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Secretory pattern of insulin-like peptide 3 and its regulation in male ruminants

雄反芻動物におけるインスリン様ペプチド 3 の分泌パターンと その調節

M. A. HANNAN

2017年
Secretory pattern of insulin-like peptide 3 and its regulation in male ruminants

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A thesis submitted in fulfillment of the requirements for the degree of
Doctor of Philosophy (PhD)

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General Introduction

Insulin-like peptide 3 (INSL3), previously known as Leydig cell insulin-like factor or relaxin-like factor, was discovered in 1993 as a novel gene product from porcine Leydig cell (Adham et al., 1993). Later in 1999, its availability in the peripheral blood of human was identified (Büllesbach et al., 1999). Because of structural similarity with relaxin-insulin family members (insulin, insulin-like growth factors and relaxin), INSL3 is considered as a member of this family. Testicular Leydig cells have been shown as sole site of INSL3 production in all studied mammalian males including human (Ivell et al., 1997), canine (Klonisch et al., 2001; Hannan et al., 2015b), caprine (Hombach-Klonisch et al., 1999; Siqin et al., 2013; Pitia et al., 2015) and rodent (Balvers et al., 1998; Sadeghian et al., 2005); follicular theca cells and luteal cells have been shown as a production site of INSL3 in female counterpart (Bamberger et al., 1999; Irving-Rodgers et al., 2002). However, INSL3 level was shown very lower in the general circulation of females than that of males (Büllesbach et al., 1999; Bay et al., 2007), indicating this as a predominant circulating peptide hormone in males (Ivell and Anand-Ivell, 2009; Bay and Andersson, 2011).

The receptor of INSL3 through which it acts is a G protein-coupled receptor called relaxin family peptide receptor 2 (RXFP2; previously called LGR8 or Great) (Ivell and Bathgate, 2007). In human, the receptor RXFP2 is expressed on meiotic and post-meiotic germ cells and, to a lower extent, on Leydig cells, suggesting both autocrine and paracrine functions of the hormone (Anand-Ivell et al., 2006b). Similar expression patterns have been confirmed within the rodent testis (Feng et al., 2007). Beyond the gonads, RXFP2 mRNA and / or protein has been described in human gubernaculum,
epididymis, vas deferens, seminal vesicle, brain, kidney, muscle, thyroid, uterus, peripheral blood cells and bone marrow (Hsu et al., 2002; Anand-Ivell et al., 2006b; Filonzi et al., 2007). The expression of this receptor is reported in several non-gonadal tissues in mice (Feng et al., 2007) and dog (Arrighi et al., 2010) as well.

In mammals, testicular descent occurs in two phases, trans-abdominal and inguinoscrotal phase. The important role of INSL3 in completing the trans-abdominal phase of testicular descent is well established, Insl3 knockout mice exhibited a cryptorchid phenotype with both testes lying in an intra-abdominal perirenal position (Nef and Parada, 1999; Zimmermann et al., 1999; Sozubir et al., 2010). INSL3 was reported essential for the thickening of gubernacular ligament (“swelling reaction”) which facilitate the translocation of the testes from the perirenal position down to a position near the internal inguinal ring (Nef and Parada, 1999). The second step of testicular descent, inguino-scrotal phase, has traditionally been regarded as androgen-dependent process (Hutson et al., 1997), however, recent evidence have suggested role of INSL3/RXFP2 signaling in addition to androgen involvement (Yuan et al., 2010). INSL3 is suggested to play a paracrine role (anti-apoptotic factor) in germ cell survival (Kawamura et al., 2004), and an endocrine role in bone metabolism (Ferlin et al., 2008) other than its crucial function in testicular descent. Not much is known regarding the other functions of INSL3 despite identification of its receptor in many other organs of the body.

INSL3 and testosterone are the two main secretory products of testicular Leydig cells. It was well documented with frequent blood sampling in male rats (Steiner et al., 1984), male lambs (Foster et al., 1978), and bulls (Stumpf et al., 1993; Finnerty et al., 1998) that
the pulsatile release of GnRH from the hypothalamus acts on the anterior pituitary to induce pulsatile secretion of luteinizing hormone (LH), which in turn stimulates the pulsatile secretion of testosterone from the testicular Leydig cells. Testosterone secretion acts as a part of negative feedback to the hypothalamus and pituitary, and thereby maintains a tight regulation of hypothalamic-pituitary-gonadal (HPG) axis (Ivell et al., 2014). However, the secretory nature of INSL3 and its temporal relationships with LH and testosterone by frequent blood sampling is yet to investigate in any mammalian males.

INSL3 has been detected in the peripheral blood from a wide variety of male species, but there have been no clear information yet regarding the regulation of this hormone secretion. Previous studies on humans have suggested that INSL3 secretion is stimulated by the long term trophic effects mediated by LH and is dependent on the status of Leydig cells differentiation (Ivell et al., 1997; Bay et al., 2005; Foresta et al., 2004; Ferlin et al., 2006; Wikström et al., 2006), and it was believed that this hormone is constitutively regulated. Exogenous stimulation of LH, either by the gonadotropin releasing hormone (GnRH) or by the human chorionic gonadotropin (hCG, LH-like activity) administration, has confirmed a significant increase of testosterone in the general circulation of bulls (D’Occhio and Aspden, 1996; Mongkonpunya et al., 1975; Sundby et al., 1975; Murase et al., 1990), male goats (Saito et al., 2012) and rams (Falvo et al., 1975). In line with this, an increase in testosterone concentrations was also observed with a single injection of hCG (5000 IU) in men, while INSL3 stimulation was not observed for daily blood samples taken up to 8 days post treatment (Bay et al., 2006; Bay and Andersson, 2011). Interestingly, INSL3 concentration was increased significantly with the same dose (5000 IU).
IU, within 4 d) (Bay et al., 2006) or even very lower dose (60 IU, 10 d after) (Roth et al., 2013) of hCG treatments when suppression of the endogenous gonadotropins was induced first. Therefore, in men, the regulation of INSL3 secretion was thought different than testosterone. However, the regulation of INSL3 by exogenous and endogenous stimulations of LH or endogenous suppression of LH have not been studied in domestic animals.

Concentrations of INSL3 in peripheral blood have been used to evaluate the Leydig cell function during ageing (Anand-Ivell et al., 2006a), cryptorchidism (Bay et al., 2007), hypogonadotropic hypogonadism (Bay et al., 2005), obesity (Foresta et al., 2009) and diabetes mellitus (Ermetici et al., 2009). Moreover, INSL3 has been suggested as a testis-specific biomarker for assessing pubertal development in humans (Ferlin et al., 2006; Wikström et al., 2006; Johansen et al., 2014), bulls (Kawate et al., 2011), male dogs (Pathirana et al., 2012) and rodents (Anand-Ivell et al., 2009). It was shown that the dynamics of the secretory patterns of plasma INSL3 in bulls (Kawate et al., 2011) and male dogs (Pathirana et al., 2012) are different during pubertal development, although both the hormones are secreted from a unique source of testicular Leydig cells; conversely, in male rats (Anand-Ivell et al., 2009) and humans (Ferlin et al., 2006; Wikström et al., 2006; Johansen et al., 2014), a similar change of INSL3 in peripheral blood during puberty was reported. However, the INSL3 profiles during pubertal development in small ruminants are yet to be elucidated.

Age-related change in scrotal circumference is a good measure of puberty. This has been discussed in detail in bulls (Kastelic, 2014). An association between sexual maturation and scrotal circumference was also reported in crossbred and British breeds
of male goats (Bongso et al., 1982; Ahmad and Noakes, 1996). The changes in scrotal circumference and plasma concentrations of androgens with age have been shown in male Shiba goats (Tani et al., 1992). In boys, an association between INSL3 concentrations in peripheral blood and testicular volume with pubertal development was reported (Johansen et al., 2014). Correlations among INSL3, testosterone and LH in peripheral blood during pubertal development were described in humans (Ferlin et al., 2006) and bulls (Kawate et al., 2011). However, the association between testicular size and peripheral levels of INSL3 during pubertal development are yet to be elucidated in any domestic male species including goat.

In this thesis, the author presents a series of in vivo studies to unveil the secretory pattern and regulation of INSL3 in male ruminants. Frequent blood samplings at 15-min intervals were performed to depict the clear secretory profile of INSL3 and its interrelationships with LH and testosterone in bulls and male goats. The GnRH and hCG was administered to stimulate LH secretion to see its effect on INSL3 secretion. Moreover, GnRH antagonist was administered to suppress LH secretion to see its effect on INSL3 secretion. A longitudinal study was done to check the secretory profile of INSL3 during pubertal development and its association with scrotal circumference over the same period. The secretory pattern and regulation of INSL3 were investigated in bulls and male goats in chapter 1 and 2, respectively. In chapter 3, the effects of a long-acting GnRH antagonist on INSL3 and testosterone concentrations and scrotal circumference were studied in male goats. Finally in chapter 4, the changes of INSL3 and testosterone, and their association with scrotal circumference during and after puberty was investigated in male goats.
Chapter 1

Secretory pattern and regulation of insulin-like peptide 3 in bulls

Introduction

According to studies on humans, secretion of insulin-like peptide 3 (INSL3) is related to the differentiation status of testicular Leydig cells and is stimulated by the long-term trophic effects of luteinizing hormone (LH) (Ivell et al., 1997; Foresta et al., 2004; Bay et al., 2005; Ferlin et al., 2006; Wikström et al., 2006). However, the process of acute regulation of INSL3 secretion is mostly unknown. Detection of INSL3 in the peripheral blood of humans (Bay et al., 2005; Anand-Ivell et al., 2006a; Büllesbach et al., 1999), dogs (Pathirana et al., 2012), and cattle (Kawate et al., 2011) indicates that INSL3 may have additional endocrine effects in mammalian males. According to recent studies from author’s laboratory, the dynamics of the secretory patterns of INSL3 and testosterone in peripheral plasma during sexual development are different between male dogs (Pathirana et al., 2012) and beef bulls (Kawate et al., 2011), although both hormones are secreted from the unique source of testicular Leydig cells. It was well documented that in many species including cattle (Stumpf et al., 1993; Finnerty et al., 1998) secretion of LH occurred in a pulsatile manner stimulating testicular Leydig cells to produce pulsatile secretion of testosterone. However, under physiological conditions the pulsatile secretory pattern of INSL3 and its relation with LH have not been elucidated.

Endogenous LH increased by gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin (hCG), which possesses LH-like activity, caused a significant
increase of testosterone in the general circulation of bulls (D’Occhio and Aspden, 1996; Mongkonpunya et al., 1975; Sundby et al., 1975; Murase et al., 1990), male goats (Saito et al., 2012), and rams (Falvo et al., 1975). In men, serum testosterone concentrations increased after hCG treatment, while INSL3 concentrations did not change (Bay and Andersson, 2011). It remains unknown whether endogenous and exogenous LH can acutely regulate the secretion of INSL3 in domestic animals.

The objectives of this chapter were to determine the temporal relationship of pulsatile secretion among LH, INSL3 and testosterone, and to monitor acute regulation of INSL3 secretion by LH using GnRH analogue and hCG in pubertal beef bulls.
Materials and Methods

Animals

Japanese Black beef bulls (n = 6) raised in an experimental beef cattle station in the Northern Center of Agriculture Technology of Hyogo Prefecture in Japan were used for the present study. The selected beef bulls had no apparent abnormalities of the reproductive status and testicular presence was checked manually to confirm the presence of both testes inside the scrotum. Body weight and scrotal circumference of the bulls were recorded monthly from 6 to 23 months of age. These bulls remained normal in appearance and health during all experiments. Bulls were kept under natural light in an open shelter covered by a roof and were maintained by ad libitum hay and concentrate to meet Japanese Feeding Standard recommendations for the beef bulls. The following experiments using bulls were performed as a part of study on evaluation of fertility in Japanese Black beef bulls which was approved by a committee of General Technological Center of Hyogo Prefecture for Agriculture. The procedures of animal experiments complied with guidelines for Proper Conduct of Animal Experiment in Academic Research Institutions in Japan.

