epididymal weight, testicular grade and mean seminiferous tubule diameter were higher in the pubertal and non-implanted cats compared to prepubertal and implanted cats, respectively ($P \leq 0.05$) (Table 5).

Table 3 *Records of male sexual behavior in the control and deslorelin implanted cats. Negative (-) and positive (+) symbols simply denote absence and presence of a behavior, respectively*

wks	Group	Behaviours			
		Marking	Mounting		Fighting
			Without intromission	With intromission	
1 – 4	Control	-	-	-	-
	Deslorelin implanted	-	-	-	-
5 – 8	Control	-	-	-	-
	Deslorelin implanted	-	-	-	-
9 – 12	Control	-	-	-	-
	Deslorelin implanted	-	-	-	-
13 – 16	Control	-	-	-	-
	Deslorelin implanted	-	-	-	-
17 – 20	Control	-	-	-	-
	Deslorelin implanted	-	-	-	-
21 – 24	Control	-	+	-	+
	Deslorelin implanted	-	-	-	-
25 – 28	Control	+	+	+	+
	Deslorelin implanted	-	-	-	-
29 – 32	Control	+	+	+	+
	Deslorelin implanted	-	-	-	-
33 – 36	Control	+	+	+	+
	Deslorelin implanted	-	-	-	-
37 – 40	Control	+	+	+	+
	Deslorelin implanted	-	-	-	-
41 – 44	Control	+	+	+	+
	Deslorelin implanted	-	-	-	-
45 – 48	Control	+	+	+	+
	Deslorelin implanted	-	-	-	-

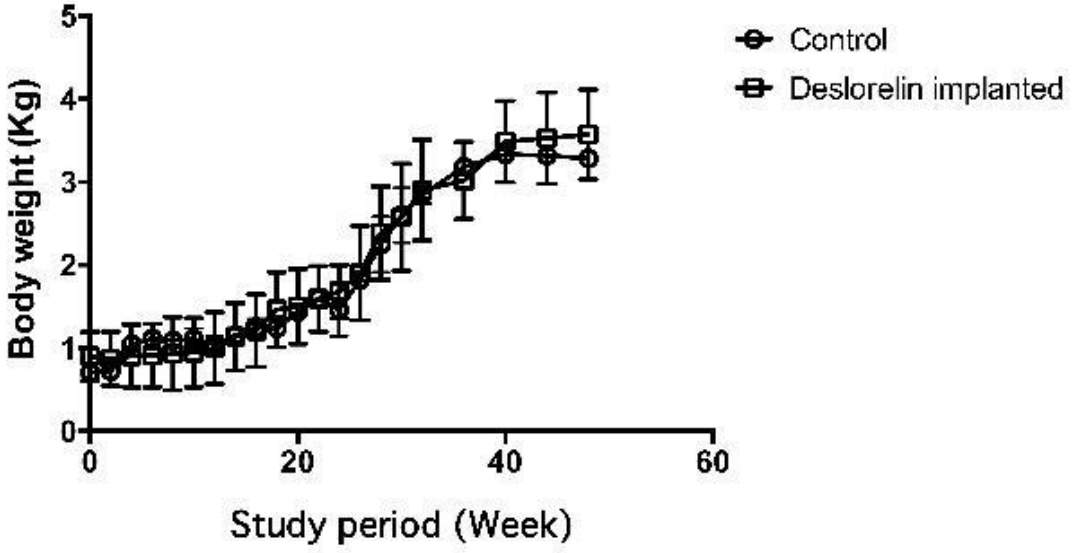

Figure 4 *The mean (± SEM) body weight (kg) of cats (n=6/group) with or without deslorelin implantation at the age of 3 months for 48 weeks.*

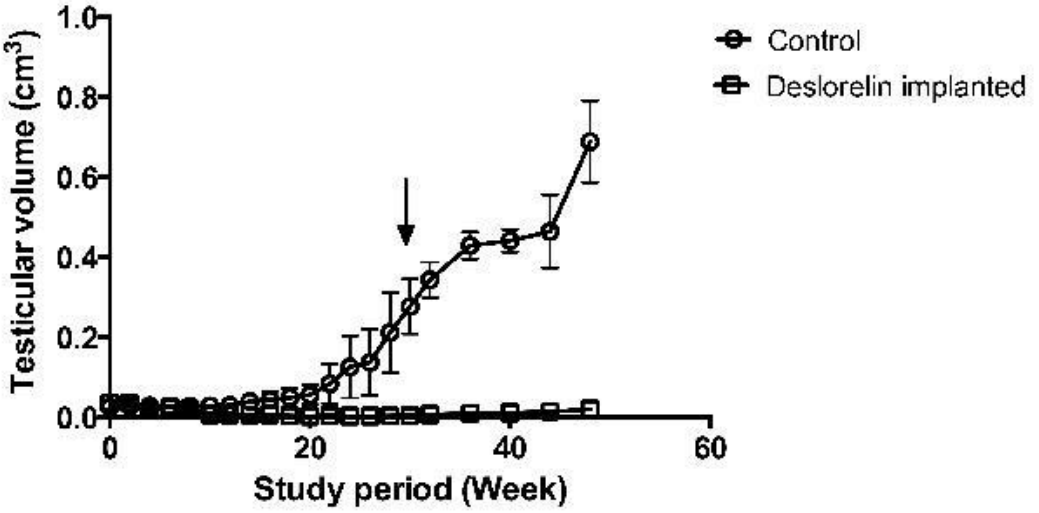

Figure 5 *The mean (± SEM) testicular volume (cm^3) in cats (n=6/group) that were either implanted with deslorelin at the age of 3 months for 48 weeks or left without implants (Controls). Black arrow indicates the week from which onwards control group has significantly higher testicular volume than the implanted group ($P \leq 0.05$).*

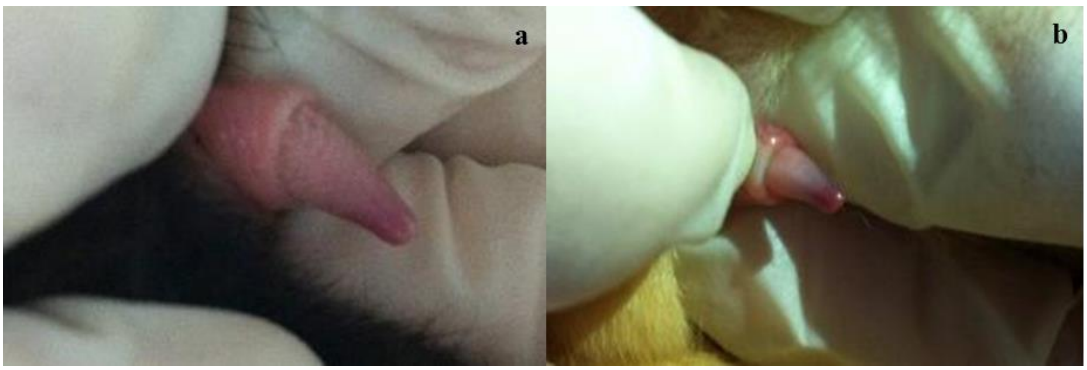

Figure 6 *Penile spines in the experimental cats. Note the presence of penile spine in the controls (a) and the absence of penile spines in Deslorelin implanted cats (b). The spines were observed from the 32th week onwards of the study period in the control cats.*

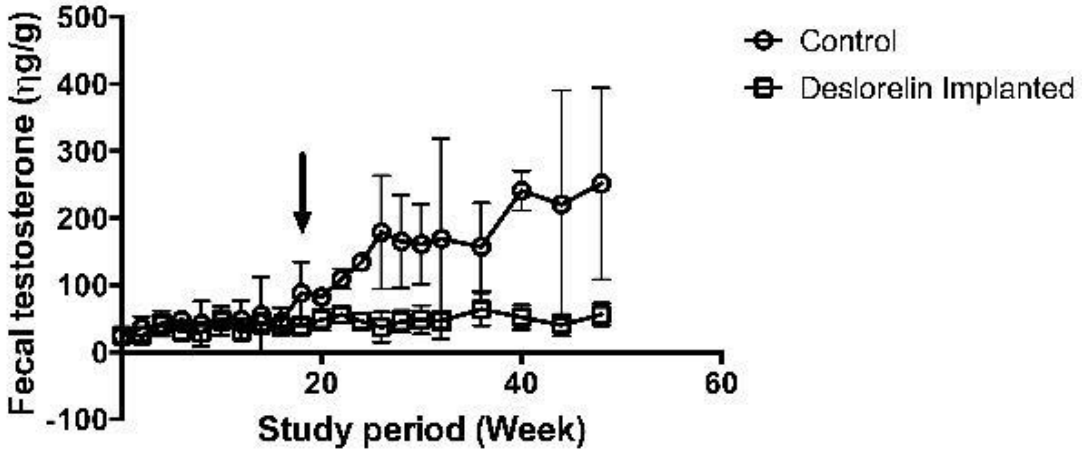

Figure 7 *The mean (± SEM) fecal testosterone concentrations (pg/mg of feces) in cats (n=6/group) that were implanted with deslorelin at the age of 3 months for 48 weeks or left without implantation (Controls). Black arrow indicates the week from which onwards control group has significantly higher fecal testosterone levels than the implanted group (P ≤ 0.05).*

Table 4 *Data on semen evaluation parameters in tomcats used in both the experiments*

Group	Evaluation Criteria			
	Volume (µl)	Motility (%)	Concentration (Sperms/ml)	Viability (%)
Prepubertal	-	-	-	-
Pubertal	17 – 65	60 – 70	$3 \times 10^6 – 20 \times 10^6$	68 – 87
Implanted	-	-	-	-
Non-implanted	10 – 73	40 – 60	$0.15 \times 10^6 – 18 \times 10^6$	49 – 72

Table 5 *Mean (± SEM) Weight (g) of the testes and epididymis, seminiferous tubule diameter (µm) and mean testicular tissue grade in different experimental groups of Experiment 1 and 2*

Experiment	Groups	Weight (g)		Seminiferous tubule diameter (µm)	Testicular tissue grade
		Testes	Epididymis		
1	Prepubertal	0.19 ± 0.04^a	0.08 ± 0.02^b	57.85 ± 8.50^b	1.33^b
	Pubertal	1.24 ± 0.20^a	0.24 ± 0.03^a	95.31 ± 3.94^a	3.83^a
2	Implanted	0.09 ± 0.02^b	0.03 ± 0.01^b	62.02 ± 2.88^b	0^c
	Non-implanted	1.54 ± 0.20^a	0.26 ± 0.03^a	98.48 ± 3.59^a	3.86^a

Values with different superscripts within a column differ significantly ($P \leq 0.05$)

3.4.1 Immunohistochemical localization of Luteinizing hormone receptor and Follicle stimulating hormone receptor

The LHR were localized in the cytoplasm of Leydic cells and FSHR were in the germ cells (Figure 8). LHR expression were higher in the non-implanted control group compared to the deslorelin-implanted group ($P \leq 0.05$) (Figure 9). However, no difference was found between pubertal and prepubertal groups (Figure 10). Moreover, no significance differences were found in the FSHR expression between either pubertal and prepubertal cats or the implanted and control cats (Figure 9 and 10).

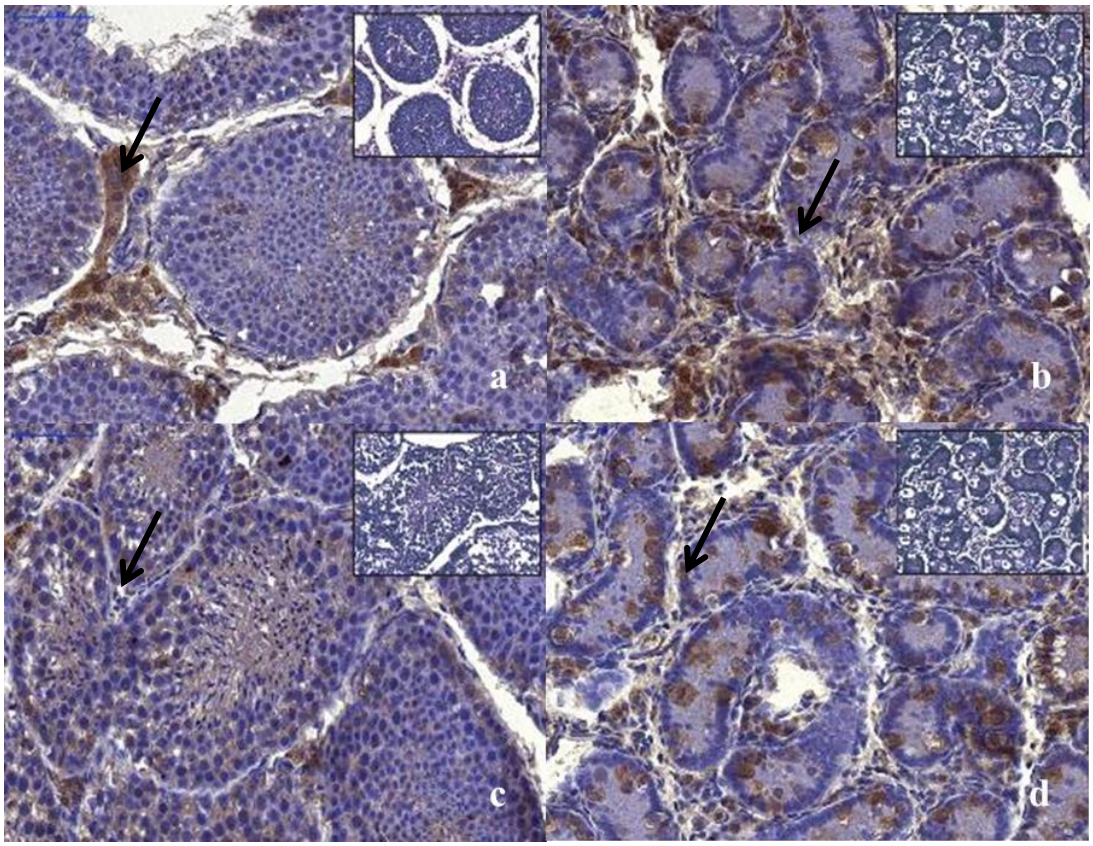

Figure 8 *The immunohistochemical localization of LHR and FSHR in the testicular tissue of non-implanted controls (a and c) and deslorelin-implanted (b and d) male cats respectively (black arrows indicates the area of immunohistochemical localization).*

