GnRH-agonist implantation of pre-pubertal male cats affects their reproductive performance and testicular LHR and FSHR expression

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Abstract

This study was conducted to investigate the effect of GnRH-agonist implantation in pre-pubertal tomcats on sexual behavior, reproductive performance and expression of testicular LHR and FSHR, and also to compare the testicular characteristics, LHR and FSHR expression between pre-pubertal and adult tomcats. In Exp1, 3 months-old tomcats (n=6/group) were either treated with or left without 4.7 mg Deslorelin implants. Semen collection and evaluation were performed just before castration at 48 wks after treatment; removed testes were analyzed for mRNA and protein expression of LHR and FSHR. We were able to collect semen from six non-treated cats, whereas in treated cats, semen was uncollectable. The results revealed that sexual behavior was absent in the implanted cats throughout the study period. Testicular volume was decreased found from 30 wks after treatment onwards in the implanted cats compared to the controls ($P < 0.05$). Semen production was found only in non-implanted cats. Testicular tissue score, seminiferous tubule diameter and LHR protein expression was found 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 Exp2, 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 differences 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 ($P < 0.05$). Testicular and epididymal weight, diameter of seminiferous tubules and the testicular grade were higher in the adult compared to pre-pubertal cats ($P < 0.05$). In conclusion, deslorelin implants suppressed protein expression of LHR and enhanced mRNA expression of FSHR along with suppression of reproductive function without any adverse effects for at least 48 wks in male cats.

Key Words: GnRH-agonist, Pre-pubertal cat, LHR, FSHR, Reproductive behaviour

Introduction

Overpopulation of cats is a serious global problem and in big towns/cities roaming of tomcats is reported to be out of owners' control. The result is unwanted pregnancies with undesirable consequences in this species. Free roaming cats without any care are also subjected to higher risks like suffering from diseases (including zoonotic diseases), malnutrition and accidents. The number of cats euthanized in shelters is also on the rise every year mainly due to overpopulation [1] which needs to be controlled in order to address the welfare problems associated with it.

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 [2] 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 [3]. Therefore, nonsurgical neutering could be a welfare-friendly and viable alternative to surgical methods of neutering [4].

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 gonadotrophins release and impairs the reproductive function [5]. 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 [5, 6] and female domestic cats [6-

8]. 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 [9-15]. Moreover, Trigg, Doyle [16] 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 wks 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 pre-pubertal cats are rare and with variable results [17, 18].

The effects of GnRH implantation on the gonadotrophins' release along with the suppression of reproductive function are well documented [5, 19]. 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 pre-pubertal male cats. Testicular morphology and expression of LHR and FSHR were also compared between pre-pubertal and adult male cats.

2. Materials and methods

2.1 Experiment design and animals

Experiment 1: Three months old tomcats that were proven to be clinically healthy and had attended a complete vaccination program were either implanted with 4.7 mg deslorelin

GnRH-agonist (Suprelorin® 4.7mg, Virbac Animal Health, France) in the interscapular area (Deslorelin implanted; n=6) or left without any implantation and served as controls (Non-implanted; n=6). The cats were housed together 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 animals were fed with a commercial diet twice daily with water always available *ad libitum*. The study had ethical approval and was performed under the license of Chulalongkorn University Laboratory Animal Center number13310056.

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

Body weight of all the cats in both the groups was recorded fortnightly until the end of the experiment (48 wks) when both the testes were collected after surgical castration. Throughout the experimental period, functional evaluations of the reproductive organs such as penile spines, testicular volume and consistency, and male sexual behavioural characteristics were monitored at 2-weekly intervals in all the cats. Presence of penile spine was taken as a criterion of puberty [5]. Length, width and depth of the scrotum/testis were measured using vernier calipers and 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) [5]. Testicular consistency was recorded by palpation by one observer and was noted as soft, firm or hard. Male sexual behaviour such as marking, mounting (with or without intromission), and fighting [20] were observed for at least 30 min at 2-weekly intervals in all the cats. Faeces were collected at 2-weekly intervals to measure testosterone concentrations. An

attempt was made to collect semen from all the cats before surgical castration by using the electro-ejaculator; which was performed 48 wks after implantation. 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.

Experiment 2: Testes were collected from 4 to 6 months old (pre-pubertal, n = 6) or 1 to 3 years old (adult, n=6) normal healthy male cats after surgical castration at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University, Thailand.

In both the experiments, after castration, weight of each testis and epididymis was recorded. Each testis was 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 for cytology/morphology and immunohistochemistry, whereas the other part was snap frozen in liquid nitrogen and stored in -80 ºC until RNA extraction.

2.2 Luteinizing hormone receptor (LHR) and Follicle stimulating hormone receptor (FSHR) Expression

Fixed testicular tissues were embedded in paraffin wax and cut into 5µm sections by a rotor microtome, applied to gelatin-coated slides and left to dry in an incubator at 37ºC. Sections were deparaffinized with Xylene (J.T. Baker, PA, USA) and rehydrated through ascending concentrations of alcohol (50%, 70%, 90%, 99.7% and 100%). The immunohistochemical staining was performed as described previously by Ponglowhapan *et al.* [21]. Briefly, the tissue sections/slides were placed in boiling 0.01M sodium citrate solution, then cooled down to room temperature for 35 mins to de-mask epitopes. Slides were then rinsed three times in phosphate buffered saline (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 and 20% (v/v) avidin solution (Avidin/Biotin blocking kit; Vector Laboratories, CA, USA). 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 primary antibodies were diluted in PBS to which 20% (v/v) biotin solution (Avidin/Biotin blocking kit; Vector Laboratories, CA, USA) was added. 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 (3 x 10 minutes). 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) were applied to the sections and incubated for 30 min. Sections were washed again three times in PBS and 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 colour 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.