Experiment 1

Experiment 1 was done to determine the temporal relationship among INSL3, LH and testosterone at 15-minute intervals sampling for an 8 hour session in beef bulls (aged, 10–11 months; n = 6). Blood sampling for all bulls was started at 10:00 AM and ended at 6:00 PM. An indwelling jugular venous catheter (Argyle™ Covidien Ltd., Dublin, Ireland) was inserted about 1 hour before the beginning of sampling. No sedation was
performed before inserting the intravenous catheter and during sampling. Head restraint by either a stanchion or a halter was not used, except during insertion of the intravenous catheter. The bulls were given access to water and hay at every 2 to 3 hours during collection of the samples. Blood samples were collected into heparinized tubes and immediately placed in ice before centrifuging (1,700 × g for 15 minute at 4°C). The plasma was decanted and stored (−30°C) until assay.

Experiment 2

A single injection of GnRH analogue (fertirelin acetate; Conceral®, Intervet, Tokyo) was given intramuscularly at a dose of 0.5 µg/kg (aged, 11–12 months; n = 6). The same beef bulls that were used in Experiment 1 were used for Experiment 2, which took place at least 1 week after completion of Experiment 1. The blood samples for assaying INSL3, LH and testosterone were collected at −0.5, 0, 1, 2, 3, 4, 5, and 6-hour after treatment. The treatment was given immediately after the 0 hour sample was drawn. Thus, blood sampling taken at −0.5 and 0 hours are pre-treatment samples. Blood samples were collected into heparinized vacutainers by jugular venipuncture and processed as above mentioned in Experiment 1.

Experiment 3

Six beef bulls that were used for Experiments 1 and 2 were also used for Experiment 3. This experiment was conducted about 6 months after completion of Experiment 2 (aged, 18–19 months). A single dose of hCG (5 IU/kg, intramuscularly; Veterinary Puberogen®, Novartis Animal Health, Tokyo) was administered. Two pre-treatment blood samples were taken at −0.5 hour and immediate before the hCG treatment (0 hour).
The sampling was then continued at 2, 4 and 8-hour on the day of treatment (Day 0) and Days 1, 2, 4, 8 and 12 of the post-treatment. Blood collection and processing of plasma were done as mentioned above in Experiment 2.

**Hormone assays**

**Extraction procedure for INSL3**

The procedure for extracting INSL3 from bovine plasma was developed by modifying earlier procedures for extracting low molecular weight proteins or peptides from serum samples (Chertov et al., 2004; Merrell et al., 2004). First, 300 µL of 0.1% trifluoroacetic acid (TFA) was added to 125 µL of bovine plasma, immediately mixed by vortexing, and then left at room temperature. After 30 minute, 200 µL of acetonitrile was added into that mixture and left overnight at 4 °C temperature. Next, the mixture was centrifuged at 15,000 × g for 20 minute at room temperature. The resulting supernatant (500 µL) was concentrated by vacuum centrifugation (Centrifugal Concentrator CC-105; Tomy Seiko Inc., Tokyo, Japan) for approximately 2 hours to make the content completely dried. Then, 125 µL of 0.05 M phosphate buffer (pH 7.5) was added to the concentrated supernatant to be used for EIA.

**INSL3 assay**

Eight-well strips (Corning Inc. Life Sciences, Lowell, MA, USA) were coated with 100 µL/well of anti-mouse IgG rabbit polyclonal antibody (MP Biochemicals, Solon, OH, USA; 5 µg/mL in 0.05 M sodium bicarbonate, pH 9.7) for 2 hours at room temperature. The wells were then drained and washed three times with 300 µL of 0.15
M sodium chloride. Next, 200 µL of assay buffer I (0.01 M phosphate buffer containing 0.15 M sodium chloride, 2% BSA and 0.02% ProClin 950, pH 7.4) was added and kept overnight at 4 °C for blocking the wells. BSA and Proclin 950 were purchased from Sigma-Aldrich, St. Louis, MO.

Anti-bovine INSL3 mouse monoclonal antibody (2-8F) and synthetic bovine INSL3 have been described (Büllesbach and Schwabe, 2002). Bovine INSL3 standards were diluted with castrated bull plasma to create various concentrations (0.31–20 ng/mL). The INSL3 standards and plasma samples were extracted simultaneously using the above procedure. Immediately before the assay, the wells were drained, and 50 µL of extracted standards (bovine INSL3) or extracted samples was added, along with 50 µL of the anti-bovine INSL3 mouse monoclonal antibody (1:1,000,000 dilution in the assay buffer I). The mixture was kept at room temperature for 2 hours. After that, 50 µL of the biotinylated canine INSL3 (2 ng/mL in assay buffer I) was added and the mixture was incubated for further 1 hour at room temperature. After the reaction, the wells were drained and washed 3 times with 300 µL of wash buffer (0.15 M sodium chloride containing 0.05% Tween 20). Then, 100 µL of the HRP-labeled streptavidin (KPL, Gaithersburg, MD; 100 ng/mL in assay buffer I) was added to the wells, which were kept for 30 minutes at room temperature. The wells were drained, washed 3 times with wash buffer, and 100 µL of substrate solution containing 3,3’,5,5’-tetramethylbenzidine (TMB; St. Louis, MO, USA) was added. After 30 minutes incubation at room temperature, the reaction was stopped by adding 100 µL of 2 M sulfuric acid, and the optical density was measured at 450 nm reference, using a microplate absorbance spectrophotometer (xMark; Bio-Rad Laboratories Inc.,
Hercules, CA, USA). The minimum detection limit of the assay was 0.31 ng/mL and the detection was reliable in the range from 0.31 to 20 ng/mL. The intra- and inter-assay coefficients of variation (CV) were 7.5% and 13.7%, respectively.

Testosterone assay

An EIA procedure to measure testosterone concentrations in bovine plasma was established, based upon a method described previously for progesterone (Kawate et al., 1997, 2000). Microtiter strip wells (Costar, Corning, NY, USA) were coated with 100 μL of anti-rabbit IgG (MP Biochemicals, Solon, OH, USA; 5 μg/mL in 0.05 M sodium bicarbonate; pH 9.7), and non specific binding sites were blocked overnight with 200 μL of 0.1% BSA, 0.02% ProClin 950 in 0.01 M phosphate buffer containing 0.15 M sodium chloride, pH 7.4 (assay buffer T). Various concentrations of testosterone standards (0.07–20 ng/mL) were diluted with the assay buffer T. Then, 125 μL of standards or bovine plasma samples were extracted with 2 mL of diethylether and the dried extract was dissolved in 125 μL of the assay buffer T by vigorous vortexing. The wells were drained, and the following were added: 50 μL of extracted testosterone standards or extracted samples, 50 μL of HRP-labeled testosterone (Cosmo Bio Co. Tokyo, Japan; 1:1500 dilution in the assay buffer T), and 50 μL of anti-testosterone rabbit polyclonal antibody (Cosmo Bio Co. Tokyo, Japan; 1:100,0000 dilution in the assay buffer T). The mixture was then incubated for 2 hours at room temperature. After the reaction, the wells were drained and washed three times with 300 μL of wash buffer. Further steps were the same as mentioned above in the INSL3 EIA. The minimum detection limit of the assay was 0.07 ng/mL. The intra- and inter-assay CVs were 6.6%
and 11.3%, respectively.

**LH assay**

Eight-well strips (Corning Inc. Life Sciences, Lowell, MA, USA) were coated with 100 µL per well of anti-rabbit IgG mouse polyclonal antibody (MP Biochemicals, Solon, OH, USA; 5 µg/mL in 0.05 M sodium bicarbonate, pH 9.7) for 2 hours at room temperature. The wells were then drained and washed three times with 300 µL of 0.15 M sodium chloride. Next, 200 µL of assay buffer (0.01M PBS, pH 7.4) supplemented with 2% bovine serum albumin (BSA; Cohn Fraction V, Sigma-Aldrich, St. Louis, MO), and 0.02% ProClin 950 (Sigma-Aldrich) was added and kept overnight at 4°C to block areas of the well that were not coated with antibody. Various concentrations of bovine LH standards (AFP11743B, NIDDK, USA; 0.31 to 40 ng/mL) were diluted with assay buffer. The plasma was centrifuged at 15,000 × g for 5 minutes at 4°C to sediment fibrin and other particles, and then supernatant was collected and diluted 2-fold with assay buffer. Immediately before the assay, the wells were drained, and 50 µL of standards or plasma samples followed by 50 µL of the anti-bovine LH antibody (Immunodiagnostik AG, Bensheim, Germany; 1: 50,000 dilution in assay buffer) were added and incubated for 2 hours while shaking (180 rpm). Thereafter, 50 µL of the biotinylated bovine LH was added (1: 50,000 dilution in assay buffer) and incubated for 1 hour. The LH (AFP11743B) was biotinylated with EZ-Link NHS-PEG₄-Biotin (Thermo Fisher Scientific, Waltham, MA USA). After the reaction, the wells were drained and washed three times with 300 µL of washing buffer (0.15 M sodium chloride containing 0.05% Tween 20). Then, 100 µL of the HRP-labeled streptavidin (KPL, Gaithersburg, MD; 100
ng/mL in assay buffer) was added to the wells and incubated for 30 minutes. The wells were then again washed three times with saline containing 0.05% Tween 20 and incubated for another 30 minutes at room temperature with 100 μL substrate solution containing 3,3’,5,5’-tetramethylbenzidine (TMB; St. Louis, MO, USA). The reaction was stopped by adding 100 μL of 2 M sulfuric acid, and the optical density was measured at 450 nm using an xMark microplate absorbance spectrophotometer (Bio-Rad Laboratories). The assay detection range was from 0.31 to 40 ng/mL. The intra- and inter-assay coefficients of variation were 4.0% and 10.7%, respectively.

**Pulse detection**

Pulses of LH, INSL3 and testosterone concentrations in plasma samples at 15-minute intervals during 8 hours were detected with Pulse XP software kindly provided by Prof. Michael L. Johnson, University of Virginia (Johnson et al., 2008). Basal concentrations of INSL3 and testosterone pulses were also determined with this software. Moreover, the increase (peak/basal concentration) of INSL3 and testosterone pulses was calculated from 15-minute interval sampling. In brief, first hormonal raw data followed by standard error (SE), sampling time point (eg, 0, 15, 30, 45-minute) and duplicate number of each sampling point used in EIA were arranged in the Excel sheet. The name of the Excel file must be ended with “.fix” (eg. Bull ID 2.fix). Thereafter, the Pulse XP software was opened and “Fix Mode” located in the File menu within the “Read a Data File” was clicked, and after that the previously selected file was opened. A new page came out with a figure based on the input raw data. Then, within the Algorithm menu, the best Algorithm type and other set-ups (eg, half-life, Bandpass filter S.D) were selected and maintained the same parameters for
detecting pulses of a specific hormone for all the animals. After that, upon clicking on “calculate”, detected peaks of pulses were shown on the figure. Next, by clicking on the “view results”, a world page showed the details of exact time points where the predicted peaks are located.