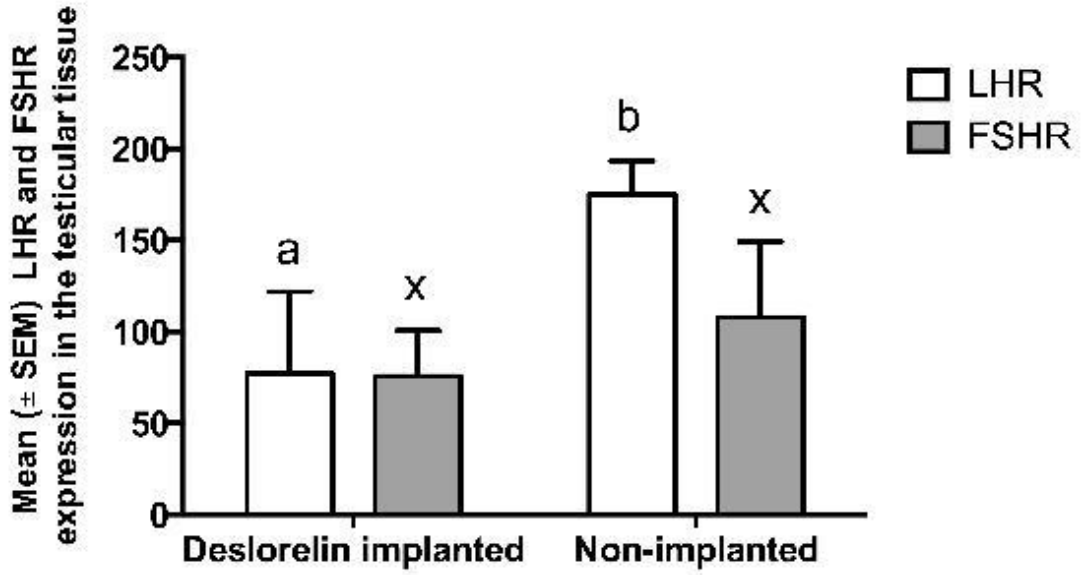

Figure 9 *The mean (± SEM) expression index for LHR and FSHR in testicular tissue of cats (n=6/group) implanted with deslorelin at the age of 3 months for 48 weeks or left without it (non-implanted). Different letters on the bar within each receptor type indicate significant differences (P ≤ 0.05) in the expression of that particular receptor between implanted and non-implanted cats.*

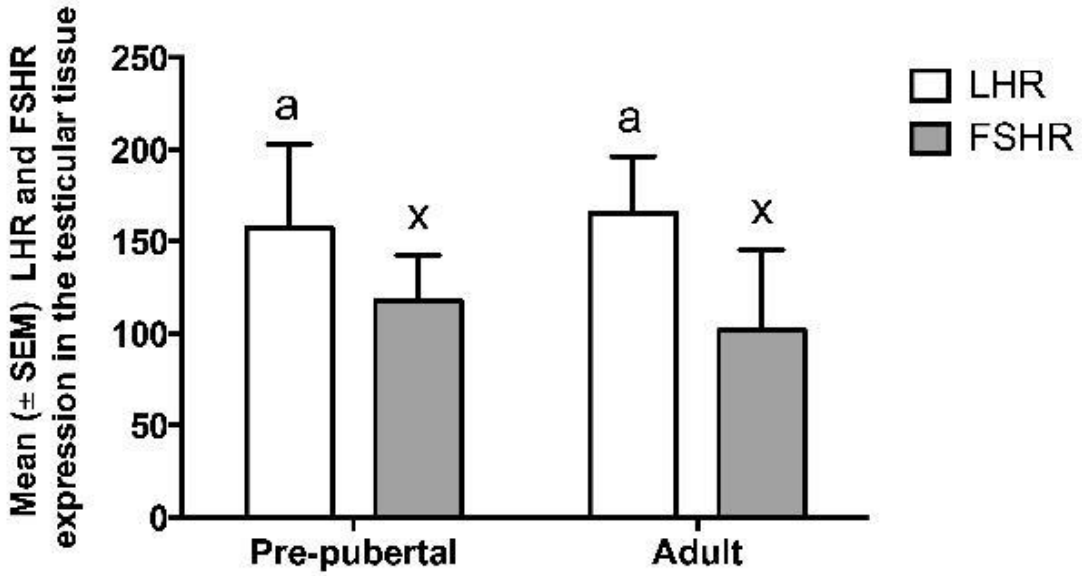

Figure 10 *The mean (± SEM) expression index for LHR and FSHR in testicular tissue of prepubertal and adult cats (n=6/group). No differences were observed in the expression of either LHR or FSHR between the prepubertal and the adult cats.*

3.4.2 Expression of Luteinizing hormone receptor and Follicle stimulating hormone receptor mRNA in the testicular tissue

LHR mRNA and FSHR mRNA were expressed in all the testicular samples collected in the two experiments. No differences were observed in the expression of LHR mRNA expression between the groups in each experiment (Figures 11 and 12). The expression of FSHR mRNA, however, was significantly higher ($P \leq 0.05$) in the prepubertal and deslorelin-implanted cats compared to adult and non-implanted cats, respectively (Figures 11 and 12).

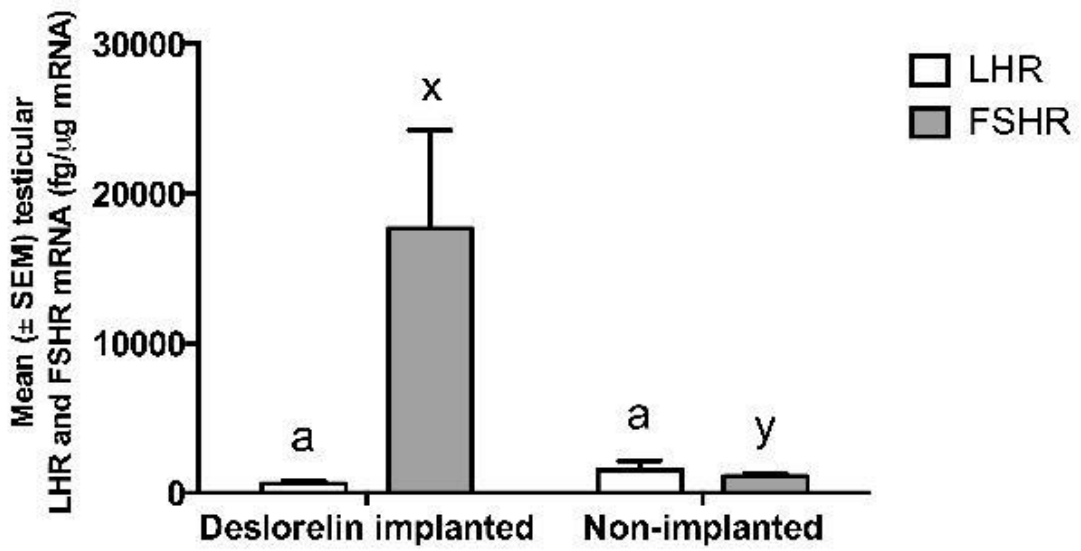

Figure 11 *The mean (± SEM) mRNA concentration for LHR and FSHR in testicular tissue of cats (n=6/group) implanted with deslorelin at the age of 3 months for 48 weeks or left without it (non-implanted). Different letters on bars for a certain receptor indicate significant differences (P ≤ 0.05).*

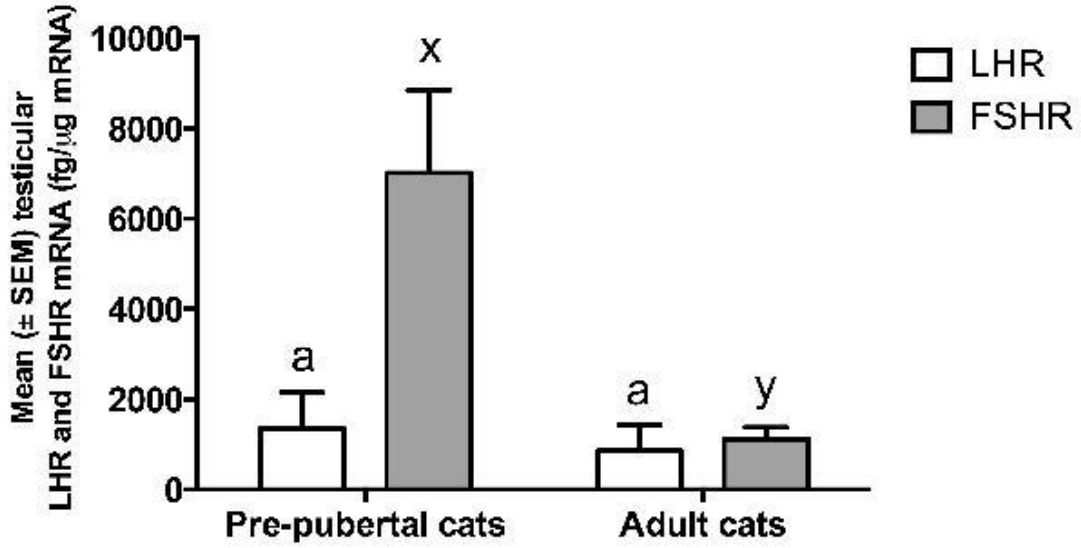

Figure 12 *The mean (± SEM) mRNA concentration for LHR and FSHR in testicular tissue of pubertal and prepubertal cats (n=6/group). Different letters on bars for a certain receptor indicates significant differences (P ≤ 0.05).*

3.5 Discussion

The objectives of this study were 1) to compare the testicular characteristics, and the LHR and FSHR expression between the prepubertal and adult tomcats, and 2) to investigate the effect of GnRH-agonist implantation on the sexual behavior, reproductive performance and the testicular LHR and FSHR expression in the prepubertal tomcats.

Deslorelin implantation which was done without any anesthesia, local or general, was very well tolerated by male prepubertal cats as has been reported in previous studies (Goericke-Pesch et al., 2011; Carranza et al., 2014). Moreover, deslorelin-implantation did not result into any rashes or oedema or infection and no lesions developed after the implantation. The sexual behaviour of the deslorelin-implanted cats was suppressed and many unwanted/undesirable behaviors such as spraying, fighting and roaming were eliminated in these cats. This suppression of behaviors resulted from deslorelin implantation was comparable with behaviors eliminated by surgical casrtration (Hart and Eckstein, 1997).

The physiology and sexual behavior of male mammals is mainly controlled by testosterone which is produced by the activation of Leydic cells after binding of LH to its receptors in the Leydic cells (Midzak et al., 2009; Smith and walker, 2014). Aromatase and 5-α reductase transform testosterone into estrogen and dihydrotestosterone respectively, and these two hormones are mainly responsible for different male behaviors and characteristics of mammals. In this study the expression of LHR protein in testicular tissue of GnRH implanted cats was noted, this could lead to the hypothesis of the absence of sexual behavior in the deslorelin-implanted group in this study. However, the original form of testosterone acts on the sertoli cells and stimulates their function and supports spermatogenesis. While FSH has an important role in the first wave of spermatogenesis in prepubertal mammals but its role in spermatogenesis in adults is not clear. Nevertheless, its role to induce the meiosis is speculated and has been reported to increase the number of spermatogonia in the seminiferous tubules (O'Shaughnessy, 2014).

Release of GnRH from the hypothalamus stimulates the gonadotropins from the pituitary gland to regulate the reproductive function. However, chronic GnRH-agonist treatment is believed to down regulate the pituitary GnRH receptors and decrease the release of gonadotropins and suppress the reproductive performance (Goericke-Pesch et al., 2013b). In this study, we have explored whether there were any changes in the expression of testicular LHR and FSHR resulting from the chronic GnRH-agonist treatment or not.