At least two sections for both positive antibody staining and negative controls were examined from each animal.

2.3 Quantification of immunohistochemical staining

The pattern and intensity of protein staining for LHR and FSHR were determined semi-quantitatively using a histochemical score (HSCORE) method. Ten fields per

section of each tissue sample were assessed blind by one assessor using a light microscope at X 200 magnification. The intensity of staining was classified on a scale of 1-3, where 1 = weak staining, 2 = moderate staining and 3 = strong staining [21, 22]. Histochemical score (H-SCORE) was assessed as percent of each level (weak, moderate or strong) of staining in each tissue area with the Image-pro plus 7.0 program (Media Cybernetics, Inc. MD, USA). An expression index (EI) was calculated for each tissue sample based on the percentage of positively stained cells and the intensity of staining using the following formula:

EI = % total stained cells x [(1 x % weak) + (2 x % medium) + (3 x % strong)]/100

A mean expression index was calculated to represent the protein expression of LHR or FSHR in each testicular section of every testis from an individual animal [22-24].

2.4 Morphology of Testes

Testes collected in both the experiments were fixed, embedded in paraffin wax, cut into 5µm sections and stained with hematoxylin and eosin. The stained sections (5 sections/testis) were evaluated for seminiferous tubules; those with normal basement membrane (basement membrane with a continuous line and germ cells well attached) [19] were considered as normal and functional. Diameter of seminiferous tubules in each tissue section was measured using ocular micrometer at X 200 magnification. Using the criteria of Novotny *et al.* [19] a total of 200 seminiferous tubules per section were classified as st0, st1, st2, st3 and st4 if they had only spermatogonia (st0), only spermatogonia and spermatocytes (st1), or with spermatids (st2), or with elongating spermatids (st3) or with elongated spermatids (st4) present in the lumen. Each tissue section was graded into 5 grades (0 to 4), based on the majority of seminiferous tubules

found in the tissue sections; 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, and Grade 4: Testicular tissue with the majority of st4 seminiferous tubules. Any pathological changes in the testes, if present, were investigated and recorded.

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

2.5.1 Extraction and reverse transcription of mRNA

Frozen testicular tissue was ground with a homogenizer at 10,000 to 20,000 RPM for 10 to 20s and used to extract the total RNA by the RNeasy mini kit (QIAGEN®, Alameda, CA, USA) following the manufacturer's instructions. Concentration and purity of the extracted RNA were assessed by spectrophotometer (ND-2000, NanoDrop,Wilmington, DE, USA). The RNA samples were stored at -70ºC before qPCR analysis.

2.5.2 Quantitative real-time PCR

Conventional PCR was performed and the PCR product was used for the preparation of standards and analyzing the optimal melting and annealing temperature for each gene [LHR, FSHR and GAPDH (reference gene)]. The thermal cycler (G-Storm Thermal Cycler, Somerset, United Kingdom) was set at the conditions of 15 min at 95ºC to activate Taq DNA polymerase, 30 cycles of 30s at 94ºC for denaturing, 90s at 57ºC for annealing, 30s at 72ºC for extension and 10 min at 72º C for the final extension. Previously published sequences of forward and reverse primers for feline LHR and FSHR, and GAPDH were used [25, 26] and are shown in Table 1. Each reaction was contained with Qiagen Multiplex PCR Kit (QIAGEN®, Alameda, CA, USA). Amplified 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 assessment.

Real-time qPCR amplification was performed using CFX96 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 (20µl) was contained with 10µl of 2x qPCR BIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd, London, United Kingdom), 0.8µl of each forward and reverse primer, 5µl of a DNA template (5ng/µl), and the volume made up to 20µl with RNase free water. 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 5s following with the optimum annealing temperature of 61.4ºC, 60ºC and 61.4ºC for 25s and melting temperature of 82ºC, 80ºC and 76ºC for 10s for GAPDH, FSHR and LHR, respectively with a gradient from 50 to 95ºC to investigate the gene expression. Standards of each gene were used as controls to determine the absolute quantity of mRNA (fg/µg of total RNA).