Statistical analysis

Analysis of variance (ANOVA) using the Generalized Estimating Equations (GEE) procedure of SPSS version 22 software (IBM, Somers, NY) was used to determine the effects of age on body weight and scrotal circumference. The average concentration of pre-treatment samples (–0.5 and 0 hours) were considered as control for GnRH and hCG treated data analyses. In addition, the increase (maximum /pre-treatment concentration) of INSL3 and testosterone concentrations after administration of GnRH and hCG was calculated. Plasma concentrations of LH, INSL3 and testosterone, and the effect of time after GnRH and hCG treatments were performed by same ANOVA mentioned above. Mean hormonal concentrations after GnRH and hCG treatments were compared with those of the control (pre-treatment) using pairwise comparisons by the least significant difference (LSD) post hoc test. Data were expressed as mean ± SEM. Differences were considered significant at P<0.05.
Results

Changes of scrotal circumference and body weight in bulls

There were age effects (P<0.001) on body weight and scrotal circumference during 6 to 23 months of age in bulls (Fig. 1-1). There was a clear increase of scrotal circumference from 6 months until around 16 months.

Changes of plasma LH, INSL3 and testosterone in blood samples taken at 15-min intervals for 8 h in bulls

INSL3 concentrations in the general circulation of beef bulls was also pulsatile, apart from the known pulsatile secretion of LH and testosterone (Fig.1-2). In six beef bulls, during the 8-hour, a total of 28, 23 and 6 pulses occurred for LH, INSL3 and testosterone, respectively. Of the 23 INSL3 pulses, 16 (69.6%) pulses peaked within 1 hour after a peak of an LH pulse. In five bulls, testosterone concentrations started increasing within 30 minutes from a peak of an LH pulse. In case of the remaining bull (Fig. 1-2, B#2), the author was unable to detect the beginning of the testosterone pulse. In this case the testosterone pulse might have started before sampling and therefore the author was unable to detect the LH pulse that induced the testosterone pulse. The frequency of LH, INSL3 and testosterone pulses during an 8-hour session was 4.7 ± 0.9, 3.8 ± 0.2 and 1.0 ± 0.0, respectively. The mean increase (peak/basal concentration) of testosterone pulses (12.9 ± 2.0 fold, n = 6) was higher (P<0.001) than those of INSL3 pulses (1.5 ± 0.1 fold, n = 23).
Changes of plasma LH, INSL3 and testosterone in response to GnRH treatment in bulls

There was an effect of time on plasma LH, INSL3 and testosterone concentrations after GnRH treatment in bulls (P<0.001). Mean plasma LH concentrations increased (P<0.01) dramatically 1 hour after treatment and reached a maximum concentration at 2-hour (Fig. 1-3A). Thereafter, the concentration slowly decreased but remained high (P<0.05) until the end of sampling at 6-hour. Mean plasma INSL3 concentrations increased (P<0.01) 1 hour after the GnRH treatment and remained high until 2-hour (P<0.05); from 3 to 4 hours INSL3 concentrations did not differ significantly when compared with the pretreatment value. However, an increase (P<0.05) of INSL3 concentrations was again observed at 5 and 6 hours after treatment (Fig. 1-3B). After GnRH treatment, mean plasma testosterone concentrations were increased (P<0.01) at all time points when compared with the pre-treatment value. Testosterone concentrations rose at 1 hour post-treatment and remained high until the end of sampling at 6-hour (Fig. 1-3C). The mean increase (maximum/pre-treatment concentration) of testosterone concentrations (7.6 ± 2.2-fold, n = 6) after administration of GnRH analogue was higher (P<0.01) than that of INSL3 (1.6 ± 0.1-fold, n = 6).

Changes of plasma LH, INSL3 and testosterone in response to hCG treatment in bulls

There was an effect of time on plasma INSL3 and testosterone concentrations after hCG treatment in bulls (P<0.001). Mean plasma INSL3 and testosterone concentrations after hCG treatment were presented in Fig. 1-4. Plasma INSL3 concentrations increased (P<0.01) 2-hour after treatment and remained high (P<0.05)
until the next sampling at 4-hour. When compared to control no significant changes occurred at 8-hour and Day 1 after treatment. However, INSL3 concentrations again increased on Days 2 through 8 (Day 2, P<0.01; Day 4, P<0.01; Day 8, P<0.05), approaching pre-treatment concentration on Day 12 (Fig. 1-4). A dramatic increase (P<0.01) of mean plasma testosterone concentrations after hCG treatment was observed from 2-hour and continued until Day 4. Thereafter, concentrations started to decrease but remained elevated (P<0.01) until Day 8, reaching basal level on Day 12 post-treatment (Fig. 1-4). The mean increase (maximum/pre-treatment concentration) of testosterone concentrations (9.7 ± 2.0-fold, n = 6) was higher (P<0.001) than that of INSL3 (1.7 ± 0.2-fold, n = 6).
Fig. 1-1. Body weight and scrotal circumference change in bulls from 6 to 23 months of age. Data are expressed as mean ± SEM (n = 6). P < 0.001 was overall age effects for body weight and scrotal circumference.
Fig. 1-2. Changes of plasma LH, INSL3 and testosterone concentrations in blood samples taken from two individual representative beef bulls (A: #1, B: #2). Blood samples were taken at 15-minute intervals for 8 hours. The peaks for INSL3 (▼), LH (►), and testosterone (○) pulses were determined by the Pulse XP software.
Fig. 1-3A. Plasma LH concentrations in response to GnRH treatment (0.5 µg/kg) in beef bulls. Data are expressed as mean ± SEM (n = 6). *P <0.05, **P <0.01 compared with the control (average of -0.5 and 0 hours of treatment).
Fig. 1-3B. Plasma INSL3 concentrations in response to GnRH treatment (0.5 µg/kg) in beef bulls. Data are expressed as mean ± SEM (n = 6). *P < 0.05, **P <0.01 compared with the control (average of -0.5 and 0 hours of treatment).
Fig. 1-3C. Plasma testosterone concentrations in response to GnRH treatment (0.5 µg/kg) in beef bulls. Data are expressed as mean ± SEM (n = 6). **P <0.01 compared with the control (average of -0.5 and 0 hours of treatment).
Fig. 1-4. Plasma INSL3 (A), and testosterone (B) concentrations in response to hCG treatment (5 IU/kg) in beef bulls. Data are expressed as mean ± SEM (n = 6). *P <0.05, **P <0.01 compared with the control (average of -0.5 and 0 hours of treatment) of the corresponding hormone.
**Discussion**

The present study was apparently the first to evaluate the secretory pattern of INSL3 at 15-minute intervals sampling in bulls. The nature of releasing INSL3 from the testicular Leydig cells into the general circulation was pulsatile with an average pulse frequency of about 4 in an 8-hour sampling session, and a temporal relationship between LH and INSL3 secretion existed. The increase of INSL3 pulses was much smaller than that of testosterone pulses.

The frequency of testosterone pulses in the present study was in accordance with the previous reports (Stumpf et al., 1993; Finnerty et al., 1998) in bulls. It was reported that LH pulses precede testosterone pulses in bulls (Stumpf et al., 1993), which has been the case not only for testosterone pulses but also for INSL3 pulses as shown in the present study. The author noticed that 70% of INSL3 pulses peaked within 1 hour after the peak of an LH pulse, indicating that in most cases INSL3 pulses were associated with LH pulses. The fewer number of testosterone pulses compared with LH pulses in an 8-hour sampling session demonstrated that not all LH pulses were capable of generating a testosterone pulse, and therefore, there might be a minimum threshold value for an LH pulse to initiate a testosterone pulse, whereas in case of INSL3 pulses, it seemed that compared with testosterone pulses a comparatively lower minimum threshold value of LH pulses is required.

Upon treatment with GnRH, the author noticed that similar to LH and testosterone, INSL3 concentrations also increased significantly within 1 hour. The increase (maximum/pre-treatment concentration) by GnRH stimulation was much lower for INSL3 than for testosterone. A similar lower increasing of INSL3 pulses than
testosterone pulses occurred under normal physiological condition in Experiment 1 with 15-minute intervals sampling. The significant increase of INSL3 concentrations within 1 hour after GnRH treatment in Experiment 2 suggested that the INSL3 secretion was acutely regulated by LH. Administration of hCG, which has LH-activity, provided additional evidence regarding this issue. After hCG treatment, a significant increase of both testosterone and INSL3 concentrations occurred that sustained over a longer interval. For both hormones, the concentrations increased shortly after treatment, remained high until Day 8, but again the increasing concentrations were much smaller for INSL3 than for testosterone. A significantly higher concentration of INSL3 and testosterone for a longer interval by hCG than GnRH treatment was probably due to the sustained longer activity of hCG (Boime and Ben-Menahem, 1999). Previously, a significant increase of testosterone in the general circulation of bulls was reported after GnRH and hCG treatments (D’Occhio and Aspden, 1996; Mongkonpunya et al., 1975; Sundby et al., 1975; Murase et al., 1990). In the present study, the simultaneous increase of INSL3 and testosterone concentrations within 1 to 2-hour after those treatments, provided another new information that LH acutely regulated the secretion of INSL3 in bull plasma. This acute regulation of INSL3 by LH in bulls was the novel finding of this present study and was in difference to previous studies in men. Bay and Andersson (2011) showed that when men were treated with hCG and peripheral blood was taken daily for 8 days, testosterone concentrations increased after hCG treatment, but INSL3 did not change, whereas other studies showed that hCG can increase INSL3 concentrations in blood after 4 or 10 days of treatment when endogenous LH secretion was inhibited by androgen analogues or GnRH antagonist (Bay et al., 2006; Roth et al., 2013).
In conclusion, the secretion of INSL3 in the general circulation of bulls occurred in a pulsatile manner. INSL3 secretion was acutely regulated by LH because plasma INSL3 concentrations increased soon after endogenous LH increase and exogenous LH stimulation. The increase of INSL3 concentrations during normal pulsatile secretion and after LH stimulation was much lower than that of testosterone.
Summary

The present study was undertaken in pubertal beef bulls to determine the temporal relationship of pulsatile secretion among LH, insulin-like peptide 3 (INSL3) and testosterone, and to monitor acute regulation of INSL3 secretion by LH using GnRH analogue and hCG. The secretion of LH, INSL3 and testosterone in the general circulation was pulsatile. The frequency of LH, INSL3 and testosterone pulses was 4.7 ± 0.9, 3.8 ± 0.2 and 1.0 ± 0.0, respectively, during the 8 hours period. Seventy percent of these INSL3 pulses peaked within 1 hour after a peak of an LH pulse had occurred. The mean increase (peak/basal concentration) of testosterone pulses was higher (P<0.001) than those of INSL3 pulses. After GnRH treatment, LH concentrations increased (P<0.01) dramatically 1 hour post-treatment and remained high (P<0.05) until the end of sampling, whereas an elevated (P<0.05) INSL3 concentration occurred at 1 and 2-hour after the treatment. Testosterone concentrations increased (P<0.01) 1 hour after the treatment and remained high until the end of sampling. After hCG treatment, an increase of INSL3 concentration occurred at 2 and 4-hour, and Days 2, 4 and 8 after treatment (P<0.05), whereas in case of testosterone, concentrations remained high (P<0.01) until Day 8 after treatment. The increase (maximum/pre-treatment concentration) of INSL3 concentrations after injecting GnRH or hCG was much lower (P<0.001) than that of testosterone. In conclusion, secretion of INSL3 in blood of bulls occurred in a pulsatile manner. The author inferred an acute regulation of INSL3 by LH in bulls because INSL3 concentrations increased immediately after endogenous and exogenous LH stimulation. The increase of INSL3 concentrations by LH was much lower than that of testosterone in bulls.
Chapter 2
Secretory pattern and regulation of insulin-like peptide 3 in male goats

Introduction

Insulin-like peptide 3 (INSL3) is a main secretory product of testicular Leydig cells in all studied mammalian species including male goats (Ivell et al., 1997; Adham et al., 1993; Siqin et al., 2013). The known major roles of INSL3 in male reproduction are to regulate the endocrine effect during embryonic development in completing the trans-abdominal phase of testicular descent in mice (Nef and Parada, 1999; Zimmermann et al., 1999), and to suppress germ cell apoptosis, a postnatal paracrine function observed in rats (Kawamura et al., 2004). INSL3 is readily detectable in the peripheral blood of a wide variety of male species including humans (Bay et al., 2005; Anand-Ivell et al., 2006a; Büllesbach et al., 1999), dogs (Pathirana et al., 2012), and cattle (Kawate et al., 2011), thus, raising the possibility of additional endocrine roles in mammalian males. Previous studies on humans have shown that INSL3 secretion is stimulated by long term trophic effects mediated by LH and dependent on the status of Leydig cell differentiation (Ivell et al., 1997; Bay et al., 2005; Foresta et al., 2004; Ferlin et al., 2006; Wikström et al., 2006). However, studies in farm species on the regulatory mechanism of INSL3 secretion, and its association with LH and testosterone have been limited. The studies described in chapter 1 demonstrated that the nature of plasma INSL3 secretion is pulsatile and is highly associated with pulsatile LH secretion in Japanese Black beef bulls (Hannan et al., 2015a). Moreover, it was previously reported from the author’s laboratory that plasma INSL3 and testosterone are secreted in a different fashion during the sexual
development in male dogs (Pathirana et al., 2012) and beef bulls (Kawate et al., 2011),
despite their common cellular source of secretion. Plasma concentrations of INSL3 in
male goats are yet to be measured. The episodic secretory pattern of LH associated with
subsequent rises in testosterone in peripheral blood have been well documented in many
male species including goats (Howland et al., 1985), however, under physiological
conditions the secretory pattern of INSL3 and its relation with LH have not been
elucidated in male goats.