FSHR is expressed in the sertoli cells of the testes and is responsible to control spermatogenesis after activation by the FSH (Nieschlag et al., 1999), whereas LHR is expressed in the Leydic cells' cytoplasm, and is responsible to stimulate the androgens' secretion (Chauvigne et al., 2014) by activating the pathway that transforms cholesterol into testosterone (Bjelic et al., 2014). In this study a significantly higher expression of FSHR mRNA was observed in the testicular tissue of deslorelin-implanted male cats compared to untreated controls. The observed increase in FSHR mRNA expression could be a result of the compensatory mechanism to compensate the suppression of the endogenous release FSH that might have induced by the deslorelin (GnRH-agonist) implantation or the suppression of deslorelin may preserve the FSHR as it is in the prepubertal stage as we could see that the expression of deslorelin implanted animals expresses in the same fashion as the FSHR expression in the prepubertal animals. FSH plays a major role and is particularly responsible for the first wave of spermatogenesis in animals reaching puberty (Meehan et al., 2000; O'Shaughnessy, 2014). However, in adult animals spermatogenesis is mainly androgen-dependent, and the role of FSH is mainly limited to support the spermatogonia (O'Shaughnessy, 2014). Therefore, absence of sperm production observed in the study in the deslorelin-implanted cats might be the result of suppressed/lower testosterone production due to the down-regulation of LHR in the Leydic cells of the implanted cats.

Even though there were no difference observed between the expression of the FSHR protein in the testicular tissue of implanted and non-implanted cats but we found lower LHR protein expression in deslorelin-implanted cats compared to the non-implanted cats, which may have been responsible for the suppression of fecal

testosterone levels and/or testosterone-dependent behaviors in the implanted cats. Both protein expression and mRNA concentration of the LHR were studied, and the results have shown that deslorelin implantation did not affect the mRNA expression but suppressed the protein expression of LHR. This simply suggests that deslorelin down regulates the LHR by interfering at the translation level without any effect on the transcription of the gene. Furthermore, the lower expression of the LHR from the Leydic cells could also be a result from the relatively lesser amount of Leydig cells and its cytoplasm seen in the histological testicular tissue observation.

It is difficult to estimate the exact time period for which the LHR in the implanted cats' Leydic cells were suppressed because the investigation of the receptors took place at the 48th wk of the deslorelin implantation. It is difficult to estimate the exact time when testosterone suppression started in the implanted cats as the fecal testosterone levels in both the deslorein implanted and control groups were same and basal at the time of start of treatment. However, the fecal testosterone levels in the treated cats remained to be basal throughout the study period, whereas in the control cats testosterone levels started to increase from the 24th week onwards of the treatment which might suggest that deslorelin treatment was certainly effective to suppress testosterone by 24th week of the treatment. Many male characteristics such as the presence of penile spines and male behaviors such as roaming, fighting and spraying are gonadal steroid hormones (especially testosterone) dependent and could be eliminated via the suppression of testosterone (Hart and Eckstein, 1997) as was obvious from the results of this study.

LH starts its activity by binding to its receptor and signals cAMP to stimulate the transport of cholesterol into mitochondria where by the activity of different enzymes it is transformed into pregnenolone, progesterone, androstenone and finally into testosterone (Midzak et al., 2009). The effect of deslorelin may not be only at the level of LH and LHR but also at the level of other sites in the pathway.

Suppression of LH secretion from long-term GnRH-agonist treatment has been reported with the chronic treatment of Goserelin in gilts (Peltoniemi et al., 1995), which supports the result in this study that the testicular LHR protein expression male cats was suppressed after the implantation of GnRH-agonist at prepubertal age.

Normally GnRH-agonist implantation presents an up-regulation effect in the first period after hormonal implantation followed by a down-regulation effect after long-term administration in pubertal tomcats (Goericke-Pesch et al., 2011). However, in our study no up-regulation effect was shown in male cats implanted with GnRH-agonist. In our study cats were implanted with GnRH-agonist at the age of 3 months when they may have an immature HPG axis that may be responsible for the absence of the up-relation effect. Infact, in another study an immature HPG axis was suspected to be the reason behind the absence of up- regulation effect (Carranza et al., 2014). Although there is limited information about the age of the HPG development in male cats but at the age of implanation in this study, we could suspect that the HPG system was not fully developed yet. An earlier study in which <4 months old dogs were implanted with GnRH-agonist also described about the immaturation of the HPG system (Trigg et al., 2006). Taken together, one may say that GnRH-agonist implantation may induce long term delay in puberty but without an up-regulation effect that could range from birth (Carranza et al., 2014) to 3 months of age.

The suppression of the reproductive performance in this study was present at least for 48 weeks, and no attempts could be made to check its reversibility due to limited time. However, irreversible of reproductive performance has been reported in male and female dogs, implanted with GnRH-agonist before they were 4 months old (Trigg et al., 2006; Sirivaidyapong et al., 2012). Although reversible reproductive performance was found in studies using 1.6 mg deslorelin in postnatal cats and 4.7 mg deslorelin in 114 days old female cats, puberty was postponed until the age of 16 months and 134 – 286 days, respectively (Carranza et al., 2014), . However, after 4.7mg deslorelin administration in 3 months old cats it still remains to be investigated.

In conclusion, the results of the present study have shown that implantation of 4.7 mg GnRH-agonist (Deslorelin®) in male cats at the age of 12 weeks suppresses the reproductive function for at least for 48 weeks without any adverse effects on the general health. Moreover, this suppression of reproductive function may be achieved partly by down-regulation of LHR in the Leydic cells while maintaining the FSHR mRNA expression at the prepubertal levels in the sertoli cells of the testis.

CHAPTER IV

THE EFFECTS OF GnRH-AGONIST IMPLANTATION ON THE REPRODUCTIVE PERFORMANCE OF PREPUBERTAL FEMALE CATS

4.1 Abstract

This study investigated the effect of GnRH-agonist (deslorelin) on the reproductive function and ovarian LHR and FSHR expression in prepubertal female cats that were either implanted with 4.7 mg Deslorelin GnRH-agonist (Group 1 (Deslorelin-implanted): n=6), left without implants (Group (Control) 2: n =18) and ovariohysterectomized at prepubertal age for the collection of reproductive tract (Group 3 (Prepubertal OVH): n =6). Body weights, fecal estradiol and sexual behavior of cats in Groups 1 and 2 were monitored for 48 weeks followed by collection of their ovaries and uteri. Ovaries and uteri were collected from the control cats (Group 2) at their follicular, luteal and inactive stage (anestrus or interestrus) (n = 6/group) of the estrous cycle. Ovaries and uteri were collected from cats in Group 3 (Prepubertal OVH) while they were still prepubertal. Both ovaries and uteri were analyzed for anatomical and histological characteristics. Ovaries were also analyzed for LHR and FSHR protein and mRNA expression. Data were statistically analyzed; body weights were compared between Groups 1 and 2 by Independent t-test and general linear model (GLM) was used to compare the ovarian weight, endometrial gland diameter, thickness of endometrium and myometrium, number of primordial, primary, secondary and antral follicles, and the mRNA and protein expression of LHR and FSHR among the experimental groups. The control cats (Group 2) had significantly higher ($P \leq 0.05$) body weight compared to implanted cats only during the 22^{nd} to 26^{th} weeks of the study period. Fecal estradiol peak and estrus behavior was observed in the control cats (Group 2) but absence in the implanted cats (Group 1). Deslorelin significantly ($P \leq 0.05$) reduced the ovarian weight and the number of antral follicles. Endometrial thickness and gland diameter were not affected by deslorelin. However, myometrial thickness of the implanted cats (Group 1) was significantly ($P \leq 0.05$) lower than control cats (Group 2) at the follicular and luteal stage. Ovarian LHR mRNA expression was significantly ($P \leq 0.05$) lower in the implanted (Group 1) compared to the control cats (Group 2) at follicular stage of

estrous cycle. FSHR mRNA and LHR protein expression did not differ among the 3 groups. FSHR protein expression was, however, significantly ($P \leq 0.05$) lower in prepubertal OVH cats (Group 3) but was not affected by Deslorelin-implantation. In conclusion, GnRH-agonist implantation of prepubertal female cats suppresses their reproductive function including the ovarian weight, follicle development, estradiol production and myometrial thickness for a period of at least 48 weeks possibly through a change in the ovarian mRNA expression of LHR.

4.2 Introduction

Overpopulation of cats has become a serious problem in big cities across the world. Surgical contraception is one of the first choices as a tool for population control although it is an invasive method with major concerns of bleeding, improper post-care management and animals with risks of anesthesia (Goericke-Pesch et al., 2013a). Previous studies have suggested that non-surgical contraception could be an alternative method of population control especially in those animals that are associated to surgical or anesthesiology risks. In this regard, GnRH-agonists have been used to suppress the pituitary gland and/or the release of gonadotropins. Non-surgical contraception in the female cat is thought to be potentially a challenge, considering the unique pattern of estrous cycle in this species (Johnston et al., 2001b). However, GnRH-agonists can be used for the purpose of contraception in mammals at a wide range of age and at different stages of estrous cycles (Munson et al., 2001; Pelican et al., 2006; Trigg et al., 2006; Toydemir et al., 2012; Goericke-Pesch et al., 2013a; Carranza et al., 2014). Previous studies in female cats have shown the ability of GnRH-agonists to suppress their reproductive tract and/or function but an up-regulation / flare-up effect was reported during the early stages of the treatment that resulted into exhibition of estrus symptoms which is undesirable (Toydemir et al., 2012; Goericke-Pesch et al., 2013a). However, such an up-regulation effect was not observed with the implantation of GnRH-agonist in the prepubertal female dogs (Trigg et al., 2006). While the absence of up-regulation effect in these dogs has been attributed to their prepubertal status, there are no reports regarding the use of GnRH-agonist treatment in prepubertal female cats. Recent studies on post-natal female cats using 1.6 mg GnRH-agonist (Deslorelin) found that puberty in the treated cats was delayed for 16 months and the number of primordial, primary, secondary and antral follicles in the treated cats were significantly lower than the controls (Carranza et al., 2014; Carranza et al., 2015). However, there is not enough information regarding how long

GnRH-agonist treatment could delay puberty in prepubertal female cats. Moreover, the underlying mechanism by which GnRH-agonist suppresses the reproductive system is not known. The objectives of this study were to investigate 1) the effect of GnRH-agonist implantation on the reproductive function, and the structures and characteristics of reproductive organs like uteri and ovaries in prepubertal female cats, 2) a possible link of these effects with the ovarian LHR and FSHR expression, and 3) to see how closely GnRH-agonist implantation maintains the prepubertal status of these parameters.

4.3 Materials and methods

4.3.1 Animals, samples and data collection

Three groups of three (3) months old prepubertal female cats were used in this study. Cats in Group 1 (n=6) were implanted with 4.7 mg Deslorelin GnRH-agonist (Suprelorin® 4.7 mg, Virbac Animal Health, France) in the inter-scapular area (Implanted), Cats in Groups 2 (Control; n =18) and 3 (Prepubertal OVH; n =6) were left without implants. To collect the ovaries and uteri, cats in Group 3 (Prepubertal OVH) were ovariohysterectomized while they were still 3 months old, i.e., at their prepubertal status, whereas the cats in Group 1 (Implanted) and 2 (Control) were housed in an open air room with natural daylight in the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Thailand. During the study period of 48 weeks they were fed with a commercial diet twice daily with water always available *ad libitum*. The study was performed under the license of Chulalongkorn University Laboratory Animal Center number 13310056.

Implanted animals were monitored for any potential adverse effects like tissue reaction and/or infection at the site of implantation for a period of one week. Any rashes, oedema, erythema of the implantation area, and other possible lesions were recorded. Body temperature was measured daily for one week after the implantation to monitor any infection and if found, intravenous blood was collected for blood profile monitoring. However, blood profile was not performed in this study due to no signs of infection was monitored.

Body weight of all the cats in Groups 1 and 2 was also recorded fortnightly throughout the experimental period. Estrus behavior (rubbing and rolling, lordosis, tail deflection, treading of the hind limbs, vocalization and the acceptance of mating with intact males) of all the cats was monitored as described previously (Johnston et al.,

2001b; Toydemir et al., 2012) throughout the experimental period of 48 weeks. Estrus detection was confirmed by vaginal cytology with a clear background with more than 80% of superficial cells (Faya et al., 2013) and/or serum estradiol concentrations more than 20 pg/ml (Johnston et al., 2001b; Munson et al., 2001) or 42.2 to 157.8 pmol/L (Axner et al., 2008). Estrous behavior was taken as an indication of the ovarian follicular activity. To confirm the ovarian activity/cyclicity, 4 cats from each of the Groups 1 and 2 were housed singly and their feces were collected at 2-day intervals for a period of 4 weeks (28^{th} - 32^{nd} week of the study period). At the end of treatment period, all the cats in Groups 1 and 2 were ovariohysterectomized to collect their ovaries and uteri. Cats in Group 2 were ovariohysterectomized when they were at their follicular (n = 6), luteal (n = 6) and inactive (n = 6) stage of the estrous cycle. Stage of the estrous cycle was determined in these cats by their behavior, vaginal cytology and serum estradiol and progesterone concentrations. Cats with serum estradiol concentrations of more than 20 pg/ml were considered to be at their follicular stage of the cycle and cats with serum progesterone concentrations of 1.5 – 20 ng/ml were considered to be at their luteal stage of the estrous cycle. Cats with serum estradiol and progesterone concentrations of < 20 pg/ml and < 1.5 ng/ml, respectively, were considered at their inactive stage (anestrus or interestrus stage of estrous cycle) (Johnston et al., 2001b). Structures present on the ovaries (follicles and corpora lutea) obtained after ovariohysterectomy were also used to confirm the stage of the estrous cycle.