2.6 Statistical analysis

Body weight and testicular volume were compared between the deslorelin implanted and non-implanted (Expt 1) animals 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 between the deslorelin implanted and non-implanted (Expt 1) and between the pre-pubertal and adult (Expt 2) animals. 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 deslorelin implanted and non-implanted cats (Expt 1) and between the pre-pubertal and adult cats (Expt 2)

3. Results

No tissue reaction and/or infection were observed after deslorelin implantation. No difference in body weight was recorded between deslorelin implanted and non-implanted cats (Figure 1). The implanted cats had significantly lower ($P<0.05$) testicular volume from wk 30 of study onwards (Figure 2). Male sexual behaviour was absent in implanted cats but was present in non-implanted cats from 28 wks onwards of the study period. However, testicular consistency was soft in both groups and remained soft in deslorelin implanted cats throughout the study while from the 28^{th} wk until the end of study period non-implanted cats had comparatively firmer testicular consistency. Penile spines in non-implanted cats were present from the 28^{th} wk onwards of study period and were absent in implanted cats throughout the study period of 48 wks. Higher faecal testosterone levels ($P<0.05$) were observed in non-implanted compared to deslorelin implanted cats from 20 wks onwards of study period (Figure 3). It was possible to collect semen from all of non-implanted cats., semen volume were 10 – 73 µl with 40 – 60% of sperm motility, 49 – 72% of sperm viability and sperm concentration at $0.15 \times 10^6 – 18 \times 10^6$ sperms/ml. Not only implanted cats failed to ejaculate but also we were not able to collect epididymal sperm from them.

Testicular and epididymal weight, testicular grade and mean seminiferous tubule diameter were higher in the adult as well as non-implanted cats compared to pre-pubertal or implanted cats ($P < 0.05$) (Table 2).

LHR and FSHR were localized in the cytoplasm of Leydig and germ cells, respectively. LHR expression was significantly higher ($p<0.05$) in the non-implanted compared to deslorelin implanted tomcats whereas no difference was found between adult and pre-pubertal animals.

FSHR expression was not different between adult and pre-pubertal cats or between implanted and non-implanted cats (Figures 4, 5).

LHR and FSHR mRNA were expressed in all the testicular samples collected in both the experiments. No differences were observed in the expression of LHR mRNA expression between the groups in either experiment. The expression of FSHR mRNA, however, was significantly higher ($P < 0.05$) in the pre-pubertal and deslorelin implanted cats compared to adult and non-implanted cats, respectively (Figures 6 and 7).

4. Discussion

The objectives of this study were to compare 1) the testicular charateristics, and both protein and mRNA expression of LHR and FSHR between pre-pubertal 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 pre-pubertal tomcats.

Deslorelin implantation which was done without any anesthesia, local or general, was very well tolerated by male pre-pubertal cats as has been reported in previous studies [5, 17]. The sexual behaviour of implanted cats was suppressed and many unwanted behaviours such as spraying, fighting and roaming were totally absent in these cats. This suppression of behaviors resulting from Deslorelin implantation was comparable with behaviours eliminated by surgical castration [27]. Moreover, suppression of physiology of the reproductive organs such as, the grade of testicular tissue, the seminiferous tubules diameter, and the weight of testes and epididymides of implanted cats, which were significantly lower compared to non-implanted cats, confirms the action of Deslorelin implantation on suppressing the function of male reproductive tract.

The physiology and sexual behavior of male mammals is mainly controlled by testosterone which is produced by the activation of Leydig cells by the LH released from the

pituitary gland [28, 29]. Aromatase and 5-α reductase transform testosterone into estrogen and dihydrotestosterone, respectively and these two hormones are considered to be responsible for the change in the male behaviour. However, testosterone itself acts on the Sertoli cells of the testis to support spermatogenesis. FSH is believed to have an important role in the first wave of spermatogenesis in pre-pubertal mammals but its role in spermatogenesis in adults remains to be confirmed. However, FSH is considered to induce meiosis during spermatogenesis process and therefore, is responsible for increasing the number of spermatogonia in the seminiferous tubules [30].

The HPG axis is activated by the release of GnRH from the hypothalamus which stimulates the release of gonadotropins from the pituitary gland to regulate the reproductive function. The chronic administration of GnRH down regulates the pituitary GnRH receptors and suppresses the release of gonadotropins and the reproductive function [31]. It is for this reason that GnRH-agonist implantation is used as an alternative to surgical castration in a number of species including felines. In the present study, we have tried to confirm this in pre-pubertal male cats and to explore whether such a GnRH therapy suppresses the reproductive function via an involvement of testicular expression of LHR and FSHR. FSHR is expressed in the Sertoli cells of the testes and is responsible to control spermatogenesis after activation by the FSH [32], whereas LHR being expressed in the Leydig cells, is responsible to stimulate androgen secretion [33] by activating the biosynthetic pathway that changes cholesterol into testosterone [34].

In the present study, the significantly higher expression of FSHR mRNA in the testicular tissue of deslorelin implanted male cats compared to untreated controls could be a result of the compensatory mechanism resulting from the suppression of the endogenous release of GnRH (and/or FSH) due to implantation of deslorelin (GnRH-agonist). It seems that translational pathway has also been affected by deslorelin implantation as no difference was observed in the

protein expression of FSHR, even though the mRNA expression of the FSHR was significantly higher in the implanted group. As in pre-pubertal mammals FSH is known to plays a major role in the first wave of spermatogenesis [30, 35] but in pubertal mammals, spermatogenesis is mainly androgen-dependent and the effect of FSH is limited mainly to support the production of spermatogonia [30], the absence of sperm production observed in deslorelin-implanted cats therefore seems to result from the suppression of testosterone production due to the downregulation of LHR in the Leydig cells of deslorelin-treated cats.

In this study, we observed that faecal testosterone concentrations and testosterone-dependent sexual behaviour were both suppressed in deslorelin implanted compared with the non-implanted cats. We also found that the LHR protein expression in deslorelin implanted cats was suppressed compared with the non-implanted cats. As testosterone production depends on the activation of LHR in the Leydig cells, it therefore, seems highly likely that the observed suppression of faecal testosterone and the testosterone-dependent behaviours may be the result of observed testicular suppression of LHR protein in the implanted cats.