Elevated LH generated by administration of GnRH or hCG (LH-like activity)
resulted in a significant increase of plasma testosterone concentrations in bulls
(D’Occhio and Aspden, 1996; Mongkonpunya et al., 1975; Sundby et al., 1975; Murase
et al., 1990), rams (Falvo et al., 1975), and male goats (Samir et al., 2015; Saito et al.,
2012). It was also shown in chapter 1 that in addition to testosterone, plasma
concentrations of INSL3 increased significantly soon after these treatments in bulls
(Hannan et al., 2015a). In men, in contrast, plasma concentrations of INSL3, not
testosterone, remained unchanged when peripheral blood was taken daily for 8 days after
hCG treatment (Bay and Andersson, 2011). Such species-specific secretion profiles may
reflect different physiological outcomes. To clarify which mechanism prevails in other
farmed animals, here the author investigated the acute regulation of plasma INSL3
concentrations by endogenous and exogenous LH in male goats.

This study was performed to check the acute regulation of plasma INSL3 secretion by
LH, and the secretory pattern of INSL3, as well as its temporal relationships with LH and
testosterone in male goats. The hypothesis was that LH would acutely regulate the plasma
INSL3 secretion in male goats as was reported in chapter 1 for bulls.
Materials and Methods

Animals

Six sexually matured male Shiba goats housed in an experimental room throughout the study at Osaka Prefecture University were used. Shiba goat, a Japanese miniature breed, shows year-round breeding and becomes sexually mature at about 4 months of age (Kano and Mori, 1982). The selected goats had no apparent abnormalities of the reproductive status and testicular presence was checked manually to confirm the presence of both testes inside the scrotum. These goats remained normal in appearance and health during all experiments. Goats were fed concentrates and hay, with water ad libitum. The following experiments were performed in accordance with the Guidelines for Animal Experimentation of Osaka Prefecture University as approved by the Animal Experiment Committee of Osaka Prefecture University.

Experiment 1

A single injection of GnRH analogue (fertirelin acetate; Conceral®; Intervet, Tokyo) was given intra-venously at a dose of 1.25 µg/kg. The goats were 8–10 months of age with a body weight of 14.8 ± 1.9 kg (n = 6). Blood samples were collected at −15 and 0 minutes and then every 15 minutes until 120 minutes followed by hourly collection until 8 hours post-treatment. The treatment was given immediately after the 0 minute sample was drawn. Thus, blood sampling taken at −15 and 0 minutes were pre-treatment samples. Blood samples were collected into heparinized syringes by jugular venipuncture and immediately transferred into glass tubes and placed in ice before centrifugation (1,700 × g for 20 minutes at 4°C). The plasma was collected and immediately stored at −30°C.
Samples were thawed just before LH, INSL3, and testosterone assays.

Experiment 2

A single dose of hCG (5 IU/kg, intravenous; Veterinary Puberogen®, Novartis Animal Health, Tokyo) was administered to monitor the effects of exogenous LH on INSL3 and testosterone secretions. The same goats (aged, 9–11 months; body weight, 16.0 ± 1.8 kg; n = 6) that were used in Experiment 1 were used for Experiment 2, which took place at least 1 month after completion of Experiment 1. Two pre-treatment blood samples were taken at 15-minute and immediately before the hCG treatment (0 minute). The sampling was then continued every 15 minutes until 120 minutes, thereafter hourly for up to 8 hours, followed by daily sampling until Day 8, and lastly on Day 12 post-treatment. Blood collection and processing were done as described in Experiment 1.

Experiment 3

Experiment 3 was done to determine the temporal relationships among INSL3, LH and testosterone at 15-minute intervals sampling for an 8-hour session. The same goats that were used for Experiments 1 and 2 were also used for Experiment 3 (aged, 13–16 months; body weight, 19.7 ± 2.3 kg; n = 6). This experiment was conducted about 4 months after completion of Experiment 2 in each goat. The scrotal circumference of goats was recorded on the same day when blood was drawn for Experiment 3. Blood sampling for all goats was started at 10 AM and ended at 6 PM. An indwelling jugular venous catheter (Argyle; Covidien Ltd., Dublin, Ireland) was inserted about 1 hour before the beginning of sampling. Blood samples were collected into heparinized syringes and
processed as outlined in Experiment 1. The animals were not sedated before inserting the intravenous catheter nor during sampling. The goats were given access to water and hay every 2 to 3 hours during the time of blood collection.

**Hormone determinations**

Plasma concentrations of INSL3 were measured using an EIA. A homologous bovine plasma EIA developed and validated in the author’s laboratory (Hannan et al., 2015a) was used. The procedures have been described in details in chapter 1. An anti-bovine INSL3 mouse monoclonal antibody (2-8F) and synthetic bovine INSL3 (Büllesbach and Schwabe, 2002) for standard in combination with biotinylated canine INSL3 were employed as described in chapter 1. Preliminary experiments showed cross-reactivity of this antibody with plasma of male goats. Amino acid sequence of the bovine INSL3 antigen and the corresponding caprine INSL3 sequence are 94.9 % identical (three out of 59 amino acids are different) (Siqin at al., 2013; Büllesbach and Schwabe, 2002). The minimum detection limit of the INSL3 EIA was 0.31 ng/mL, and the detection was reliable in the range from 0.31 to 20 ng/mL. The intra-assay and interassay coefficient of variation (CVs) were 5.9% and 15.1%, respectively. Plasma INSL3-levels in castrated goats were below the detection limit, whereas very high concentrations of INSL3 were detected in intact goats. To keep the concentrations within the range of standards, the sample plasma was diluted 20-fold using 0.05 M phosphate buffer (pH 7.5) before extracting INSL3.

Plasma testosterone concentrations were determined by an EIA using the procedure previously described in chapter 1 for bulls. An anti-testosterone rabbit polyclonal
antibody and horseradish peroxidase (HRP)-labeled testosterone (Cosmo Bio Co., Ltd., Tokyo) were used. The minimum detection limit was 0.07 ng/mL, and the reliable detection range for testosterone EIA was 0.07 to 20 ng/mL. The intra-assay and interassay CVs were 4.1% and 15.8%, respectively.

The EIA procedure used to measure LH in bull plasma as described in chapter 1 was also used for LH in goat plasma except that an anti-ovine β LH antibody (NIDDK-anti-ovine LH Beta-1; 1: 16,000 dilution in assay buffer) was used instead of anti-bovine LH. The assay detection range was from 0.31 to 40 ng/mL. The intra-assay and interassay CVs were 7.1% and 9.2%, respectively.

**Pulse detection**

Pulses of LH, INSL3 and testosterone concentrations in plasma samples taken at 15-min intervals during 8 h in male goat were detected with Pulse XP software kindly provided by Prof. Michael L. Johnson, University of Virginia (Johnson et al., 2008). Basal concentrations of INSL3 and testosterone pulses, and the increase (peak/basal concentration) of those hormones were also determined with the Pulse XP software. The procedure has been described in chapter 1.

**Statistical analysis**

The time-dependent effects after GnRH and hCG treatments were determined by ANOVA using the GEE procedure of SPSS version 22 software (IBM, Somers, NY). The average concentration of pre-treatment samples (−15 and 0 minutes) was used as controls and the increase (maximum per pre-treatment concentration) of INSL3 and
testosterone concentrations after administration of GnRH analogue and hCG was calculated. Mean hormonal concentrations after GnRH and hCG treatments were compared with those of the control (pre-treatment) using paired t-test. The difference of mean scrotal circumference per body weight, and mean plasma INSL3 and testosterone concentrations during 8 hours sampling between male goats and bulls was determined by a one-way ANOVA using the Generalized Linear Models (GLM) procedure of SPSS version 22 software (IBM, Somers, NY). The data of bulls were used from chapter 1. Data were expressed as mean ± standard error of the mean. Differences were considered significant at P<0.05.
Results

After GnRH treatment of male goats a significant time-dependent increase of plasma LH, INSL3 and testosterone concentrations (P<0.001) were observed. Mean plasma LH concentrations started to increase after 30 minutes of treatment and peaked at 105 minutes (Fig. 2-1A). Thereafter, the concentrations started to decrease but remained elevated until 120 minutes post-treatment (45, 60, 90, and 105 minutes, P<0.05; 30, 75 and 120 minutes, P<0.01). Mean plasma INSL3 concentrations also increased 30 minutes after treatment compared to control and remained high until 75 minutes (P<0.05; Fig. 2-1B). Thereafter, concentrations slowly approached basal level. The time-dependent mean plasma testosterone concentrations showed a significant increase at 30 minutes after GnRH treatment and rose quickly to maximum concentration at 60 minutes. Thereafter, concentrations started to decrease slowly and remained high compared with the pre-treatment concentration until 4 hours (30, 45, 120 minutes, and 3 and 4 hours, P<0.05; 60 minutes to 105 minutes, P<0.01). Testosterone concentrations approached basal level at 5 hours and continued until the end of sampling (Fig. 2-1C). The mean increase (maximum per pre-treatment concentration) of INSL3 concentrations (2.5 ± 0.4 fold, n = 6) was lower (P<0.01) than that of testosterone (7.8 ± 2.1 fold, n = 6).

After administration of hCG, the time-dependent effect of the mean plasma INSL3 and testosterone concentrations (P<0.001) are shown in Fig. 2-2AB. Significant increase of INSL3 plasma concentrations was first observed after 30 minutes and continued until 8 hours. For all time points, except at 60 minutes and 4 hours, the increase was statistically significant. From Day 1 INSL3 plasma levels showed a dramatic increase over control reaching the maximum concentration on Day 4, thereafter, concentrations
declined but remained significantly elevated until the end of sampling on Day 12 (75 to 120 minutes, and 5, 7 and 8 hours, and Days 1, 2, 4 to 6 and 12, P<0.05; 30 and 45 minutes, and 3 and 6 hours, and Days 3, 7 and 8, P<0.01) (Fig. 2-2A).