4.3.1.1 Vaginal cytology

Vaginal cytology was performed by using a small cotton swab moistened with normal saline. The swab was introduced cranially into the vagina (about 0.5 inches cranially from the vestibule); cells from the vagina were scraped on to the swab. The swab was then rolled on a clean and dry glass slide, air-dried and stained with diff-quick stain. The stage of the estrous cycle was diagnosed from the cytology by light microscope at 40x into follicular, luteal or interestrus stage. Cells found in the vaginal smear of each stage (Johnston et al., 2001a);

Follicular stage; the follicular stage was categorized into proestrus and estrus stages.

Proestrus; when the vaginal smear had 70 – 80 percent intermediate cells, 10 – 30 percent parabasal cells, 0 – 40 percent superficial cells and about 30 neutrophils per 100 epithelial cells

Estrus; when the vaginal smear had 60 – 90 percent nucleated and anucleated superficial cells, 0 – 25 percent intermediate cells, 0 – 3 percent of parabasal cells and 0 – 10 neutrophils per 100 epithelial cells

Luteal stage; luteal stage or the diestrus period was defined when the vaginal smear had about 50 percent parabasal cells and 50 percent intermediate cells

Inactive stage; when the vaginal smear had few parabasal or intermedaite cells or other cells with clear back ground

4.3.1.2 Morphology of female reproductive organs

After ovariohysterectomy, ovarian weight, structures present on the ovary and their morphological appearance were recorded. The ovaries and uteri were divided into two parts; one part was fixed in 4% (w/v) paraformaldehyde for 48 to 72 hours and then stored in 70% ethanol until processing, whereas the other part was snap frozen with liquid nitrogen and stored at -80 ºC until RNA extraction. Fixed uterine and ovarian tissues were embedded in paraffin wax and cut into 5 µm sections by a rotor microtome, applied to gelatin-coated slides and left to dry in an incubator at 37ºC, then stained with hematoxylin and eosin staining.

Histological investigation of the uterus was performed under light microscope, five sections per uterine horn and five fields per section at 40X magnification were captured for the measurement of the thickness of endometrium and myometrium and five fields per section at 200X magnification for the measurement of the uterine gland diameter. Different types of follicles (primordial, primary, secondary and antral) and corpora lutea were counted from 5 sections per ovary and 5 fields per section under light microscope at 100X magnification. The number of different types of follicles were recorded per mm^2 of the ovarian cortex area.

4.3.1.3 Estradiol measurement in fecal and serum samples

Blood samples were collected for serum estradiol and progesterone once before OVH at the end of the study period and sent to Bangkok R.I.A. group clinical laboratory for serum estradiol level determination by radio immunoassay (RIA) method,

which is consistently used for serum steroid hormone detection in felid species (Axner et al., 2008). Serum estradiol and progesterone were used to confirm the stage of estrous along with the estrous behaviours, the vaginal cytology and the structures on the ovaries.

After collection, fecal samples were stored in a freezer at -20° C. Fecal estradiol concentrations were measured by the Khao Kheow open zoo laboratory in Bangkok, Thailand, using enzyme immune assay (EIA) method as described previously (Kinoshita et al., 2011). Fecal estradiol was used to analyze the ovarian function of the treated (Group 1) and controls (Group 2).

4.3.2 Luteinizing hormone receptor (LHR) and Follicle stimulating hormone receptor (FSH) Expression

The localization and expression of testicular LHR and FSHR was done by immunoistochemical staining as described in chapter II, section 2.4.

4.3.3 mRNA expression of LHR and FSHR RNA in the ovarian tissue

The method of quantitative real-time polymerase chain reaction (qPCR) was used to measure the mRNA expression of the testicular LHR and FSHR as described in chapter II, section 2.5.

4.3.4 Statistical analysis

The statistical analysis was performed using SAS (SAS Institute INC., 2002). Body weight was compared between the deslorelin-implanted (Group 1) and control cats (Group 2) using Independent t-test. Simple presence of estrogen peaks in a cat was taken an indication of the ovarian follicular activity.

The ovarian weight, the endometrial gland diameter, the thickness of endometrium and myometrium, the number of primordial, primary, secondary and antral follicles, the mRNA, and the protein expression of LHR and FSHR were tested for normality using unvariate procedure and were compared among 3 groups. i.e., deslorelin-implanted (Group 1), control cats at follicular, luteal and anestrus stages of estrous cycle (Group 2) and prepubertal OVH (Group 3) using general linear model (GLM) procedure. Least-squared means were obtained from each group and were compared by using least significant difference test. The number of CLs was compared among groups using Wilcoxon rank sum test. The statistical analysis of *P* value 0.05 or less was indicated as significant difference.

4.4 Results

Following GnRH-agonist implantation, no adverse effects were observed in any of the implanted cats. The control cats (Group 2) had significantly higher ($P \leq 0.05$) body weight compared to implanted cats (Group 1) but only during the 22nd to 26th weeks of the study period. However, for rest of the treatment period no difference was observed in the body weight of cats between these two groups (Figure 13).

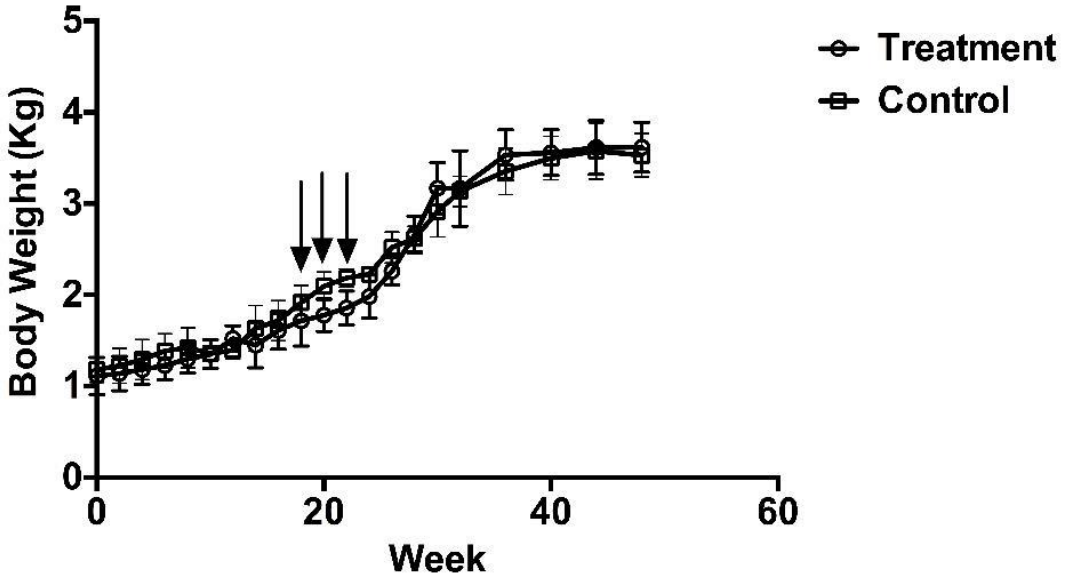

Figure 13 *The mean (± SEM) body weight (kg) in cats with or without deslorelin implantation. Cats were implanted with deslorelin at the age of 3 months for a period of 48 weeks. Black arrow indicates the weeks (22nd to 26th) that the implanted group has significantly higher (P ≤ 0.05) bodyweight than the control group.*

No estrus behavior was observed in deslorelin-implanted cats (Group 1) throughout the experimental period, whereas estrus was observed in all the control cats in group 2 during the experimental period. Among the control group (Group 2), 2 cats started to show signs of estrus from the 10th week after the start of the treatment,

whereas rest of the other control cats showed signs of estrus from the 12th week after the start of the study (June 2012).

Figure 14 shows the fecal estradiol concentrations from 28th to 32nd week of the treatment period in the deslorelin-implanted (Group 1) and the control (Group 2) cats. While the control cats showed estradiol peaks, no estradiol peak was observed in the deslorelin-implanted cats.

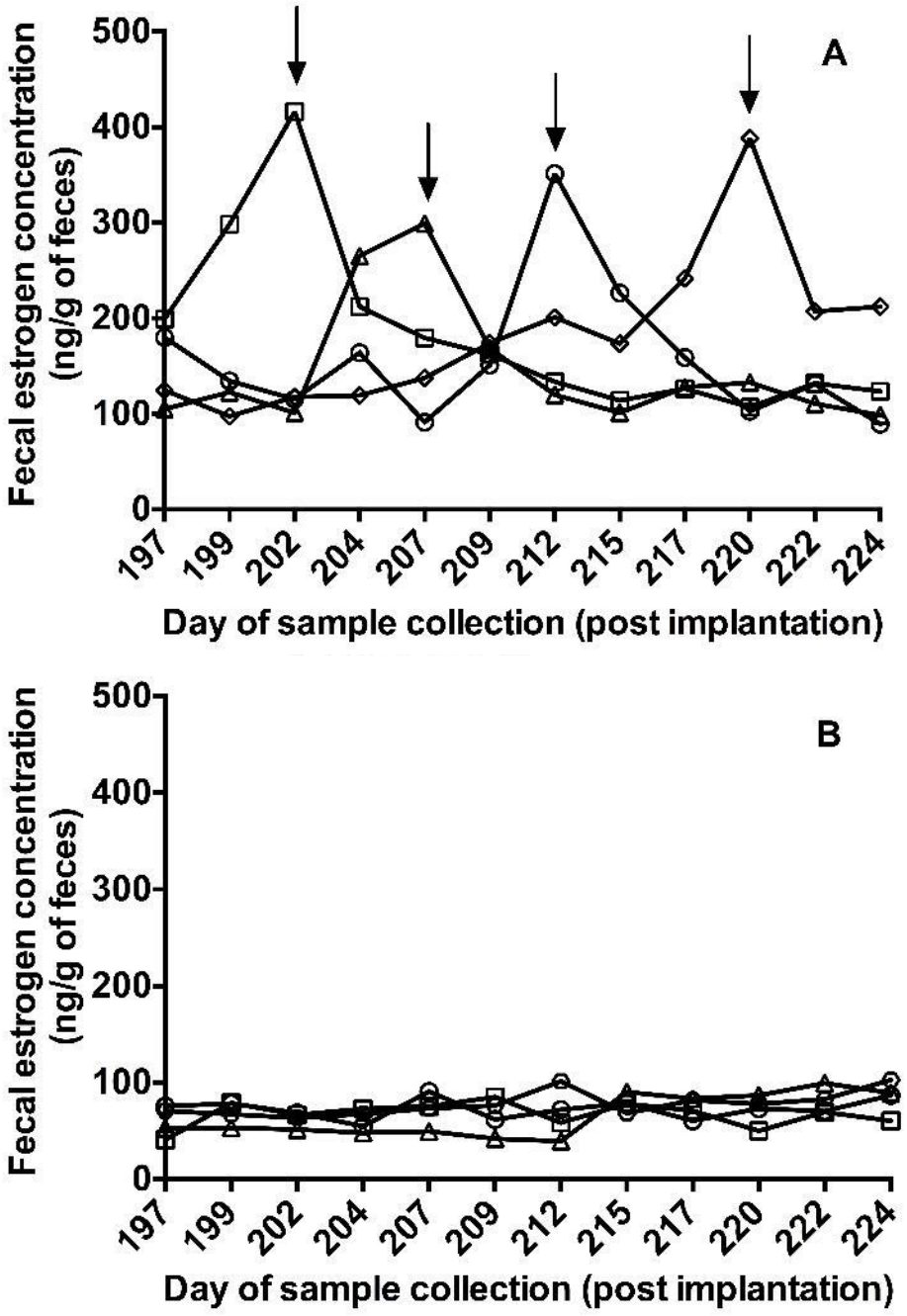

Figure 14 *The mean (± SEM) fecal estrogen concentrations (ng/g of feces) in cats with (B) or without (A) deslorelin implantation. Cats were implanted with deslorelin at the age of 3 months for a period of 48 weeks. Black arrows indicate estrogen peaks of control cats.*

The ovarian weight of the implanted cats (Group 1) was significantly lower ($P \leq 0.05$) than that of the prepubertal OVH (Group 3) and the control cats (Group 2) at all stages of their estrus cycle. Significantly lower ($P \leq 0.05$) ovarian weight was found in prepubertal OVH cats compared to control cats at luteal and estrus but no significant difference was found in prepubertal OVH cats compared to control inactive stage of the estrous cycle (Table 6). The number of primordial follicles did not differ among the different groups. Number of primary follicles was significantly higher ($P \leq 0.05$) in implanted cats compared with control cats at all the stages of the estrous cycle. However, it did not differ from that in prepubertal OVH (Group 3) cats (Table 6). The number of secondary follicles did not differ between deslorelin-implanted (Group 1) and other experimental groups. The number of antral follicles was, however, significantly lower ($P \leq 0.05$) in implanted cats compared to other groups except the luteal stage cats (Table 2). The number of corpora lutea (CL) in the control cats at luteal stage was significantly higher ($P \leq 0.05$) compared to implanted, prepubertal OVH (Group 3) and control (Group 2) cats at the follicular and inactive stages of the estrous cycle (Table 6).