Both mRNA and protein expression of the LHR were studied in deslorelin-implanted and control cats. Deslorelin suppressed the LHR protein expression but was without any effect on the LHR mRNA. These results suggest that deslorelin downregulates the LHR by interfering at the translational level but do not interfere transcription of the gene. Surprisingly, no difference was observed in the protein or mRNA expression of LHR between the adult and pre-pubertal cats. This may indicate that at the age of 3 months cats already had active Leydig cells that are capable to produce testosterone. It is difficult to estimate the exact time period after which deslorelin might have been effective to suppress the LHR in the implanted cats because the testes were collected only 48 wk after the implantation. However, there was no difference in the faecal testosterone concentrations between the two groups at the time of implantation but from wk20 onwards testosterone concentrations in the implanted group started to be

significantly lower than the controls. This may suggest that the LHR suppression might have resulted sometimes within the 20 wks period of deslorelin implantation. However, it remains to be determined whether testosterone suppression in deslorelin-implanted cats was the result of LHR suppression only or was a result of the combined effect of suppression in the LH concentrations and LHR. Moreover, this study was not designed to investigate how the deslorelin implantation directly or indirectly might have affected the biosynthetic pathway of testosterone production that may involve changes in the cAMP to stimulate the transport of cholesterol into the mitochondria and changes in the activities of different enzymes responsible for the pregnenolone, progesterone, androstenone and finally testosterone production [29]. We speculate that the effect of deslorelin may not be only at the level of LH production and/or testicular LHR expression but may also be at other sites in the biosynthetic pathway of testosterone production. A suppression of pulsatile LH secretion from long-term GnRH-agonist (Goserelin) treatment has already been reported in gilts [15] and several male characteristics such as the presence of penile spines and male behaviours such as roaming, fighting and spraying are gonadal steroid hormones (especially testosterone) dependent and could be eliminated via the suppression of testosterone [27].

Normally GnRH-agonist implantation presents an upregulation effect in the first period after hormonal implantation followed by a downregulation effect after long-term administration in pubertal tomcats [5]. However, in this study we did not observe any upregulation effect when male cats were implanted with GnRH-agonist at the age of 3 months possibly because of an immature HPG axis at this age [17].

This study was not designed to investigate whether the observed suppression of the reproductive function could be reversed or not. However, non-reversibility of reproductive function has been reported in dogs that were implanted with GnRH agonist before the age of 4 months [16, 36]. Reversibility of reproductive function has been reported in studies using 1.6

mg deslorelin in post-natal cats and 4.7 mg deslorelin in 114 days old female cats, puberty was postponed until the age of 16 months and 134 to 286 days, respectively [17, 18] . However, in this study it remains to be seen whether reproductive function could be reinstated or not, nevertheless, the results obtained show that reproductive function remained suppressed for a period of at least 48 wks after 4.7 mg deslorelin administration at the age of 3 months.

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 wks suppresses the reproductive function for at least for 48 wks 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 Leydig cells while maintaining the FSHR expression at the pre-pubertal levels in the Sertoli cells of the testis.

5. Acknowledgement

This study was supported by the Royal Golden Jubilee Ph.D. programme, the 90th Aniversary of Chulalongkorn University (Ratchadaphiseksomphot Endowment Fund) and the Research Unit of Obstetric and Reproduction in animals of Chulalongkorn University, Bangkok, Thailand. We are thankful to Dr Zhangrui Cheng for laboratory assistance and Dr Em-on Olanratmanee for help in statistical analysis of the data.

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Table 1: Description of forward and reverse primers for GAPDH as housekeeping gene and feline LH receptor (LHR) and FSH receptor (FSHR) as target genes.

Gene	Primer sequence (5′–3′)	Length (bp)	Reference
GAPDH	F: GGAGAAAGCTGCCAAATATG	20	[25] and [26]
	R: AGGAAATGAGCTTGACAAAGTGG	23	
LHR	F: CTAATGCCTTTGACAACCTAATA	23	[25]
	R: CCCATTGAATGCATGACTTTGTA	23	
FSHR	F: CATGCTGCTAGGCTGGATCTT	21	[25]
	R: CTTGGCGATCTTGGTGTCACT	21	

Table 2: Mean ± SEM values for weight (g) of the testes and epididymides, seminiferous tubule diameter (μm), and grade of testicular tissues obtained from tomcats in both the experiments.

Expt	Groups	Weight (g)		Seminiferous tubule diameter (μm)	Testicular tissue grade
		Testes	Epididymides		
1	Implanted	0.09 ± 0.02^{a}	0.03 ± 0.01^{a}	62.02 ± 2.88^{a}	0.00 ± 0.00^{a}
	Non-implanted	1.54 ± 0.20^{b}	0.26 ± 0.03^{b}	98.48 ± 3.59^{b}	3.86 ± 0.17^{b}
2	Adult	1.24 ± 0.20x	0.24 ± 0.03^{x}	95.31 ± 3.94^{x}	3.83 ± 0.41^{x}
	Prepubertal	0.19 ± 0.04^{y}	0.08 ± 0.02^{y}	57.85 ± 8.50^{y}	1.33 ± 0.21^{y}

Values within a column with different superscript letters (a, b and x, y) in an experiment differ significantly ($P < 0.05$).