After hCG administration time-dependent changes of the mean plasma testosterone concentrations occurred in two phases. The onset of the first phase occurred at 15 minutes, thereafter testosterone concentrations increased dramatically up to 45 minutes, maintained at the same level until 75 minutes and then slowly decreased before reaching a plateau at the 4-hour time point. The onset of the second phase occurred at Day 1 and peaked on Day 3 before declining. Compared with control testosterone levels remained significantly elevated for all time points between 15 minutes and 6 days (Days 4 to 6, P<0.05; 15 minutes to Day 3, P<0.01) (Fig. 2-2B). The mean increase (maximum per pretreatment concentration) of INSL3 concentrations (4.6 ± 1.3-fold, n = 6) was lower (P<0.01) than that of testosterone (12.1 ± 2.3-fold, n = 6).

Fig. 2-3 shows two examples of plasma LH, INSL3 and testosterone concentrations at 15-minute intervals. The plasma secretory pattern of all 3 hormones was pulsatile, and a total of 33, 28 and 13 pulses were recorded for an 8-hour sampling session for LH, INSL3 and testosterone, respectively. Twenty out of 28 (71%) of these INSL3 pulses peaked within 1 hour after a peak of an LH pulse. Testosterone pulses were noticed within 30 minutes from a peak of an LH pulse in 11 out of 13 (85%) testosterone pulses. The magnitude of the remaining two testosterone pulses was very low and no defined LH pulse preceding those testosterone pulses could be identified. The frequency of pulses for LH, INSL3 and testosterone during an 8-hour session was 5.5 ± 0.6, 4.7 ± 0.5 and 2.2 ± 0.5, respectively. The mean increase (peak per basal concentration) of INSL3 pulses
(2.1 ± 0.1 fold, n = 28) was lower (P<0.01) than that of testosterone pulses (4.3 ± 2.2 fold, n = 13).

Table 2-1 shows a comparison of the mean scrotal circumference, body weight and scrotal circumference per body weight as well as mean plasma concentrations of INSL3 and testosterone at 15-minute intervals during 8 hours sampling in male goats (n = 6), and in bulls (n = 6) of chapter 1. For goats the scrotal circumference per body weight was 14.4 times higher (P<0.001) than for bulls, whereas the mean plasma INSL3 concentrations in goats was 9.3 times (P<0.001) and the mean testosterone concentrations was 3.2 times higher (P<0.01) than in bull.
Fig. 2-1A. Plasma LH concentrations in response to GnRH treatment (1.25 µg/kg) in male goats. Data are expressed as mean ± standard error of the mean (n = 6). *P <0.05, **P <0.01 compared with the control (average of –15 and 0 minutes of pretreatment).
Fig. 2-1B. Plasma INSL3 concentrations in response to GnRH treatment (1.25 µg/kg) in male goats. Data are expressed as mean ± standard error of the mean (n = 6). *P <0.05 compared with the control (average of –15 and 0 minutes of pretreatment).
Fig. 2-1C. Plasma testosterone concentrations in response to GnRH treatment (1.25 µg/kg) in male goats. Data are expressed as mean ± standard error of the mean (n = 6). *P <0.05, **P <0.01 compared with the control (average of –15 and 0 minutes of pretreatment).
Fig. 2-2A. Plasma INSL3 concentrations in response to hCG treatment (5 IU/kg) in male goats. Data are expressed as mean ± standard error of the mean (n = 6). *P < 0.05, **P < 0.01 compared with the control (average of –15 and 0 minutes of pretreatment).
Fig. 2-2B. Plasma testosterone concentrations in response to hCG treatment (5 IU/kg) in male goats. Data are expressed as mean ± standard error of the mean (n = 6). *P < 0.05, **P < 0.01 compared with the control (average of –15 and 0 minutes of pretreatment).
Fig. 2-3. Changes of plasma LH, INSL3 and testosterone concentrations in blood samples taken from two individual representative male goats (A: #1, B: #2). Blood samples were taken at 15-minute intervals for 8 hours. The peaks for INSL3 (●), LH (●), and testosterone (○) pulses were determined by the Pulse XP software.
Table 2-1. Mean scrotal circumference (SC), body weight (BW), scrotal circumference per body weight (SC/BW) as well as mean plasma concentrations of INSL3 and testosterone at 15-minute intervals sampling in male Shiba goats (n = 6) and beef bulls (n = 6; Hannan et al., 2015a). *P <0.01, **P <0.001 compared with the same parameter of bull.

<table>
<thead>
<tr>
<th>Animal</th>
<th>SC (mm)</th>
<th>BW (kg)</th>
<th>SC/BW</th>
<th>INSL3 (ng/mL)</th>
<th>Testosterone (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>213.3 ± 10.8</td>
<td>19.7 ± 2.3</td>
<td>11.3 ± 0.8**</td>
<td>43.9 ± 10.3**</td>
<td>5.4 ± 1.9*</td>
</tr>
<tr>
<td>Bull</td>
<td>241.2 ± 9.5</td>
<td>308.5 ± 12.5</td>
<td>0.8 ± 0.0</td>
<td>4.7 ± 0.7</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Goat/Bull</td>
<td>–</td>
<td>–</td>
<td>14.4</td>
<td>9.3</td>
<td>3.2</td>
</tr>
</tbody>
</table>
Discussion

There is an apparent difference in the regulation of INSL3 secretion between men and bulls (Hannan et al., 2015a; Bay and Andersson, 2011), the only two species studied in this detail. It was therefore decided to investigate the male goat to elucidate whether or not the endocrine regulation is similar to the one observed in bull. In this chapter, the time-dependent secretion of INSL3 in association with LH and testosterone in the peripheral blood of male goats was studied. Plasma levels of LH and testosterone have been reported elsewhere (Samir et al., 2015; Saito et al., 2012; Tani et al., 1992) and were confirmed in the present study. In the male goat, the pulsatile secretory patterns of LH, INSL3 and testosterone at 15-minute intervals for an 8-hour session were mostly in accordance with those in beef bulls of chapter 1; however, a 9.3-fold higher mean plasma concentrations of INSL3 was observed in the male goat than in bulls of chapter 1. Moreover, the ratio of scrotal circumference per body weight was 14.4-times higher in male goats than bulls. Therefore, it is suggested that this higher absolute INSL3 concentration was due to the higher proportion of testis per body weight in male goats than that in bulls. This high concentration of INSL3 in the peripheral blood of male goats was surprising. It is the highest among all mammalian species (Bay et al., 2005; Anand-Ivell et al., 2006a; Büellesbach et al., 1999; Pathirana et al., 2012; Kawate et al., 2011) determined so far, implying the urgency of exploring important endocrine roles of this hormone in male goats.

The present pulsatile secretory pattern of LH associated with subsequent pulsatile secretion of testosterone in male goats were similar to that reported in bulls (Stumpf et al., 1993; Finnerty et al., 1998) and male lambs (Foster et al., 1978). An LH-induced rise
in testosterone was also evident in male goats (Howland et al., 1985). Moreover, 71% peak of INSL3 pulses was observed within 1 hour after a peak of an LH pulse, implying an additional positive association between LH and INSL3. The higher number of INSL3 pulses compared with testosterone pulses during an 8-hour session in this chapter was in agreement with that of chapter 1 in bulls (Hannan et al., 2015a). Thus, it was assumed that in addition to well-known testosterone pulses, INSL3 pulses might also be following LH pulses. The higher number of INSL3 pulses compared to testosterone pulses within a specific period of time indicated that a comparatively lower threshold value of an LH pulse was sufficient to generate an INSL3 pulse.

The hypothesis that LH would acutely regulate INSL3 secretion in male goats was supported by LH stimulation or substitution. Previously, it has been reported that in male goats testosterone concentrations increased significantly after GnRH and hCG treatments (Samir et al., 2015; Saito et al., 2012). In this chapter, the stimulation of endogenous LH by a GnRH analogue induced immediate increase of INSL3 concentrations, besides the expected changes in testosterone concentrations. Moreover, after administration of hCG, an exogenous LH, the increase of INSL3 and testosterone concentrations persisted longer. In GnRH and hCG treatments, the increase (maximum per pre-treatment concentration) of INSL3 concentrations was lower than that of testosterone. The persistence of the significant increase of INSL3 and testosterone concentrations to about 1 week after hCG treatment compared to a few hours after GnRH treatment for both hormones was likely due to a longer half-life of hCG in circulation (Biome and Ben-Menahem, 1999). The significant increase of INSL3 release just 30 minutes after endogenous and exogenous LH stimulation implied that LH acutely regulated INSL3 secretion. This was further
supported by normal physiological conditions where 71% of INSL3 pulses peaked within 1 hour after an LH pulse peak had occurred. Such acute regulation of INSL3 by LH in male goats was consistent with the data of chapter 1 in bulls (Hannan et al., 2015a). In men, however, daily peripheral blood sampling until 8 days after hCG treatment revealed that testosterone concentrations increased after treatment, whereas INSL3 concentrations remained unchanged (Bay and Andersson, 2011). Conversely, other studies in men showed that serum INSL3 concentrations increased 4 or 10 days after hCG treatment when endogenous LH secretion was suppressed by androgen analogues or GnRH antagonist (Bay et al., 2006; Roth et al., 2013), although none of these study attempted any frequent sampling after hCG treatment.

In conclusion, the secretion of INSL3 in blood occurred in a pulsatile manner immediately after LH pulses in male goats as it does in beef bulls. The INSL3 concentration was acutely increased by endogenous and exogenous LH in male goats, although the rise of INSL3 by LH was lower than that of testosterone. In addition, the absolute concentration of INSL3 in plasma of male goats was much higher than that in bulls, in accordance with the far larger proportion of testis to body size in goats.
Summary

It was shown in chapter 1 that in bulls the secretion of INSL3 in blood occurred in a pulsatile manner and was acutely regulated by LH. In this chapter, in order to check whether or not this similar endocrine regulation existed in small ruminants, the acute regulation of plasma INSL3 and its temporal relationships with LH and testosterone were examined in male goats. After GnRH treatment, mean plasma concentrations of all 3 hormones increased (P<0.05) dramatically from 30 minutes and remained high until 120 minutes (LH), 75 minutes (INSL3) and 4 hours (testosterone) post-treatment. After hCG treatment, mean plasma INSL3 concentrations increased (P<0.05) from 30 minutes and remained elevated until the end of sampling on Day 12. An increase (P<0.05) in mean plasma testosterone concentrations occurred from 15 minutes and remained high until Day 6. The mean increase (maximum per pre-treatment concentration) of INSL3 concentrations after administration of GnRH and hCG was lower (P<0.01) than that of testosterone. The secretory pattern of LH, INSL3 and testosterone in the general circulation was pulsatile with a frequency of 5.5 ± 0.6, 4.7 ± 0.5 and 2.2 ± 0.5, respectively, during the 8-hour period. Twenty out of 28 (71%) of these INSL3 pulses peaked within 1 hour after a peak of an LH pulse. The mean increase (peak per basal concentration) of INSL3 pulses (2.1 ± 0.1 fold, n = 28) was lower (P<0.01) than that of testosterone pulses (4.3 ± 2.2 fold, n = 13). In conclusion, secretion of INSL3 in blood occurred, like in bulls, in a pulsatile manner soon after LH pulses in male goats. The absolute concentrations of INSL3 in male goats was higher than that reported in other mammals. INSL3 concentrations were acutely increased by endogenous and exogenous LH in male goats, but the rise of INSL3 was lower than that of testosterone.
Chapter 3
Effects of a long-acting GnRH antagonist on insulin-like peptide 3, testosterone and scrotal circumference in male goats

Introduction

Insulin-like peptide 3 (INSL3) concentrations in the peripheral blood change with age or testicular abnormality in humans (Bay et al., 2005; Anand-Ivell et al., 2006a; Büllesbach et al., 1999), dogs (Pathirana et al., 2012), cattle (Kawate et al., 2011), and goats (Hannan et al., 2016), implying that the blood INSL3 concentrations can be used as a biomarker for Leydig cell function.