There were no differences in the uterine gland diameter between the implanted and prepubertal OVH (Group 3) or Control (Group 2) cats at any stage of the estrous cycle. Prepubertal OVH cats, however, had significantly lower ($P \leq 0.05$) endometrial gland diameter than the follicular and luteal cats (Table 6). The endometrial glands in the implanted, prepubertal OVH and inactive cats had a single layer of cuboidal epithelium, a single layer of columnar epithelium was presented in the control cats at the follicular and luteal stages with greater height and abundant secretion in the glands (Figure 15). There were no significant differences in the endometrial thicknesses between implanted cats and prepubertal OVH (Group 3) or the control (Group 2) cats. However, the implanted cats had significantly lower ($P \leq 0.05$) myometrial thickness compared to control cats at the follicular and luteal phases but similar to prepubertal OVH and inactive control cats (Table 6).

Table 6 *Mean ± SEM ovarian weight (g), numbers of structures (follicles and corpora lutea) per mm^2 of ovarian tissue, uterine gland diameter (µm), endometrial and myometrial thickness (µm) in deslorelin-implanted, control (follicular, luteal and anestrus) and prepubertal female cats.*

Groups	Ovarian weight (g)	Numbers (SEM) / mm^2 of ovarian tissue					Endometrial gland diameter (µm)	Endometrial thickness (µm)	Myometrial thickness (µm)
		Primordial follicle	Primary follicles	Secondary follicles	Antral follicles	Corpora lutea			
Deslorelin implanted	0.03±0.00^{a}	310.33±45.49^{a}	32.33±4.88^{a}	4.00±1.15^{ab}	1.33±0.67^{a}	0.00±0.00^{a}	30.45±2.37^{ab}	374.54±14.6^{a}	361.90±20.19^{ad}
Controls (Follicular)	0.12±0.01^{b}	234.33±45.12^{a}	16.33±5.04^{b}	8.67±2.04^{a}	8.33±1.09^{bc}	0.67±0.67^{a}	40.44±5.22^{a}	515.85±115.2^{a}	733.57±153.29^{b}
Controls (Luteal)	0.13±0.02^{b}	204.67±30.14^{a}	10.33±2.39^{b}	4.00±1.03^{b}	4.67±0.42^{ab}	7.33±0.99^{b}	40.75±6.17^{a}	506.43±114.3^{a}	705.36±142.90^{bc}
Controls (Anestrus)	0.08±0.01^{c}	249.00±57.50^{a}	11.33±2.91^{b}	6.33±1.67^{ab}	6.67±1.98^{bc}	0.00±0.00^{a}	32.60±4.11^{ab}	439.37±40.3^{a}	504.16±67.94^{acd}
Pre-pubertal	0.08±0.01^{c}	344.67±64.63^{a}	25.33±5.28^{ab}	9±3.61^{ab}	9.00±1.69^{c}	0.00±0.00^{a}	25.13±7.56^{b}	398.32±50.7^{a}	392.27±56.80^{d}

Values with different superscripts within a column differ significantly ($P \leq 0.05$)

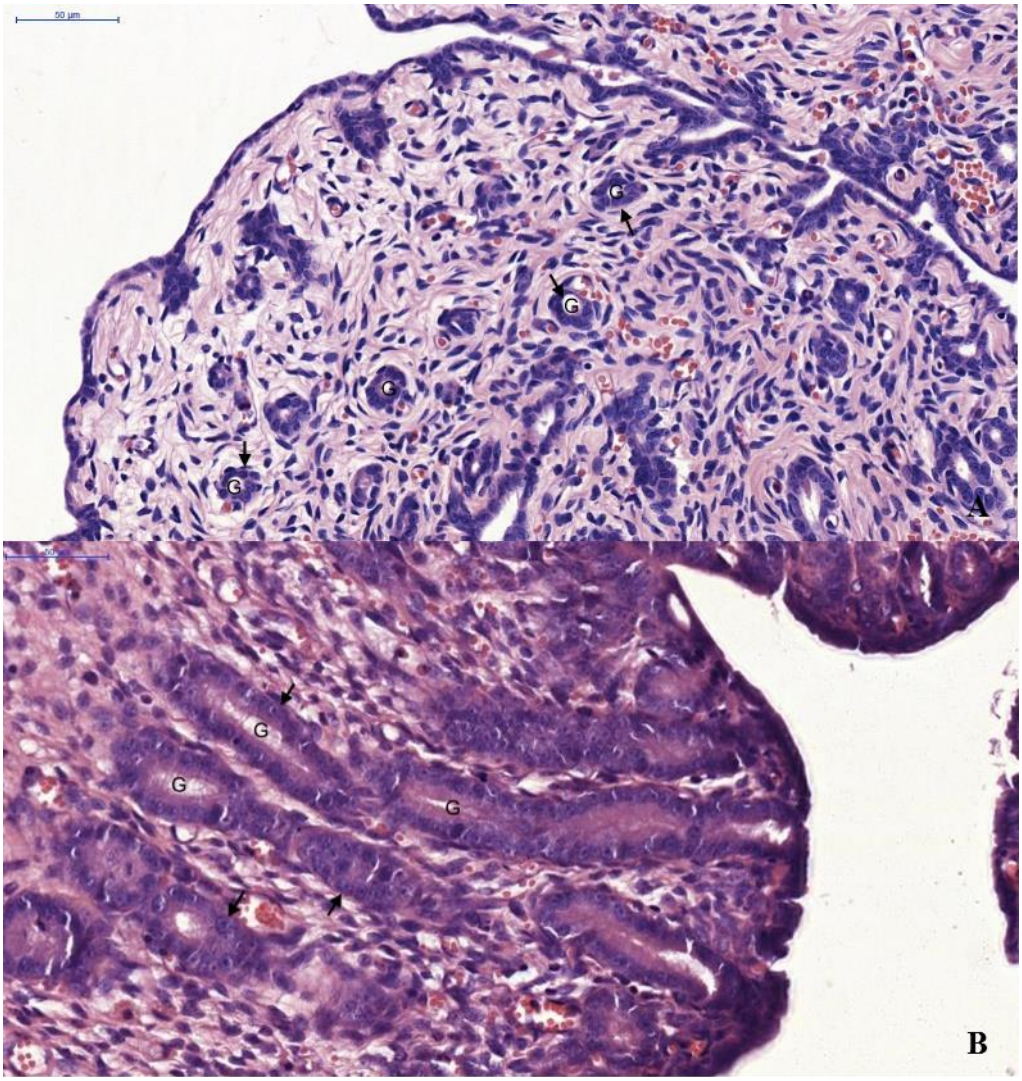

Figure 15 *The histological appearance of the uterine glands and secretion of a deslorelin-implanted cat (A) and a control cat at follicular stage (B). Abundant secretion in the uterine gland of the control cat was found compared with the deslorelin-implanted cat. The letter "G" indicates the uterine glands.*

Protein expression of LHR was observed in the cytoplasm of theca cells, interstitium of the ovarian tissue and granulosa cells of large antral follicles only, whereas the FSHR protein expression was found in the granulosa cells of antral follicles only. No difference was observed in the protein expression of the LHR among different experimental groups of cats studied (Figure 16). Prepubertal OVH (Group 3) cats had significantly lower ($P \leq 0.05$) protein expression of FSHR compared to the Deslorelin-implanted (Group 1) and the control (Group 2) cats at the luteal stage of the estrous cycle. However, there was no difference in the protein expression of FSHR among the deslorelin-implanted (Group 1) and control (Group 2) cats at the follicular and inactive stages of the estrous cycle (Figure 17).

Ovarian LHR mRNA expression was significantly lower ($P \leq 0.05$) in the deslorelin-implanted cats (Group 1) than the control cats (Group 2) at the follicular stage of the estrous cycle. However, there was no difference in the mRNA expression of LHR among the deslorelin-implanted, prepubertal OVH (Group 3) and control (Group 2) cats at their luteal or inactive stages of the estrous cycle (Figure 18). Moreover, no difference was observed in the mRNA expression of FSHR between the deslorelin-implanted (Group 1), prepubertal OVH (Group 3) and the control (Group 2) cats (Figure 19).

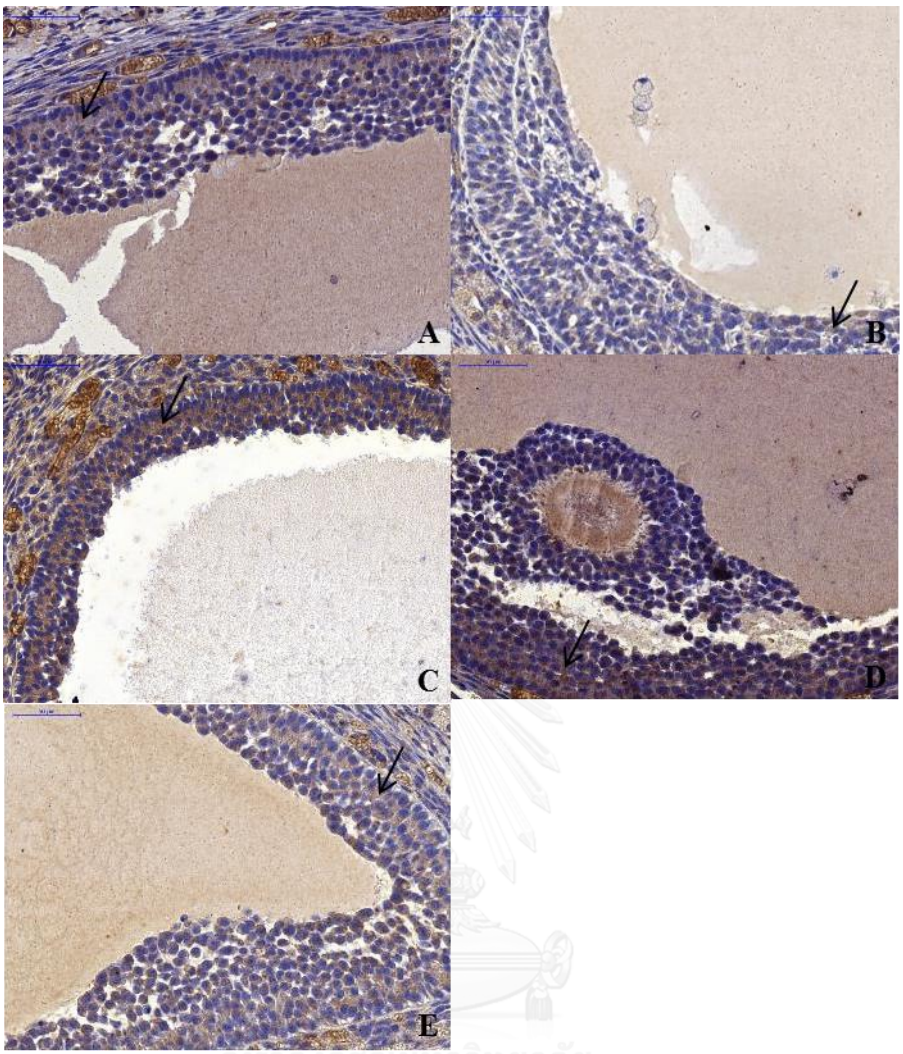

Figure 16 *The immunohistological staining of FSHR protein in the granulosa cells (the letter "G" indicates the granulosa cells) of antral follicles in the ovarian tissue of deslorelin implanted (A), prepubertal (B) and control cats in follicular (C), luteal (D) and anestrus (E) stages of estrous cycle. Black arrows show the immunohistological staining of the FSHR in the cytoplasm of granulosa cells."*

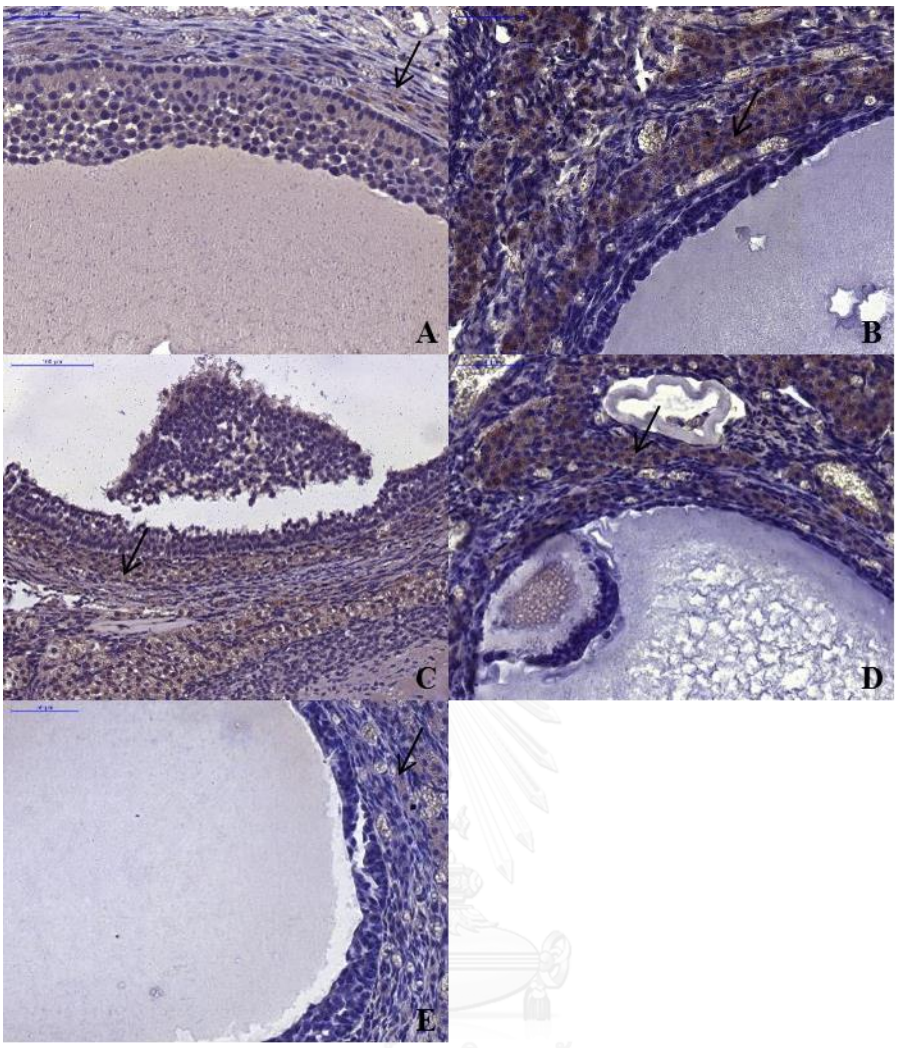

Figure 17 *The immunohistological staining of LHR protein in the cytoplasm of theca cells, interstitium of the ovarian tissue and granulosa cells of large antral follicles (the letter "G", "T" and "I" indicates the granulosa cells, theca cells and the intersitium respectively) in the ovarian tissue of deslorelin implanted (A), prepubertal (B) and control cats in follicular (C), luteal (D) and anestrus (E) stages of the estrous cycle. Black arrows show the immunohistological staining of the LHR in the cytoplasm of the cells."*