Figure legends

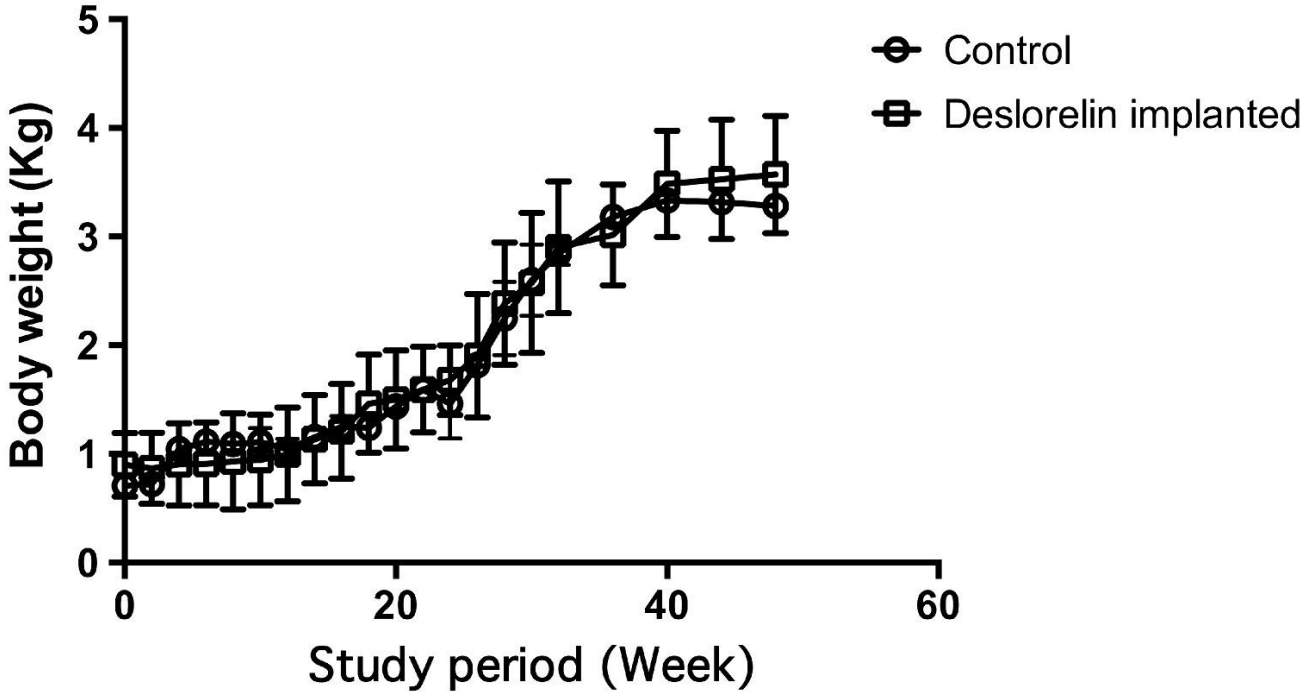
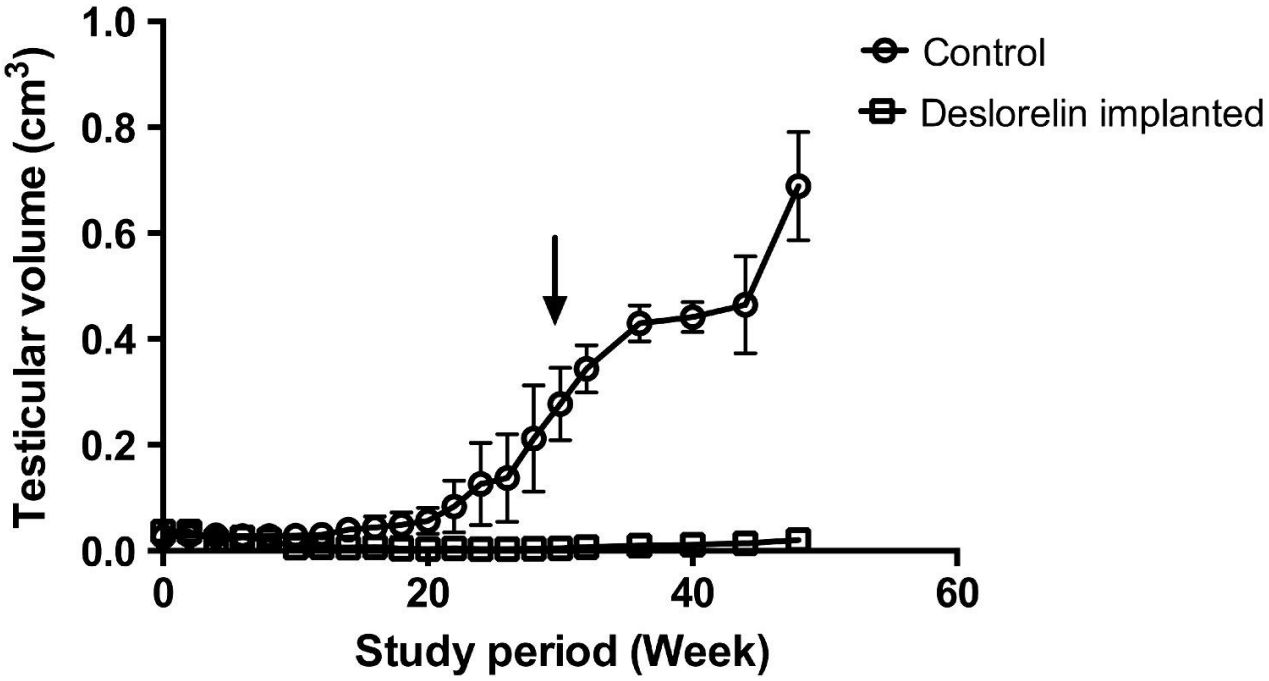