The production of INSL3 is dependent on the state of Leydig cells and is stimulated by the long-term trophic effects of LH as shown in humans (Ivell et al., 1997; Bay et al., 2005; Foresta et al., 2004; Ferlin et al., 2006; Wikström et al., 2006). It was demonstrated in chapters 1 and 2 that the nature of INSL3 discharges into the peripheral blood is pulsatile and LH pulses preceded the INSL3 pulses in bulls (Hannan et al., 2015a) and male goats (Hannan et al., 2016). Moreover, it was showed in those chapters that after stimulation of LH secretion by a GnRH analogue or hCG, a significant increase in plasma concentrations not only of testosterone but also INSL3 were observed in bulls (Hannan et al., 2015a) and male goats (Hannan et al., 2016). The effect of LH suppression by administering a GnRH antagonist or a combination of androgen and progestin in men indicated a decline in serum INSL3 (Bay et al., 2006; Roth et al., 2013). However, the effects of GnRH antagonist treatment on plasma concentrations of INSL3 are yet to be elucidated in male domestic animals including goats.
Degarelix acetate (Jiang et al., 2001), a long-acting GnRH antagonist, which is clinically used for treatment of prostate cancer in men, suppresses testosterone secretion for more than one month by a single administration without allergic reactions (Steinberg, 2009; Boccon-Gibod et al., 2011). Moreover, a reduction of testicular weight after degarelix acetate treatment was shown in male rats (Broqua et al., 2002). However, the effects of this GnRH antagonist on testicular size in domestic male animals including goat remain unknown.

The aim of this chapter was to investigate the effects of a long-acting GnRH antagonist treatment on secretory patterns of plasma INSL3 and testosterone in the immediate phase, and plasma concentrations of these hormones and scrotal circumference in the long-term in male goats.
Materials and Methods

Animals

Six sexually matured male Shiba goats (aged, 13–16 months; body weight, 19.7 ± 2.3 kg) housed in an experimental room throughout the study at Osaka Prefecture University, were used. Shiba goat, a Japanese miniature breed, becomes sexually mature at about 4 months of age and shows breeding all the year round (Kano and Mori, 1982; Tani et al., 1992). Before selecting for the experiment, examination of the goats showed no apparent abnormalities of the reproductive status and both testes, checked manually, were located inside the scrotum. Goats were fed concentrates and hay, with water ad libitum. This study was performed in accordance with the Guidelines for Animal Experimentation of Osaka Prefecture University as approved by the Animal Experiment Committee of Osaka Prefecture University.

Hormonal treatment and blood sampling

A schematic presentation of the protocol is presented in Fig. 3-1. The blood sampling was done at 15-minute intervals for an 8-hour session in three sets (Days −5, 0 and 3). On Day −5, blood sampling for all goats was started at 10 AM and ended at 6 PM. An indwelling jugular venous catheter (Argyle; Covidien Ltd., Dublin, Ireland) was inserted about 1 hour before the beginning of 15-minute intervals sampling. Blood samples were collected into heparinized syringes and immediately transferred into glass tubes and placed on ice before centrifugation (1,700 × g for 20 minutes at 4°C). The separated plasma was immediately stored at −30°C until assays were conducted. The animals were neither sedated before inserting the intravenous catheter nor during sampling. The goats
were given access to water and hay every 2 to 3 hours during 15-minute intervals sampling. On Day 0, at 2 hours (Hour 0) after start of 15-minute intervals sampling, a single injection of long-acting GnRH antagonist (degarelix acetate; Gonax®, Astellas Pharma Inc., Tokyo; 4 mg/kg; subcutaneously) was given and collection of blood samples was continued for further 6 hours. On Day 3, another session of 15-minute intervals sampling for an 8 hour was performed. The time of insertion of jugular venous catheter as well as blood sampling and processing on Days 0 and 3 were done as described above for Day –5. In addition, to study the long-term effect of the treatment, a daily blood sample was taken by jugular venipuncture from Day 0 (before treatment) to Day 7, followed by twice weekly until 9 weeks and finally at week 10 post-treatment. The blood samples were taken until 6 weeks after the GnRH antagonist treatment from 6 animals but continued 6.5 to 10 weeks from 4 animals.

Scrotal circumference

Scrotal circumference was recorded by a single experienced investigator before and after a long-acting GnRH antagonist treatment on each male goat (n = 6) to observe any changes after the treatment. The first measurement was performed on Day 0 (day of treatment) before treatment and continued every two weeks until week 10.

Hormone assays

Plasma concentrations of INSL3 were determined by an EIA as described in chapter 2. An anti-bovine INSL3 mouse monoclonal antibody (2-8F) and synthetic bovine INSL3 (Büllesbach and Schwabe, 2002) for standard were employed in combination
with biotinylated canine INSL3 as described (Hannan et al., 2016). The minimum
detection limit of the INSL3 EIA was 0.31 ng/mL, and the detection was reliable in the
range from 0.31 to 20 ng/mL. The intra-assay and interassay coefficient of variation
(CVs) were 7.4% and 14.5%, respectively.

Plasma testosterone concentrations were determined by an EIA using the procedure
previously described in chapter 1 in bulls. An anti-testosterone rabbit polyclonal
antibody and horseradish peroxidase (HRP) -labeled testosterone (Cosmo Bio Co., Ltd.,
Tokyo) were used. The minimum detection limit was 0.07 ng/mL, and the reliable
detection range was 0.07 to 20 ng/mL. The intra-assay and interassay CVs were 3.6%
and 15.7%, respectively.

An EIA procedure to measure LH in bull plasma described in chapter 1 was also used
for LH in goat plasma except that an anti-ovine β LH antibody (NIDDK-anti-ovine LH
Beta-1; 1: 16,000 dilution in assay buffer) was used instead of anti-bovine LH. The assay
detection range was from 0.31 to 40 ng/mL. The intra-assay and interassay CVs were
4.6% and 11.8%, respectively.

Pulse detection

Pulses of LH, INSL3 and testosterone in plasma samples taken at 15-minute intervals
were detected with Pulse XP software kindly provided by Prof. Michael L. Johnson,
University of Virginia (Johnson et al., 2008). The procedure has been described in chapter
1. After identifying the pulses and their peaks, the nadir of each pulse was determined
from the raw data (by considering the preceding lower value of the defined peak of that
pulse). This information was used to calculate the pulse amplitude (peak – nadir).
**Statistical analysis**

The blood samples taken at 15-minute intervals for 8 hours on Day –5 and first 2 hours on Day 0 (Hours -2 to 0, before GnRH antagonist treatment) were combined and considered as pre-treatment (total 10 hours), and blood samples taken after treatment on Day 0 (Hours 1 to 6) was considered as post-treatment Day 0 (total 6 hours). Blood samples taken at 15-minute intervals for 8 hours on 3 days after treatment was considered as post-treatment Day 3 (total 8 hours). The effects of time on hormonal concentrations, pulse frequency (per hour) and pulse amplitude (peak – nadir) of plasma LH, INSL3 and testosterone before (combined pre-treatment Days -5 and 0) and after (post-treatment Day 0 and post-treatment Day 3) GnRH antagonist treatment were determined by ANOVA using the GEE procedure of SPSS version 22 software (IBM, Somers, NY). Differences in hormone concentrations, pulse frequency and pulse amplitude on post-treatment Day 0 and post-treatment Day 3 compared with pre-treatment were analyzed using pairwise comparisons of the GEEs procedure by the LSD post hoc test. The mean hormonal concentrations of each goat’s based on 15-minute intervals sampling on post-treatment Day 3 was divided by mean hormonal concentrations of pre-treatment (combined pre-treatment Days -5 and 0) of that same goat, and obtained mean result was considered as mean decreasing rate. The mean decreasing rate between INSL3 and testosterone was compared using paired t-test.

In long-term sampling, the effects of time on plasma INSL3 and testosterone concentrations as well as on scrotal circumference from pre-treatment to 10 weeks post-treatment of GnRH antagonist treatment were determined by same ANOVA mentioned above. The hormonal concentrations on post-treatment Day 1 was divided by that of pre-
treatment (a daily sample taken immediately before treatment on Day 0) for each goat and obtained mean result was considered as mean decreasing rate. Mean hormonal concentrations and scrotal circumference as well as mean decreasing rate after GnRH antagonist treatment were compared with those of the pre-treatment using paired \( t \)-test. Data were expressed as mean ± standard error of the mean. Differences were considered significant at P<0.05.
Results

The goats remained normal in appearance and health during the study. The effect of degarelix acetate, a long-acting GnRH antagonist, on the plasma secretory pattern of LH, INSL3 and testosterone was determined on Days –5, 0 and 3. In order to evaluate the pulsatile secretion properties of these hormone blood samples were collected at 15-minute intervals. Data obtained from a representative male goat are shown in Fig. 3-2ABC. A difference between pre-treatment and post-treatment periods was noticed for all 3 hormones in terms of concentrations, pulse frequency (per hour) and pulse amplitude (peak – nadir).

The mean plasma concentrations, pulse frequency and pulse amplitude of LH, INSL3 and testosterone based on sampling at 15-minute intervals during pre-treatment (Day –5 and first two hours on Day 0) and post-treatment Days 0 and 3 of GnRH antagonist are presented in Fig. 3-3ABC. Before and after the GnRH antagonist treatment, a significant differences was observed for mean concentrations, pulse frequency and pulse amplitude of plasma LH, INSL3 and testosterone (P<0.05).

A reduction (P<0.05) in mean plasma LH concentrations was observed on post-treatment Days 0 and 3 compared to pre-treatment (Fig. 3-3A). Mean plasma INSL3 concentrations did not differ significantly between pre-treatment and post-treatment Day 0, but a lower (P<0.01) concentration was observed on post-treatment Day 3 compared to pre-treatment (Fig. 3-3A). A reduction (P<0.05) of mean plasma testosterone concentrations was observed on post-treatment Days 0 and 3 compared to pre-treatment (Fig. 3-3A). The mean decreasing rate of INSL3 concentrations (0.52 ± 0.05) on post-treatment Day 3 compared to pre-treatment was lower (P<0.01) than that of testosterone
(0.16 ± 0.04).

The mean plasma LH pulse frequency (per hour) after GnRH antagonist treatment was lowered (P<0.05) on post-treatment Days 0 and 3 compared to pre-treatment (Fig. 3-3B). The mean plasma INSL3 pulse frequency, compared to pre-treatment, did not differ significantly on post-treatment Day 0, but was decreased (P<0.01) on post-treatment Day 3 (Fig. 3-3B). A decline (P<0.01) in mean plasma testosterone pulse frequency was observed on post-treatment Days 0 and 3 compared to pre-treatment (Fig. 3-3B).

There was a reduction (P<0.05) of mean plasma pulse amplitude of LH on post-treatment Days 0 and 3 compared to pre-treatment (Fig. 3-3C). A decline (P<0.01) in mean plasma pulse amplitude of INSL3 was exhibited on post-treatment Day 3, but not on post-treatment Day 0, compared to pre-treatment (Fig. 3-3C). Mean plasma testosterone pulse amplitude was reduced (P<0.01) on post-treatment Days 0 and 3 compared to pre-treatment (Fig. 3-3C).

The long-term effect of GnRH antagonist treatment on the mean plasma INSL3 and testosterone concentrations was assessed for 10 weeks (Fig. 3-4AB). Significant differences between before and after GnRH antagonist treatment were observed for plasma INSL3 and testosterone concentrations (P<0.001). On post-treatment Day 1, a reduction (P<0.05) in mean plasma INSL3 concentrations, compared to pre-treatment, was observed which continued until Day 3 and thereafter maintained at an almost constant level until 8.5 weeks before reaching to pre-treatment levels (Fig 3-4AB).