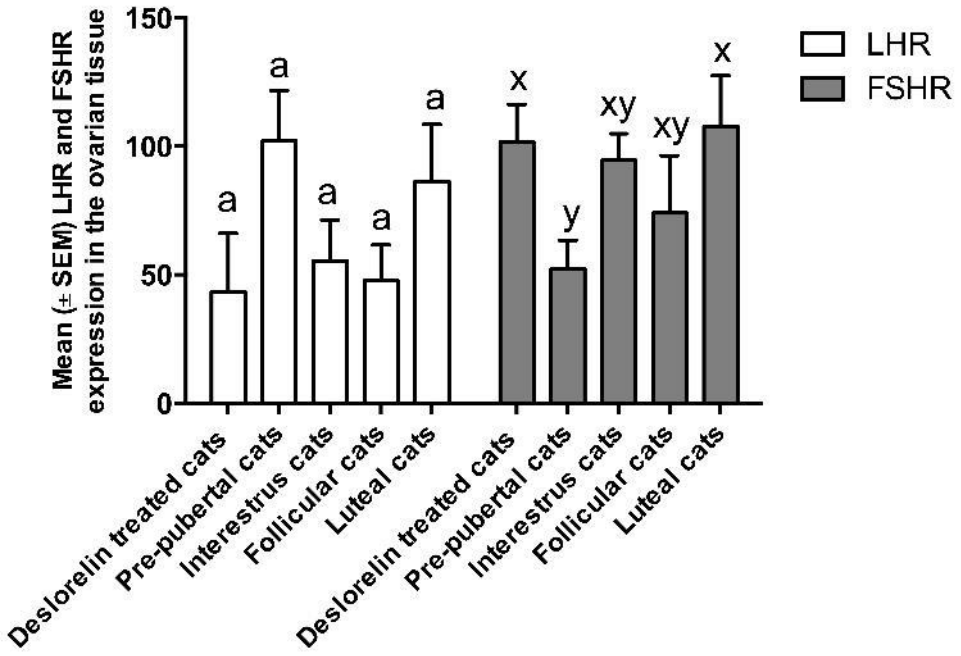

Figure 18 *The mean (± SEM) level of protein expression for the ovarian LHR and FSHR in cats that were either prepubertal (n = 6) or implanted with deslorelin (n =6) at the age of 3 months for 48 weeks or non-implanted controls at the interestrus, follicular and luteal phase (n = 6/phase) of their estrous cycle. Different letters on bars for a certain receptor indicate significant (P ≤ 0.05) differences.*

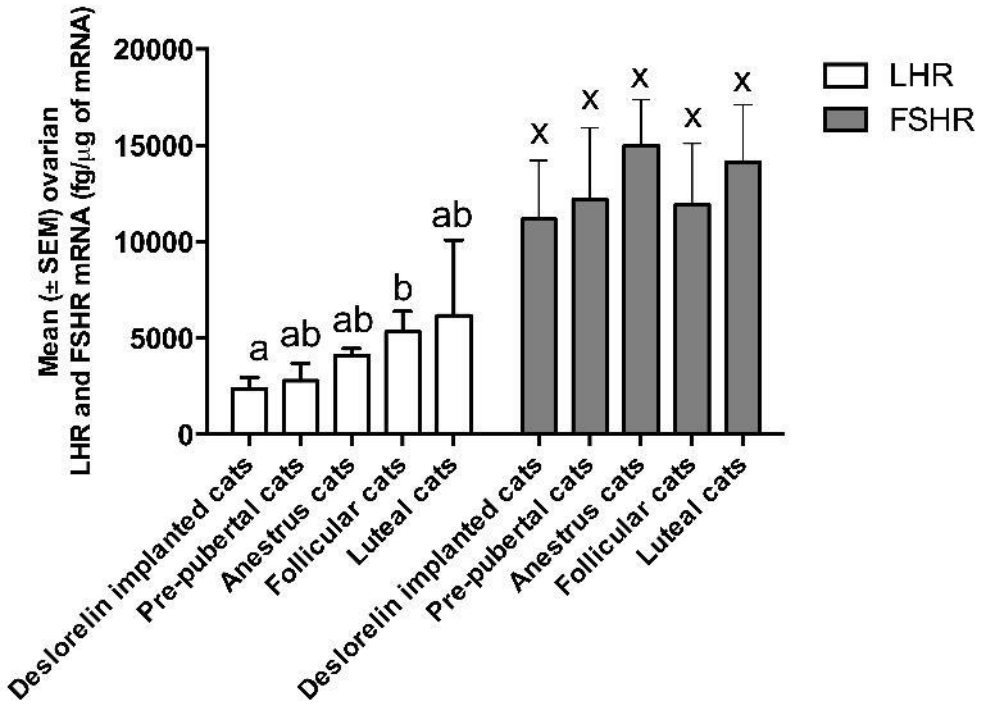

Figure 19 *The mean (± SEM) mRNA (fg/µg of RNA) for the ovarian LHR and FSHR in cats that were either prepubertal (n = 6) or implanted with deslorelin (n =6) at the age of 3 months for 48 weeks or non-implanted controls at the interestrus, follicular and luteal phase (n = 6/phase) of their estrous cycle. Different letters on bars for a certain receptor indicate significant (P ≤ 0.05) differences.*

4.5 Discussion

This study was designed to investigate the effects of the treatment of prepubertal female (3 months old) cats with 4.7mg GnRH-agonist (Deslorelin) on their reproductive function and the ovarian expression of LHR and FSHR. Moreover, to see how closely GnRH-agonist implantation maintains the prepubertal status of the parameters studied, tissues were also obtained and analysed from the prepubertal animals.

The prepubertal female cats at the age of 3 months tolerated the Deslorelin-implantation very well without any local or general anesthesia; no adverse effects were observed in the implanted animals. This supports the previous studies in which tolerance of deslorelin-implantation has been reported in prepubertal cats (Risso et al., 2012; Carranza et al., 2014). General health of the implanted (Group 1) and the control animals (Group 2) was also monitored by recording the weekly body weights of the cats throughout the study period. The body weight of deslorelin-implanted and control cats did not differ from the control cats during most of the study period of 48 weeks except during the 20^{th} to the 26^{th} week when body weight of the treated cats was found to be significantly lower than that of the controls. This difference in the body weight observed only during the specific period of 6 weeks might have resulted from the individual difference of each cat in the study. Fecal estradiol in felid species is considered to be an indication of the ovarian activity (Brown et al., 1994). The presence of fecal estradiol peaks in the control cats (Group 2) and the absence of such peaks in the deslorelin-implanted cats (Group 1) simply proves that Deslorelin ceases the ovarian activity in prepubertal cats. Moreover, unlike the previous studies (Brown et al., 1994) we did not notice any up-regulation or flare up effect because of the deslorelin implantation. In addition to the fecal estradiol, ovarian activity was also monitored in these animals at the end of study period by recording the ovarian weight and the number of ovarian structures including follicles and corpora lutea. No difference was observed in the number of primordial follicles between the experimental groups which may indicate that all the experimental groups had the same potential of follicle development. In the deslorelin-implanted group, however, follicle growth seemed to be arrested at the primary follicle stage with little growth to the secondary stage and almost negligible growth to the antral stage of follicle development. This was also evidenced by the significantly lower ovarian weight recorded in this group. In the control groups (Group 2), follicle growth basically reflected the stage of the estrous cycle. Understandably though, the highest number

of antral follicles was observed in the prepubertal OVH group (Group 3) that may be due to an absence of ovulation in this group. As expected, the number of corpora lutea was the highest in the luteal group and was found only in few in stance in follicular phase queens with no evidence of ovulation or CL in the prepubertal OVH (Group 3) or deslorelin-implanted (Group 1) animals. The corpora lutea in control cats (Group 2) which never expose to copulation may indicate the evidence of spontaneous ovulation in domestic cats which may occur (Bristol-Gould and Woodruff, 2006; Brown, 2006). Gonadotropins play an important role to control the overall activity of female reproductive tract including the ovarian activity or the process of folliculogenesis. Follicle growth is stimulated by the rising levels of FSH while LH would normally remain at basal levels until the initiation of the process of ovulation and/or luteinization (Orosz et al., 1992). Gonadotropins act by the binding to their receptors on the ovarian cells, so a change in either the gonadotropins' concentrations or the number of gonadotropins' receptors may modulate the effects of the gonadotropins on the follicular development, ovulation and/or luteinization (Saint-Dizier et al., 2007). FSH binds to its receptors to stimulate the follicle growth and development, and LH binds with the LHR to stimulate the luteinization process after copulation (Bristol-Gould and Woodruff, 2006). LHR in the interstitium is also found to be responsible in modulating the production of steroid hormones which are necessary for the functions of the reproductive tract (Saint-Dizier et al., 2007).

The mRNA expression of LHR in the Deslorelin implanted cats (Group 1) were significantly lower than in the control animals (Group 2) at their follicular phase while there were no differences in the LHR mRNA expression between the deslorelin-implanted cats and the control animals during the luteal and inactive stages. This may suggest that receptors for LH are activated in the control cats during their follicular stage of the estrous cycle in concomitant with an increase in the LH release itself (Bristol-Gould and Woodruff, 2006). It is worth noting that mRNA expression of LHR was not different during the other stages (luteal and inactive) of the estrous cycle when LH release remains basal with mild fluctuation (Bristol-Gould and Woodruff, 2006). Although LH release was not monitored in this study, it is well-documented that GnRH administration does cause suppression of LH in almost all the species including dogs (Wright et al., 2001; Ponglowhapan, 2011) and cats in which it was monitored from the decreased levels of estradiol (Toydemir et al., 2012). In this study LH suppression by deslorelin implantation, seems to be quite effective and consistent, and might have resulted in the lower expression of LHR mRNA observed in the deslorelin-implanted cats (Group 1). However, the observed lack of difference in the

LHR mRNA expression between the prepubertal OVH (Group 3) and control (Group 2) cats suggests that LHR synthesis does occur during the prepubertal period but it remained suppressed in the deslorelin-implanted cats. In spite of the differences observed in the LHR mRNA, no difference was observed in the protein expression of the LHR between the deslorelin-implanted and the control cats, even at their follicular stage of the estrous cycle. The activation of LH, however, normally occurs after its release induced by copulation or may spontaneously release in cats with spontaneous ovulation (Bristol-Gould and Woodruff, 2006) especially in laboratory maintained cats which was reported to be depended on the individual conditions and housing of the cats (Brown, 2006).

FSHR is normally detected in the granulosa cells of antral follicles and is considered to play a role in the follicular development. The fact that the number of antral follicles was significantly higher in the prepubertal OVH (Group 3) than the deslorelin-implanted (Group 1) cats in spite of significantly lower expression of FSHR protein in the prepubertal OVH females, simply points out to the fact that the ligand itself plays the critical role required for the follicle development rather than changes in its receptor expression. There might be a suppression of the FSH which makes the ligand in complete due to FSHR is still expressed without any different between the deslorelin-implanted cats and the control prepubertal cats in all stages of estrous cycle. However, a lack of difference in the FSH mRNA between these groups may suggest that translation was compromised in the prepubertal animals.