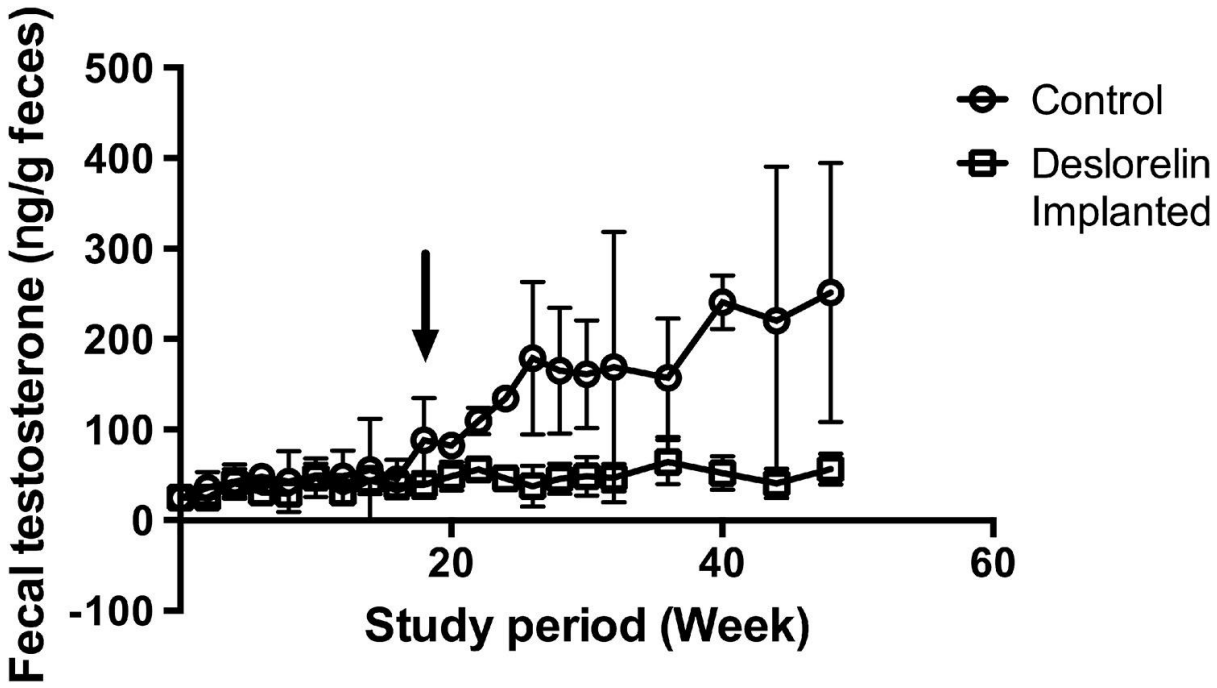
Figure 1: The mean (± SEM) body weight (kg) in cats (n=6/group) with or without deslorelin implantation. Cats were implanted with deslorelin at the age of 3 months for a period of 48 weeks.



503

504 **Figure 2:** The mean (± SEM) testicular volume (cm^3) in cats (n=6/group) with or without
505 deslorelin implantation. Cats were implanted with deslorelin at the age of 3 months for a
506 period of 48 weeks. Black arrow indicates the week that the control group has significantly
507 higher ($p < 0.05$) testicular volume than the implanted group.