Compared with pre-treatment levels mean plasma testosterone concentrations decreased (P<0.05) to a very low level on the following day and maintained the similar
low level until 8.5 weeks post-treatment. After that, concentrations returned to pre-treatment levels (Fig 3-4AB). The mean decreasing rate of INSL3 (0.70 ± 0.05) concentrations 1 day after treatment compared to pre-treatment was lower (P<0.01) than that of testosterone (0.25 ± 0.10).

The scrotal circumference was measured before and after GnRH antagonist treatment over a period of 10 weeks (Fig. 3-5). A significant effect of time before and after degarelix acetate administration was observed (P<0.001). The first significant reduction (P<0.01) in mean scrotal circumference was recorded 4 weeks after the treatment, and the reduction continued gradually until the end of the experiment at week 10.
Fig. 3-1. Diagrams of experimental designs for frequent blood sampling (A) and long-term study (B). The blood was taken at 15-minute intervals for 8 hours on Days –5, 0 and 3. A single injection of a long-acting GnRH antagonist (degarelix acetate; 4 mg/kg; subcutaneously) was given at 2 hours (Hours 0) after start of blood sampling at 15-minute intervals on Day 0 in male goats (A). A separate blood sample was taken immediate before treatment and continued up to Day 7, followed by twice weekly until 9 weeks and finally at week 10 post-treatment (n = 6 up to 6 weeks; n = 4 from 6.5 to 10 weeks) (B). Scrotal circumference was measured before treatment and continued every two weeks until 10 weeks post-treatment (n = 6).
Fig. 3-2A. Changes of plasma LH, INSL3 and testosterone secretions in blood samples taken at 15-minute intervals for 8 hours on Day –5 of a long-acting GnRH antagonist treatment in an individual representative male goat. The peaks for INSL3 (▲), LH (←), and testosterone (○) pulses were determined by the Pulse XP software.
Fig. 3-2B. Changes of plasma LH, INSL3 and testosterone secretions in blood samples taken at 15-minute intervals for 8 hours on Day 0 of subcutaneous administration of degarelix acetate, a long-acting GnRH antagonist (4 mg/kg) in an individual representative male goat. The treatment was given immediately after 2-hour sampling. The peaks for INSL3 (•), LH (○), and testosterone (△) pulses were determined by the Pulse XP software.
Fig. 3-2C. Changes of plasma LH, INSL3 and testosterone secretions in blood samples taken at 15-minute intervals for 8 hours on 3 days after (Day 3) a long-acting GnRH antagonist treatment in an individual representative male goat. The peaks for INSL3 (●), LH (▲), and testosterone (○) pulses were determined by the Pulse XP software.
Fig. 3-3A. Changes of mean plasma LH (A), INSL3 (B) and testosterone (C) concentrations, in response to degarelix acetate, a long-acting GnRH antagonist (4 mg/kg; subcutaneously) treatment in male goats. The treatment was given subcutaneously immediately after 2-hour sampling on Day 0. The blood samples taken at 15-minute intervals for 8 hours on Day –5 and first 2 hours on Day 0 were combined and considered as pre-treatment (total 10 hours) and blood sampling taken after treatment on Day 0 (hours 2.25 to 8) was considered as post-treatment Day 0 (total 6 hours) and blood sampling taken at 15-minute intervals for 8 hours on 3 days after treatment was considered as post-treatment Day 3 (total 8 hours). Data are expressed as mean ± standard error of the mean (n = 6). *P<0.05, **P<0.01 compared with the pre-treatment of the corresponding hormone.
Fig. 3-3B. Changes of mean plasma LH (A), INSL3 (B) and testosterone (C) pulse frequency, in response to degarelix acetate, a long-acting GnRH antagonist (4 mg/kg; subcutaneously) treatment in male goats. The treatment was given subcutaneously immediately after 2-hour sampling on Day 0. The blood samples taken at 15-minute intervals for 8 hours on Day –5 and first 2 hours on Day 0 were combined and considered as pre-treatment (total 10 hours) and blood sampling taken after treatment on Day 0 (hours 2.25 to 8) was considered as post-treatment Day 0 (total 6 hours) and blood sampling taken at 15-minute intervals for 8 hours on 3 days after treatment was considered as post-treatment Day 3 (total 8 hours). Data are expressed as mean ± standard error of the mean (n = 6). *P<0.05, **P<0.01 compared with the pre-treatment of the corresponding hormone.
Fig. 3-3C. Changes of mean plasma LH (A), INSL3 (B) and testosterone (C) pulse amplitude, in response to degarelix acetate, a long-acting GnRH antagonist (4 mg/kg; subcutaneously) treatment in male goats. The treatment was given subcutaneously immediately after 2-hour sampling on Day 0. The blood samples taken at 15-minute intervals for 8 hours on Day –5 and first 2 hours on Day 0 were combined and considered as pre-treatment (total 10 hours) and blood sampling taken after treatment on Day 0 (hours 2.25 to 8) was considered as post-treatment Day 0 (total 6 hours) and blood sampling taken at 15-minute intervals for 8 hours on 3 days after treatment was considered as post-treatment Day 3 (total 8 hours). Data are expressed as mean ± standard error of the mean (n = 6). *P<0.05, **P<0.01 compared with the pre-treatment of the corresponding hormone.
Fig. 3-4A. Mean plasma INSL3 and testosterone concentrations in response to degarelix acetate, a long-acting GnRH antagonist (4 mg/kg; subcutaneously) treatment in male goats. A daily blood sample was taken from Day 0 (before treatment) to Day 7, followed by twice weekly until 6 weeks post-treatment. Data are expressed as mean ± standard error of the mean. *P < 0.05 compared with the pre-treatment of the corresponding hormone (n = 6).
Fig. 3-4B. Mean plasma INSL3 and testosterone concentrations in response to degarelix acetate, a long-acting GnRH antagonist (4 mg/kg; subcutaneously) treatment in male goats. Data of Day 0 (pre-treatment) and twice weekly blood from 6.5 to 9 weeks and finally at week 10 post-treatment are shown. Data are expressed as mean ± standard error of the mean. *P < 0.05 compared with the pre-treatment of the corresponding hormone (n = 4).
Fig. 3-5. Changes of scrotal circumference in response to degarelix acetate, a long-acting GnRH antagonist (4 mg/kg; subcutaneously) treatment in male goats. Scrotal circumference was recorded before treatment and followed every biweekly until week 10. Data are expressed as mean ± standard error of the mean (n = 6). *P <0.05 compared with the pre-treatment.
Discussion

The consequences of suppressing LH on INSL3 secretion have been studied only in humans (Foresta et al., 2004; Bay et al., 2006; Roth et al., 2013). To the best of the author’s knowledge, there is no report of examining secretion of INSL3 after suppressing endogenous LH in domestic animals. Here, for the first time the author showed that INSL3 secretion in peripheral blood is suppressed by the long-acting GnRH antagonist, degarelix acetate, in male goats.

The mean plasma concentrations of INSL3, based on mean concentrations of 15-minute intervals sampling, was lower on post-treatment Day 3, but not significantly suppressed at post-treatment Day 0. According to a daily sampling schedule, a significantly lower concentration was observed already 1 day after the GnRH antagonist treatment. In case of testosterone, a profound reduction was noticed within a few hours of treatment. Simultaneously, with the decline of plasma concentrations of INSL3 and testosterone, the pulse frequency and amplitude of these hormones also decreased within 3 days after treatment, and again in case of testosterone, these reduction was quicker and greater than for INSL3. The suppression of plasma INSL3 within 3 days of LH deprivation by GnRH antagonist indicated that secretion of this hormone was acutely regulated by LH in male goats as we have recently shown by exogenous and endogenous stimulation of LH in the same species (Hannan et al., 2016). Moreover, the slower decrease of INSL3 compared to testosterone implies that the degree of dependence on LH is less for INSL3 than for testosterone, although LH is the regulator of both. The reduction of plasma INSL3 secretion in the present study was in agreement with reported lower serum INSL3 concentrations after gonadotropins deprivation in men (Foresta et
al., 2004; Bay et al., 2006). However, the studies in men have shown that suppression of endogenous LH resulted in a marked decline in serum INSL3 at post-treatment Day 10 or later (Foresta et al., 2004; Bay et al., 2006; Roth et al., 2013). Because none of these studies reported sampling prior to this time point, the exact onset when the suppression of INSL3 secretion occurred remained uncertain. The present data are valuable with respect to elucidate the acute regulation of INSL3 secretion by endogenous LH more precisely in male goats.

The long-term sampling in the present study revealed that the suppression of plasma concentrations of INSL3 and testosterone persisted for 8.5 weeks in response to the long-acting GnRH antagonist (degarelix acetate) treatment and both hormonal concentrations returned to pre-treatment levels at week 9. Blache et al. (1997) reported a suppression of LH and testosterone secretion after treatment with a short-acting GnRH antagonist, antarelix, in rams, and showed that the secretion of LH and testosterone was suppressed for only 9 hours post treatment. Conversely, treatment with degarelix has been repeated monthly for more than 1 year for the management of prostate cancer in men (Steinberg, 2009; Boccon-Gibod et al., 2011). In male dogs, a single dose of another long-acting GnRH antagonist, acyline, decreased serum concentrations of testosterone for 9 days after administration, and thereafter on Day 14 concentrations gradually began to increase and a clear rebound above baseline could also be seen at the end of the follow-up period of the study on day 29 (Gobello, 2012; García Romero et al., 2009). In the present study, a quick and long-lasting suppression of testosterone and INSL3 after a single injection, with subsequent recovery of the hormonal concentrations, implies a potential application of this antagonist by means of reversible long-term chemical castration in male goats.
Further studies with long-term treatments are required to establish the best approach of a chemical castration therapy in domestic male animals.

The scrotal circumference increased rapidly until 5 months of age but thereafter did not change substantially as shown previously in male Shiba goats (Tani et al., 1992). In this study, in addition to acute hormonal suppression, a reduction in scrotal circumference in goats was observed from 4 to at least 10 weeks after treatment, suggesting that the reduction in testicular size was subsequent to peripheral hormonal suppression. The author speculated that the reduction of scrotal circumference could be due to reduction of testicular Leydig cell number and disturbance in the spermatogenesis process because of deprivation of gonadotropins. Because the present study was discontinued after 10 weeks of GnRH antagonist treatment, the author does not know whether or not complete recovery of testis size in goats will occur and how long the recovery will take to approach testes size at a final steady state. As suggested by experiments with degarelix acetate in male rats, it could be possible that testis size and function can be recovered in male goats. The authors showed that in rats degarelix acetate caused a remarkable decrement of testicular weight on Day 45 which was largely restored to pretreatment values on Day 102 (Broqua et al., 2002).

In conclusion, the acute regulation of INSL3 by LH was confirmed by reduction of plasma INSL3 concentrations, pulse frequency and amplitude within 3 days after a long-acting GnRH antagonist (degarelix acetate) treatment in male goats. The suppression of testosterone was more rapid than that of INSL3 and persisted for 8.5 weeks for both hormones, and subsequently the concentrations returned to pre-treatment levels. A significant reduction in testicular size was also observed. The quick, long-lasting and
transient suppression of testosterone and INSL3 after a single injection implies a potential application of this antagonist as a mean for reversible long-term chemical castration in male goats.
Summary

It was shown that plasma INSL3 concentrations increased soon after endogenous and exogenous stimulations of LH in bulls and male goats in chapters 1 and 2, respectively. However, the effects of LH suppression on INSL3 secretion are unknown in domestic animals. In this chapter, the effects of a long-acting GnRH antagonist (degarelix acetate; 4 mg/kg) on the secretions of plasma INSL3 and testosterone were studied in two phases, an immediate and a long-term phase in male goats (n = 6; aged, 13–16 months). During the immediate phase, blood was taken at 15-minute intervals for 8 hours on Days –5, 0 and 3. The GnRH antagonist was administered after 2-hour sampling on Day 0. Moreover, a daily blood sample was taken from Days 0 to 7, followed by twice weekly until 9 weeks and finally at week 10. The scrotal circumference was recorded before treatment and continued biweekly until week 10.