The endometrial glands are responsible for uterine secretions that nourish the early embryos during pregnancy (Chatdarong et al., 2005) and they are also active during certain stages of the estrous cycle. The significantly lower endometrial gland diameter in the prepubertal OVH (Group 3) cats suggests their lower activity in these animals compared to the control cats (Group 2) during their follicular and luteal phase of the estrous cycle. Although the endometrial gland diameter of deslorelin-implanted cats (Group 1) was not lower than that of the control cats (Group 2) but epithelium of the endometrial glands of the deslorelin-implanted cats consisted of a single layer of cuboidal cells while the control cats during their luteal and follicular stages of the estrous cycle had a single layer columnar epithelial cells with greater height and abundant secretion in the glands. This simply suggests a negative effect of deslorelin-implantation on the structure/morphology and function/activity of the endometrial glands. No differences were observed in the endometrial thickness between the deslorelin-implanted (Group 1) and the control cats (Group 2) during any stage of the estrous cycle but the myometrial thickness of the implanted cats was significantly

lower than that of control cats during their luteal and follicular phases of the estrous cycle. Moreover, myometrial thickness of the prepubertal OVH (Group 3) cats was also significantly lower than that of the control cats during their follicular and luteal phases of the estrous cycle. The lower thickness of myometrium in the implanted cats may be a result of suppressed estrogen concentrations in these animals as assembly of the myometrial fiber filaments is said to be induced by estrogens (Kelly and Verhage, 1981). Nevertheless, results of this study suggest that the endometrial glands and the myometrium of the deslorelin-implanted cats (Group 1) do not seem to be fully functional compared to the control cats (Group 2) with active ovaries. These data therefore support the notion that both the myometrium and the endometrial glands are not quite functional due to the suppression of ovarian function induced by the GnRH-agonist implantation.

In conclusion, the results of this study have demonstrated that GnRH-agonist implantation of prepubertal female cats is capable of suppressing their ovarian weight, follicle development, estrogen production as well as myometrial thickness for a period of at least 48 weeks without any serious adverse effects. Associated to the suppression of estrogen production and other ovarian functions sexual behaviour in deslorelin treated cats were also suppressed, and this suppression of reproductive function by the deslorelin-implantation seems to have partly been achieved through changes in the ovarian mRNA expression of LHR. These studies could be extended to investigate both the actual duration of suppression of reproductive function beyond the 48 weeks and other possible mechanism(s) by which GnRH-agonist treatment could act apart from the ovarian LHR and FSHR expression.

CHAPTER V

GENERAL DISSCUSSION AND CONCLUSION

The main objective of this study was to investigate the effects of GnRH-agonist implantation on the age at puberty, sexual behavior and reproductive tract development and function in prepubertal male and female cats. Effects of GnRH-agonist implantation were also studied on the gonadal LH and FSH receptors. The GnRH-agonist used for implantation was deslorelin 4.7 mg which suppressed sexual behavior and reproductive function in both prepubertal male and female cats for at least 48 weeks.

Deslorelin implants did not cause any adverse effects when inserted in the inter-scapular area using aseptic technique without local or general anesthesia. Absence of any lesions, edema or infection may be due to the aseptic technique involved and probably also due to enough space available in the inter-scapular area for the implants that were about 10 mm long and 3 mm wide. Such an absence of adverse effects have already been reported in male prepubertal cats (Goericke-Pesch et al., 2011; Carranza et al., 2014).

The deslorelin implantation also did not affect the body weight of implanted cats and their general health. Moreover, in male cats, deslorelin implants suppressed the protein expression of LHR and enhanced mRNA expression of FSHR along with suppression of reproductive function. In female cats, the ovarian weight, follicle development, estradiol production and myometrial thickness was suppressed by the effect of the deslorelin implantation possibly through a change in the ovarian mRNA expression of LHR.

5.1 Body Weight and General Health

In this study, the general health of both male and female cats that were implanted with 4.7 mg deslorelin device remained normal. There was neither acute nor chronic reaction form the implantation. The body weight was also monitored.

However, no significant difference was observed in the body weight of deslorelin implanted male cats and control male cats except for a short period of time when significantly lower body weight was recorded in deslorelin-implanted female cats and control female cats. Overall, it can be said that deslorelin implantation in prepubertal male and female cats does not cause any serious adverse effects to the cats.

5.2 Sexual behavior

The sexual behavior of implanted cats was suppressed and many unwanted behaviors such as spraying, fighting and roaming were totally absent in these cats. This suppression of behaviors resulting from deslorelin implantation was similar as observed after surgical castration or ovariohysterectomy in male and female cats, respectively (Hart and Eckstein, 1997). It is well known that sexual behavior in male and female cats is controlled by the sex steroid hormones like testosterone and estradiol. Estradiol controls the estrus behavior in female cats and testosterone controls the sexual behavior and the sexual characteristics such as the penile spines in male cats (Johnston et al., 1996; Johnston et al., 2001b; Johnston et al., 2001c). Continuous administration of GnRH or its agonists results in the downregulation of pituitary GnRH receptors and suppression of pituitary gonadotropins like LH and FSH (Padula, 2005). The suppression of gonadotropins then is likely to affect both the reproductive function and sexual behavior possibly through an effect on the gonadal LH and/or FSH receptors. In this study we did not observe changes in gonadotropins. However, gonadal steroid hormones like testosterone and estradiol were measured and were found to be suppressed by deslorelin implantation. We believe that the observed suppression of sexual behavior in implanted male and female cats might have resulted from the suppressed of gonadal steroid hormones. The sexual behavior and reproductive function are related and complimentary to each other and this study also investigated the changes in reproductive function.

5.3 Reproductive function

Gonadotropins are known to be the key hormones to regulate reproductive function in both male and female cats (Saint-Dizier et al., 2007; O'Shaughnessy, 2014;

Thompson and Kaiser, 2014). GnRH-agonist devices has higher potential compared to GnRH. The long-term administration of GnRH-agonist causes the desensitization of pituitary GnRH receptors rendering the GnRH ineffective (Gobello, 2007). Gonadotropins, whose release is regulated by GnRH also become suppressed from the long-term GnRH-agonist administration. As described above, gonadotropins were not monitored in this study. To monitor any changes in gonadotropins, ideally their concentrations need to be measured in the blood samples. However, the frequency required for the blood sampling needs to be high enough to monitor pulsatile release of LH, e.g, at 12 minutes intervals for 4-6 h. Considering the invasiveness nature of the technique to collect blood samples and the total amount of blood drawn on any particular day prohibited us such type of sampling in pet animals. Alternatively, activity of gonadotropins can be assessed indirectly by assessing the fertility, reproductive function and/or status of gonadal steroid hormones. In this study it was decided to adopt the 2nd approach and simply monitored the changes in the reproductive tract and function by simple morphology/histology, gonadal testosterone and estradiol concentrations in the feces of male and female cats respectively. Moreover, the sexual behaviors in male and female cats, which are known to be under the influence of reproductive steroid hormones (Hart and Eckstein, 1997), was also monitored.

In male cats, the criteria for reproductive function evaluation were testosterone concentrations in the feces, male reproductive behaviours (copulatory behavior, erection and ejaculation), secondary male chatacteristics (the penile spine) and the changes in the morphology of the reproductive tract (the testicular volume). The results of the study have shown that all the parameters included in these criteria were significantly diminished in the male cats that were treated with 4.7 mg deslorelin since the age of 3 months compared to non-treated male cats. Moreover, nearly all the parameters in the criteria observed in treated cats and control cats were recorded to be similar to the prepubertal and adult cats, respectively. Semen production is one of the most important functions of the male reproductive tract and the deslorelin-treated male cats were found to fail in the semen collection process (induced by electro ejaculator and epididymal semen collection). The testicular weight, testicular tissue score and the mean diameter of seminiferous tubules in the deslorelin-implanted cats

were also found to be reduced from the effect of deslorelin implantation. This simple fact that deslorelin-treated cats had similar type of reproductive function as the prepubertal cats suggests that the GnRH-agonist suppressed the reproductive function to the extent of prepubertal stage.

In female cats, criteria that were used to evaluate the reproductive function were estradiol concentrations in the feces to confirm the ovarian cycle, the estrous behavior confirmed by vaginal cytology, the acceptation of coitus. In this study, all of the control cats showed estrus behavior along with estradiol peaks and accepted coitus. The deslorelin-implanted cats did not show any estrous behavior, had no estradiol peaks and did not accept coitus from the male cats during the study period. Moreover, ovarian weight and the numbers of antral follicles and the myometrial thickness was also reduced from effect of deslorelin implantation. Long-term administration of GnRH-agonist in mammals including cats and dogs has already been shown to result in the suppression of reproductive function (Trigg et al., 2006; Goericke-Pesch et al., 2011; Goericke-Pesch et al., 2013a) similar to the results obtained in this study which clearly demonstrate that implantation of 4.7 mg deslorelin in both male and female cats at the age of 3 months could fully suppress their reproductive function at least for 48 weeks (or until at least 15 months old). However, prolonged efficacy of these implants needs to be tested.

5.4 Reproductive Tract Morphology

The gonadal tissue weight of both male and female deslorelin implanted cats was found to be significantly lower compared to the control cats. The testicular tissue score and the mean diameter of the seminiferous tubules of deslorelin-implanted cats were noted to be lower compared to the control cats but was not different compared to the prepubertal male cats. This confirms the effect of deslorelin implantation on suppressing the development of the reproductive tract of the male cats possibly through a decrease in testosterone production.

In our study, we also found that the ovarian tissue of prepubertal cats had a higher ratio of primordial, primary and secondary follicles compared to antral follicles

than ovarian tissue of adult female cats in follicular, luteal and interestrus stages. The lowest number of antral follicles was observed in the deslorelin-implanted group that may be due to an absence of ovulation in this group. As expected, the number of corpora lutea was the highest in the luteal group with no evidence of ovulation or CL in the prepubertal or deslorelin-implanted animals. The diameter of antral follicles in prepubertal cats are smaller than antral follicle of adult cats in all stages which might be the reason of the lower expression of FSHR at the granulosa cells of antral follicles. The histological study of the uterus also found that the epithelium of the endometrial glands of the deslorelin-implanted cats consisted of a single layer of cuboidal cells while the control cats during their luteal and follicular stages of the estrous cycle had a single layer of columnar epithelial cells with greater height and abundant secretion in the glands, which was suggested that there was negative effect of deslorelin implantation suppressing the function of the endometrial glands. Moreover, it was noted that the myometrial thickness of implanted female cats was significantly lower compared to control cat at follicular and luteal stage which was suggested that it may be the result of suppressed estrogen concentrations in these animals as assembly of the myometrial fiber filaments is said to be induced by estrogens (Kelly and Verhage, 1981). The endometrial glands and the myometrium of the deslorelin-implanted cats in this study do not seem to be fully functional compared to the control cats with active ovaries, which was suggested to be from the effect of the suppression of ovarian function induced by the GnRH-agonist implantation.

5.5 The GnRH-agonist implantation and the HPG axis

The HPG axis is composed of hypothalamus, the pituitary gland and the gonads. GnRH is secreted by the hypothalamus, it binds to pituitary GnRH receptors and stimulates release of gonadotropins from the anterior pituitary. The gonadotropins act on the gonads after binding to their receptors. Previous studies have shown that serum LH and FSH decreases to almost base line after long-term continuous administration of GnRH or its agonists (D'Occhio et al., 2000; Herbert and Trigg, 2005). As deslorelin implanted cat had a continuous long-term supply of GnRH-agonist, on the basis of the

literature cited above it can be speculated that their pituitary GnRH-receptors would have been desensitized/down-regulated and this would have resulted in the complete suppression of gonadotropins release along with downstream effects like morphological changes in the reproductive tract, suppression of reproductive function as well as of gonadal LHR and FSHR.

In this project we have studied both mRNA and protein expression of LHR and FSHR and the presence of the mRNA of the receptors. The protein expression of the receptors indicate that the receptors are ready to function after binding to their ligands. The expression of mRNA of the receptors, however, does not indicate that a receptor is functional, rather it is an indication that potential for protein expression of that receptor exists and mRNA could be translated into protein under the appropriate conditions. So it is possible that mRNA may or may not translated into protein.

In this study, deslorelin-implanted male cats had lower protein expression of LHR without any effect on the mRNA expression. This may simply suggests that deslorelin or continuous long-term GnRH-agonist treatment does not affect the mRNA of the LHR but decreases the rate of its translation into protein to carry out its effect. The suppression of LHR observed in this study could be one of the reasons that the fecal testosterone in the deslorelin-implanted cats was significantly lower than the control cats, because the production of testosterone partially depends to LH and its receptor expression (Tilbrook and Clarke, 2001; Midzak et al., 2009).

The mRNA of FSHR was significantly higher in deslorelin implanted male cats compared with unimplanted control cats and also in prepubertal male cats compared with adult male cats. This may imply that FSHR are expressed in deslorelin treated cats in the same fashion as in prepubertal cats. The FSHR mRNA that is expressed significantly higher could be the result of a mechanism to compensate the suppression of endogenous FSH from the GnRH-agonist implantation.

In female cats, we only found significance difference in the mRNA expression of LHR between the deslorelin-implanted and the control cats during their follicular stage. The basal/lower levels of fecal estradiol observed in deslorelin-implanted cats could be explained on the basis either the suppression of the LH itself as the suppression of the LHR mRNA is not likely to explain the basal levels of fecal estradiol

observed in deslorelin-implanted cats. Or alternatively, degree of variation observed in the data may indicate that the technique used to measure the protein expression (IHC) being semi-quantitative in nature was not good enough to give a better estimate or the number of samples tested was not big enough to give an accurate measure.

5.6 LHR and FSHR expression

It is well known that LH and FSH regulate reproductive activity in both male and female mammals and are important hormones for fertility; stimulate follicular development in female mammals, regulate spermatogenesis in male mammals along with stimulation of the production of sex steroid hormones in both sexes (Thompson and Kaiser, 2014). Apart from the experiments on deslorelin implanted and control cats, a parallel experiment was conducted to compare the expression of the LHR and FSHR between prepubertal and adult normal cats without any treatment. The objective was that the data obtained from these two experiment will allow us determine and understand the effect of deslorelin-imlants more clearly.