508



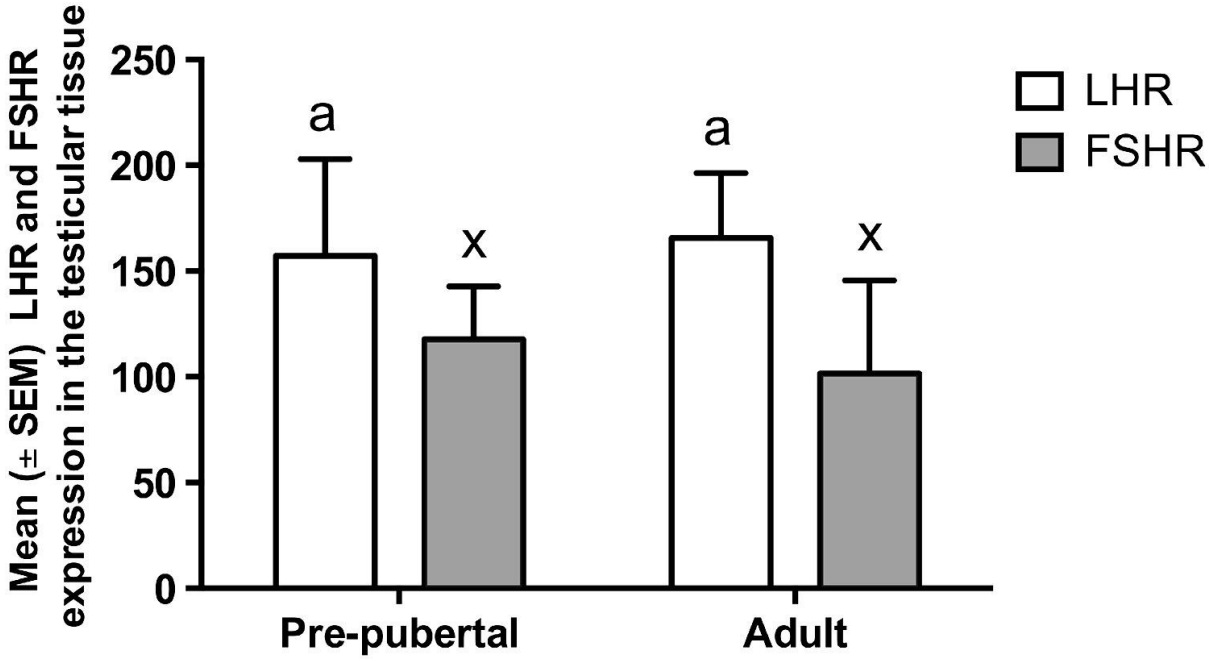
509

510 **Figure 3.** The mean (± SEM) faecal testosterone levels (ng/g of faeces) in cats (n=6/group)
511 with or without deslorelin implantation. Cats were implanted with deslorelin at the age of 3
512 months for a period of 48 weeks. Black arrow indicates the week when the control group
513 started to have significantly higher faecal testosterone levels than the implanted group ($P < 0.05$).

514

515

516



517

518 **Figure 4.** The mean expression index (± SEM) for LHR and FSHR in testicular tissue of

519 pubertal and pre-pubertal cats (n=6/group). Different letters on bars for a certain receptor

520 indicate significant differences ($P \leq 0.05$).

521

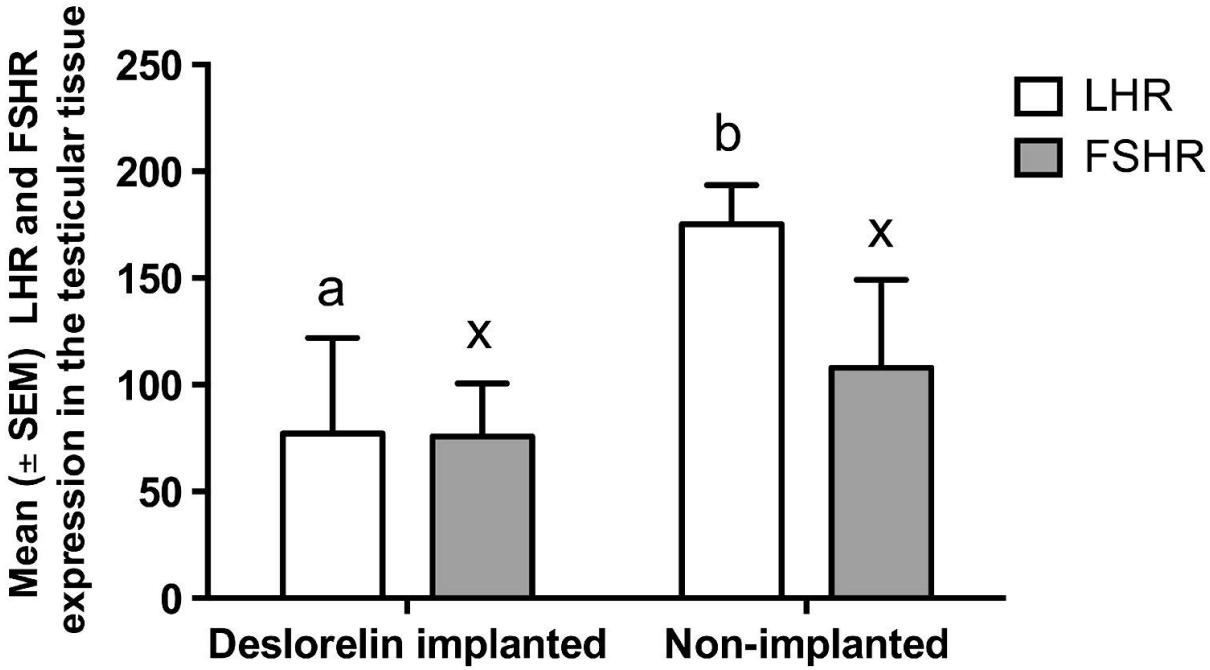


Figure 5. The mean expression index (± SEM) for LHR and FSHR in testicular tissue of cats (n=6/group) with or without deslorelin implantation. Cats were implanted with deslorelin at the age of 3 months for 48 weeks. Different letters on bars for a certain receptor indicate significant differences ($P \leq 0.05$).