In male cats, no difference of LHR protein expression and mRNA was observed between prepubertal and adult cats but we found that prepubertal male cats had significantly higher FSHR mRNA concentration compared to adult cats. However, no difference was observed in the protein expression of FSHR between prepubertal and pubertal male cats. The higher concentration of FSHR mRNA in prepubertal male cats may indicate its role in the development of the gonads or refer to the role of FSH in the early stages of spermatogenesis, as FSH is known to be important to increase the number of spermatogonia in prepubertal mammals (O'Shaughnessy, 2014). In a previous study in postnatal mouse, they also found that FSHR was expressed at the highest levels at about 7-21 days postnatal which was suspected to be related to the proliferation of sertoli cells in the testicular tissue (Ahda and Soeharso, 2003).

Female cats used in this experiment were either prepubertal or adults at different stages of estrus cycle (follicular stage, luteal stage and interestrus). We found that, the protein expression of FSHR in the ovarian tissue of female cats in luteal phase was significantly higher than in prepubertal female cats. In a previous study, they found

that FSHR expression was mainly limited to the granulosa cells of early antral follicles to preovulatory stage of follicles and in some of the atretic follicles. Moreover, antral follicles with higher diameters are found to have higher expression of LH and FSH receptors (Saint-Dizier et al., 2007).

5.7 Comparation between the effect of GnRH-agonist and GnRH-antagonist in cats

GnRH-antagonist is a competitive blocker with the GnRH to the GnRH receptors. Acycline is a GnRH-antagonist analog used in dogs and cats to suppress the reproductive function (Valiente et al., 2007; Garcia Romero et al., 2009; Garcia Romero et al., 2012). GnRH-antagonist suppresses the reproductive tract by blocking the GnRH receptors and therefore of the endogenous release of GnRH. Reproductive suppression is almost immediately observed after the administration of GnRH-antagonist, while the action of GnRH-agonist needs a delay up to about 2 weeks after the surge of the reproductive tract activation in adult animals. This is mainly due to activation first followed by downregulation of the pituitary GnRH receptors and/or gonadotropins release in case of GnRH-agonist. This will lead to an up-regulation of gonadotropins (LH and FSH) and after a period of time (about 7-14 days) after a continuous administration of agonist that the pituitary GnRH receptors will be desensitized/downregulated and result in the decrease of serum gonadotropins. Whereas in case of GnRH-antagonists there is only occupation of pituitary GnRH receptors with immediate blocking of gonadotropins' release (Herbert and Trigg, 2005; Padula, 2005; Gobello, 2007). However, the knowledge on GnRH-antagonist analogs in cats and dogs is limited and their development has been lagged behind the GnRH-agonist development (Padula, 2005). Moreover, acycline, which is a single dose usage GnRH-antagonist, is one of the only analog of GnRH-antagonist used in dogs and cats, while there are many analogs and a wide range of usage of GnRH-agonist nowadays. Although GnRH-agonist has about 2 weeks of delay in contraception after the flare up effect which is problematic in the aspect of fertility control (Herbert and Trigg, 2005; Padula, 2005). However, our study done on prepubertal cats and other previous studies on prepubertal dogs clarify that the usage of the long-term GnRH-agonist

(Deslorelin) administration in prepubertal dogs and cats could eliminate the flare up effect (Trigg et al., 2006; Sirivaidyapong et al., 2012) and still has the potential to offer good contraception. GnRH-agonist also has a wide range of analogs that could be used for different purposes, such as single dosage for the usage of reproductive management and the long term device of GnRH-agonist such as Deslorelin, for the usage in treatment of diseases involved with hormones of the reproductive tract and for the purpose in contraception (Padula, 2005).

5.8 Concluding remarks

Results of this study have shown that

1. GnRH-agonist (4.7 mg Deslorelin) could be used in prepubertal male and female cats less than 3 months old to suppress the function of reproductive tract without any adverse and/or flare up effect.
2. In prepubertal male cats, reproductive function starts to be active at the age of about 5 months and is fully functional by the age of 7 months. However, with deslorelin-implantation it can be suppressed for a period of at least 48 weeks.
3. The reproductive tracts of the deslorelin-implanted male cats also had exhibited an absence of penile spines, the lower testicular volume and the absence of sexual behavior (copulatory behavior, spraying, erection and ejaculation).
4. Deslorelin implantation suppresses the protein expression but does not interfere the LHR mRNA concentrations in male cats (Figure 20).
5. Deslorelin implantation of prepubertal male cats up-regulates FSHR mRNA concentration similar to that observed in the prepubertal animals thus maintaining the function of FSHR at the prepubertal levels in sertoli cells. In prepubertal female cats, deslorelin implantation in suppresses the reproductive functions without any adverse or flare up effects.
6. Deslorelin implantation affect both the anatomical and functional aspects of the reproductive tract as well as the protein and mRNA expression of the LHR and FSHR.
7. Deslorelin implantation were found to delay puberty for at least 48 weeks confirmed by the basal levels of fecal estradiol and significantly lower ovarian weight compared to the control cats. Numbers of primary follicles in the treated cats were also significantly higher than the control cats, which shows that ovary of the implanted cats remained similar to the ovaries of prepubertal cats.

8. Down-regulation of the protein expression of LHR was found along with a trend of lower concentration of LHR mRNA in the ovarian tissue of deslorelin-implanted cats (Figure 20).

In summary, 4.7 mg deslorelin could be successfully used in prepubertal cats to achieve contraception (at least for 48 weeks). However, the reversibility of the reproductive function is one of the concerns and therefore, this may be questioned for its use for permanent contraception and population control. Nevertheless, it could be effectively used for suppression of reproductive function for a certain period of time, or to prevent the problem of unwanted litters both in shelter animals and owned animals without any risk of flare-up effect.

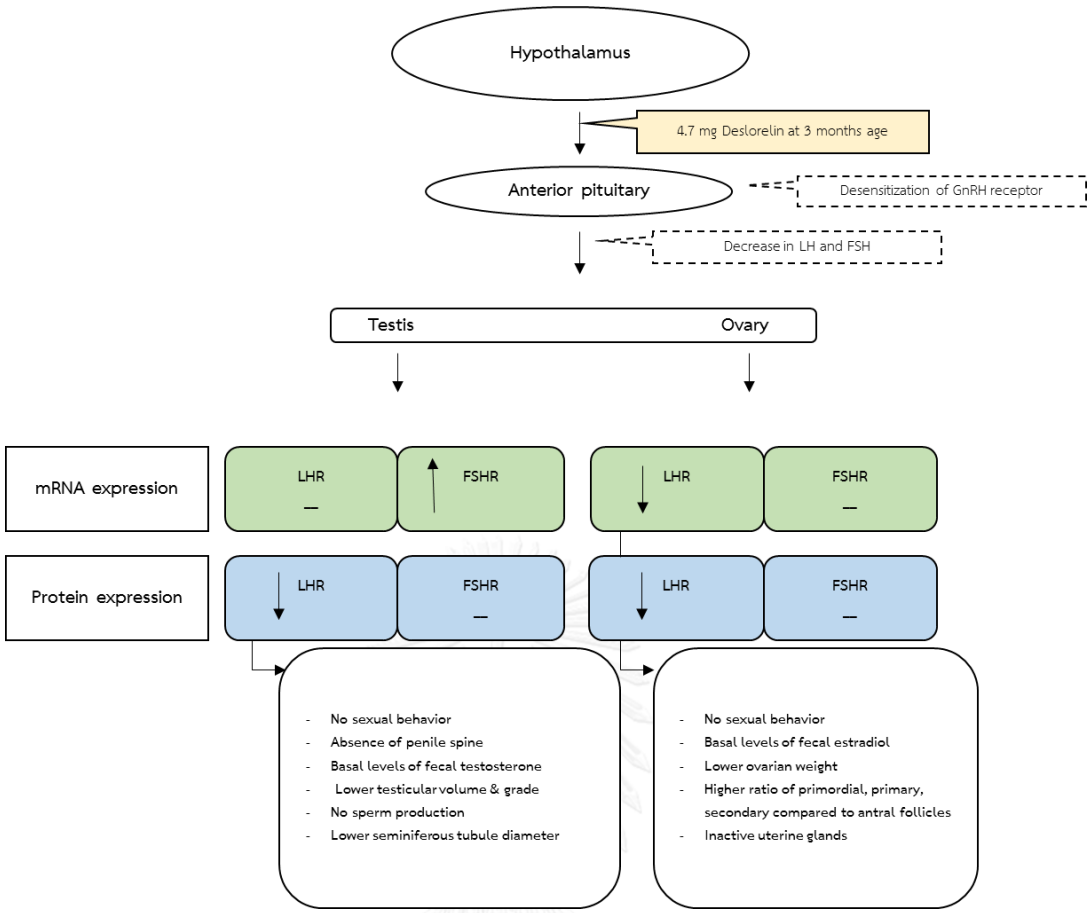

Figure 20 *A summary pathway of the effects of the GnRH-agonist implantation in prepubertal male and female cats on their testicular LHR and FSHR and the effect to their reproductive development and function.*

5.9 Suggestions for further investigation

In this study, effects of the GnRH-agonist implantation on the anatomical and functional aspects of the reproductive system including the underlying mechanism had been defined in prepubertal male and female cats. Further studies could investigate the actual duration of the effects of deslorelin-implantation in prepubertal male and female cats beyond 48 weeks. Mechanism of action of the deslorein-implantation was partly determined in this study, whereas other parts of the mechanism such as the effect of deslorelin-implantation on the pituitary GnRH receptor expression, changes in the gonadotropins and the downstream signaling after binding of gonadotropins to their gonadal receptors leading up to testosterone and/or estradiol synthesis, remain to be investigated.

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APPENDIX

List of publications and conference proceedings

1. Mehl N.S., Khalid M., Srisuwatanasagul S., Swangchan-uthai T., Sirivaidyapong S. GnRH-agonist implantation of prepubertal male cats affects their reproductive performance and testicular LHR and FSHR expression. 2015. Theriogenology. 85: 841 – 848.

2. Sirivaidyapong S., Mehl N.S., and Trigg T.E. Delay of Puberty and Reproductive Performance in Male Dogs Following the Implantation of 407mg and 9.4mg GnRH-agonist Deslorelin at Early Prepubertal Aged. 2012. Reproduction in Domestic Animal. 47(Supplement 6): 400 – 402.

3. Mehl N.S., Srisuwatanasagul S., Sirivaidyapong S. A Preliminary Study on The Effect of GnRH-agonist (4.7mg Deslorelin) Implantation in Tomcats at Early Prepubertal Age. **RGJ Seminar Series XCIX "Innovative Reproductive Technology for Wildlife."** 20th November 2013. Khaokeaw open zoo, Chonburi, Thailand. (Oral presentation)

3. Mehl N.S., Srisuwatanasagul S., Sirivaidyapong S. A Preliminary study on the delay of puberty in female cats following GnRH-agonist (4.7mg Deslorelin) implantation at early early prepuberty age. **The 2nd symposium of the Thai Society for Animal Reproduction and Society for Reproduction and Development.** 20-21 March, 2014. Bangkok, Thailand. (Poster presentation)

4. N.S. Mehl, M. Khalid, E. Olanratmanee, S. Sirivaidyapong. GnRH-agonist implantation of prepubertal male cats does not alter their testicular tissue FSHR expression. **International Conference on Veterinary Science 2014.** 16 - 18 Dec 2014. Bangkok, Thailand. (Poster presentation).

5. N.S.Mehl, M. Khalid, S. Srisuwatanasagul, and S. Sirivaidyapong GnRH-agonist implantation in prepubertal female cats does not alter their gonadal LHR protein expression. **14th Chulalongkorn University Veterinary Conference.** 20-22 April 2015. Thailand. (Poster presentation).

6. N.S.Mehl, M. Khalid, T. Swangchan-Uthai, and S. Sirivaidyapong. **GnRH-agonist implantation of prepubertal male cats does not alter their testicular LHR mRNA expression.** RGJ-Ph.D. Congress XVI "ASEAN: Emerging Research Opportunities." 11 - 13 June 2015. Pattaya, Chonburi, Thailand. (Poster presentation).

7. Mehl N.S., Srisuwatanasagul S., Sirivaidyapong S., Khalid M. GnRH-agonist implantation of prepubertal male cats affects their testiculatal LHR expression. **The 40th World Small Animal Veterinary Association Congress (WSAVA 2015).** 15 - 18 May 2015. Bangkok, Thailand. (Poster presentation).

VITA

Ms. Nicole Sirisopit Mehl was born on the 25th of December 1986 in Bangkok province, Thailand. She graduated high school from Chulalongkorn University Demonstration school in 2005, graduated with her bachelor degree of Doctor of Veterinary Medicine (D.V.M.) with 2nd degree of honor in 2011. She received a scholarship from the Royal Golden Jubilee (RGJ) Ph.D. program of Thailand Research Fund for her Ph.D. program of Theriogenology at the Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her field of interest was about techniques of non-surgical contraception in cats in order to help solve the problem of overpopulation nowadays. Her study was focused on the effect of 4.7 mg Deslorelin (GnRH-agonist) on the reproductive performance in prepubertal male and female cats.