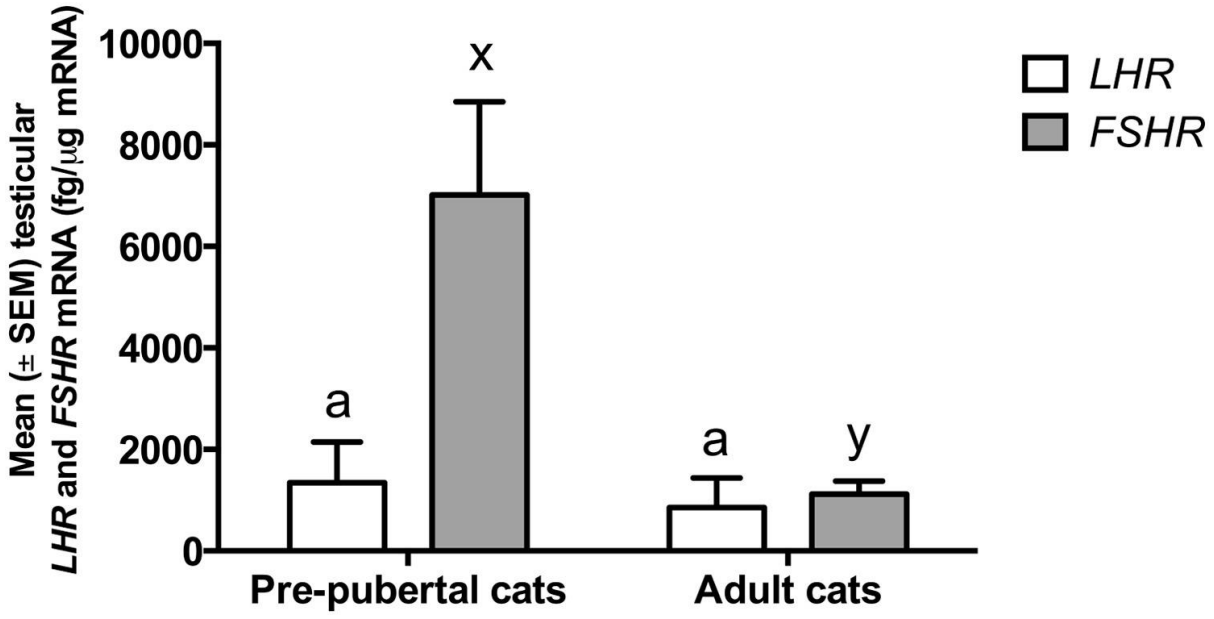
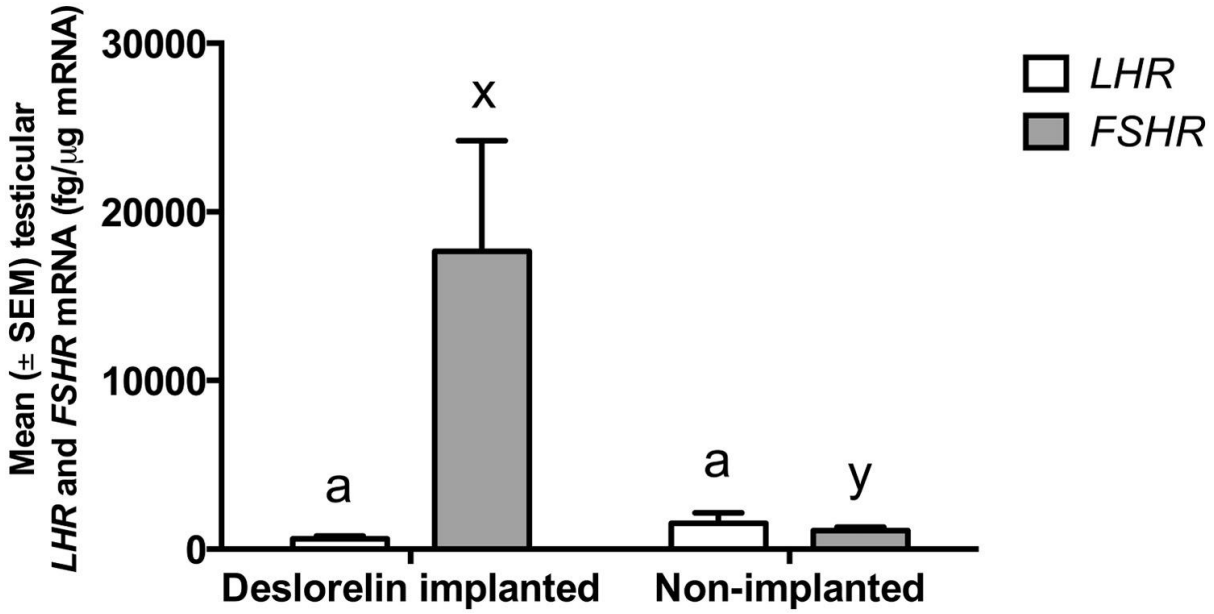


Figure 6. The mean mRNA concentration (fg/μg mRNA) (± SEM) for LHR and FSHR in testicular tissue of pubertal and pre-pubertal cats (n=6/group). Different letters on bars for a certain receptor indicate significant differences ($P \leq 0.05$).



536

537 **Figure 7.** The mean mRNA concentration (fg/μg mRNA) (± SEM) for LHR and FSHR in

538 testicular tissue of cats (n=6/group) with or without deslorelin implantation. Cats were

539 implanted with deslorelin at the age of 3 months for 48 weeks. Different letters on bars for a

540 certain receptor indicate significant differences ($P \leq 0.05$).

541