Concentrations of LH, INSL3 and testosterone in plasma were determined by EIA and the pulsatile nature of secretion analyzed using pulse XP software. The mean concentrations, pulse frequency (per hour) and pulse amplitude (peak – nadir) of plasma LH and testosterone reduced from pre-treatment to post-treatment Days 0 and 3 (P<0.05). A decline in mean concentrations, pulse frequency and pulse amplitude of INSL3 was exhibited on post-treatment Day 3 compared to pre-treatment (P<0.01). During long-term sampling, a decline (P<0.01) in plasma testosterone and INSL3 concentrations was observed 1 day after treatment and remained lower until 8.5 weeks post-treatment, and thereafter returned to pre-treatment levels. A reduction in scrotal circumference was recorded 4 weeks after treatment and remained lower until 10 weeks post-treatment (P<0.05).
In conclusion, the acute regulation of INSL3 by LH was confirmed by reduction of plasma INSL3 levels within 3 days after GnRH antagonist treatment in male goats. Although the onset of suppression of testosterone was more rapid than that of INSL3, the low levels persisted for 8.5 weeks for both hormones, and subsequently the concentrations returned to pre-treatment levels. A significant reduction in testicular size was also observed. The quick, long-lasting and transient suppression of testosterone and INSL3 after a single injection implies a potential application of this antagonist in reversible long-term chemical castration in male goats.
Chapter 4

Changes of insulin-like peptide 3 and testosterone, and their association with scrotal circumference during and after puberty in male goats

Introduction

Insulin-like peptide 3 (INSL3) is secreted by Leydig cells in the testis and by follicular theca cells in the ovary (Ivell and Bathgate, 2002). This peptide hormone has been used as a testis-specific biomarker for assessing pubertal development (Ferlin et al., 2006; Wikström et al., 2006; Johansen et al., 2014; Kawate et al., 2011; Pathirana et al., 2012; Anand-Ivell et al., 2009), but no data are available regarding the secretory profile of INSL3 during pubertal development in small ruminants. Previous research clarified that the dynamics of the secretory patterns of plasma INSL3 in bulls (Kawate et al., 2011) and male dogs (Pathirana et al., 2012) are different during pubertal development, and a similar change of INSL3 in peripheral blood during puberty was reported in male rats (Anand-Ivell et al., 2009) and humans (Ferlin et al., 2006; Wikström et al., 2006; Johansen et al., 2014). Though the roles of INSL3 during sexual development in males are still unknown, the author speculated that species-specific secretion profiles during sexual development may reflect different physiological outcomes.

Age-related change in scrotal circumference is a good measure of puberty. This has been discussed in detail in bulls (Kastelic, 2014). An association between sexual maturation and scrotal circumference was also reported in crossbred and British breeds of male goats (Bongso et al., 1982; Ahmad and Noakes, 1996). The changes in scrotal circumference and the plasma concentrations of androgens (testosterone and
androstenedione) with age have been shown in male Shiba goats (Tani et al., 1992). In boys, an association between INSL3 concentrations in peripheral blood and testicular volume with pubertal development was reported (Johansen et al., 2014). Correlations among INSL3, testosterone and LH in peripheral blood during pubertal development were described in humans (Ferlin et al., 2006) and bulls (Kawate et al., 2011). However, the association between testicular size and peripheral levels of INSL3 during pubertal development are yet to be elucidated in any domestic male species including goat.

The purpose of the present study was to determine the age-related changes in the plasma concentrations of INSL3 and testosterone and their association with scrotal circumference during pubertal development in male goats. The author’s goal was to clarify the mechanisms underlying the changes in plasma INSL3 concentrations in small ruminants, with an eye toward INSL3 as a potential biomarker.
Materials and Methods

Animals

Male Shiba goats (n = 5) housed in an experimental room throughout the study at Osaka Prefecture University were used. Body weight was measured every 2 weeks from week 10 to week 52 of each goat's life. Shiba goats, a Japanese miniature breed, show year-round breeding and become sexually mature at about 4 months (16 weeks) of age (Tani et al., 1992; Kano and Mori, 1982). The selected goats were normal at birth and had no apparent abnormalities of reproductive status and remained healthy throughout the study. Before the goats were selected for the study, the presence of both testes inside the scrotum was confirmed by manual palpation. The goats were fed concentrates and hay, with water ad libitum. The study was performed in accordance with the Guidelines for Animal Experimentation of Osaka Prefecture University as approved by the Animal Experiment Committee of Osaka Prefecture University.

Blood sampling and scrotal circumference

Blood samples and scrotal circumference measurement were done every 2 weeks intervals starting at week 10 and ending at week 52. Scrotal circumference was recorded by a single experienced investigator. Blood samples were collected into heparinized syringes by jugular venipuncture between 10:00 and 11:00 AM and immediately transferred into glass tubes and placed in ice before centrifugation (1,700 × for 20 minutes at 4°C). The plasma was collected and immediately stored at −30°C. The samples were thawed just prior to the INSL3, testosterone and LH assays.
**Hormone assays**

The plasma concentrations of INSL3 were measured by a modified time-resolved fluorescence immunoassay (TRFIA), based on the procedure described by Pathirana et al. (2012). Briefly, microtitration plate wells (96 wells per plate; DELFIA 1244–550; Wallac Oy, Turku, Finland) were coated with 100 µL of anti-mouse IgG rabbit polyclonal antibody (5 µg/mL in 0.05 M sodium bicarbonate; pH 9.7) for 2 hours at room temperature. The wells were then drained and washed with 300 µL saline (0.15 M sodium chloride). Next, 200 µL of assay buffer (0.01M phosphate buffer containing 0.15M sodium chloride, pH 7.4) supplemented with 2% bovine serum albumin (BSA; Cohn Fraction V, Sigma-Aldrich, St. Louis, MO), and 0.02% ProClin 950 (Sigma-Aldrich) was added and kept overnight at 4°C to block the remaining active sites. Prior to the assay, goat plasma was diluted 20-fold using 0.05 M phosphate buffer (pH 7.5) to keep the samples within the range of the standards. The wells were drained, and 50 µL of synthetic bovine INSL3 (Büllesbach and Schwabe, 2002) for the standards or 50 µL of samples and 50 µL of anti-bovine INSL3 mouse monoclonal antibody (2-8F) (Büllesbach and Schwabe, 2002) were dispensed and incubated for 2 hours at room temperature. Then, 50 µL of biotinylated canine INSL3 (2 ng/mL in assay buffer) was added and incubated for 1 hour at room temperature. After the reaction, the wells were drained and washed four times with 400 µL of wash buffer (0.05 M Tris-HCl containing 0.15M sodium chloride, pH 7.8) supplemented with 0.1% Tween 20 (Sigma-Aldrich). After the washing step, 100 µL of Eu-labeled streptavidin (100 ng/mL in DELFIA assay buffer; PerkinElmer, Boston, USA) was added to the wells and incubated for 30 minutes at room temperature. The wells were then washed five times with 400 µL of the wash
buffer, and 100 µL of enhancement solution (PerkinElmer, Boston, USA) was dispensed into each well. The plate was then shaken (80 rpm) for 15 minutes at room temperature. Finally, time-resolved fluorescence was measured using an ARVO multilabel counter (PerkinElmer, Wallac Oy, Finland). The minimum detection limit of the INSL3 EIA was 0.078 ng/mL, and the detection was reliable in the range from 0.078 to 20 ng/mL. The intra-assay and interassay coefficients of variation (CVs) were 2.6% and 9.4%, respectively.

The plasma testosterone concentrations were determined by an EIA using the procedure as described in chapter 1 for bulls (Kawate et al., 2011). An anti-testosterone rabbit polyclonal antibody and horseradish peroxidase (HRP)-labeled testosterone (Cosmo Bio Co., Ltd., Tokyo) were used. The minimum detection limit was 0.07 ng/mL, and the reliable detection range was 0.07 to 20 ng/mL. The intra-assay and interassay CVs were 5.2% and 18.7%, respectively.

An EIA procedure to measure LH in bull plasma as described in chapter 1 was used to measure the LH in goat plasma except that an anti-ovine β-LH antibody (NIDDK-anti-ovine LH Beta-I; 1: 16,000 dilution in assay buffer) was used instead of anti-bovine LH. The assay detection range was from 0.31 to 40 ng/mL. The intra-assay and interassay CVs were 2.1% and 13.2%, respectively.

**Statistical analyses**

The age of the male goats was divided (during pubertal development) into three periods based on changes of scrotal circumference as shown in Fig. 4-1. There was an apparent increase of mean scrotal circumference up to week 34, a period assigned as
During the time following week 34 up to the end of the sampling at week 52, no changes in scrotal circumference were observed; the author considered this period as the post-pubertal period. The pubertal period was again divided into an early pubertal period (the steep increasing period of scrotal circumference, up to 22 weeks), and a late pubertal period (the moderate increasing period, 22 to 34 weeks). Because of late recruitment, the author had data of only two goats from weeks 10 to 12. These data were not included in the age-related analyses but were included when the data were grouped into different pubertal stages.

The effects of time on the biweekly changes in scrotal circumference and body weight as well as the plasma INSL3, testosterone and LH concentrations were examined by conducting ANOVA using the GLMs procedure of SPSS ver. 22 software (IBM, Somers, NY). The effects of time on changes with the three pubertal periods (early, late and post-pubertal) of those data were also examined by the same ANOVA. The differences in hormone concentrations and scrotal circumference data among the early, late and post-pubertal periods were compared by conducting pairwise comparisons of the GLM procedure by using the LSD as a post hoc test. The best regression curves between scrotal circumference and hormone concentrations and between pairs of hormone concentrations were estimated using the Curve Estimation procedure of Regression Analysis of SPSS ver. 22 software (IBM, Somers, NY). The data are expressed as mean ± standard error of the mean. Differences were considered significant at P<0.05.
Results

The age-related changes in the mean scrotal circumference and body weight (Fig. 4-1) and mean plasma concentrations of INSL3, testosterone and LH (Fig. 4-2) recorded from weeks 14 to 52 in the five male goats are shown. There was a significant effect (P<0.05) of time on scrotal circumference, body weight, and the plasma concentrations of INSL3, testosterone and LH. A steep increase in the mean scrotal circumference was observed up to week 22, followed by a moderate increase until week 34, before the scrotal circumference reached a plateau. A slow and steady increase in the mean body weight was observed throughout the period (Fig. 4-1).

The mean plasma INSL3 concentrations moderately increased throughout the sampling period. The mean plasma testosterone concentration showed a sharp increase from the very beginning of the measurement until week 26. Thereafter, the concentrations fell to a lower level and then continued at the same level before starting a further gradual increase from week 42 to reach a maximum level at week 46, maintaining this high level until the end of the study. The mean plasma LH level showed an initial peak at week 16 that was followed by a gradual decline until week 20 before it rose again slowly until the end of the experiment (Fig. 4-2).

The changes in the mean plasma concentrations of INSL3, testosterone and LH as well as the scrotal circumference data during the early, late and post-pubertal periods are presented in Fig. 4-3AB. There was a significant effect (P<0.001) of pubertal period on the plasma concentrations of all three hormones and the scrotal circumference. The mean plasma INSL3 concentrations increased significantly (P<0.01) from the early pubertal to late and post-pubertal periods, but they did not differ between the late pubertal and post-
pubertal periods (Fig. 4-3A). There was no significant difference in the mean plasma testosterone concentrations between the early and late pubertal periods, but a dramatic increase (P<0.01) was observed during the post-pubertal period compared to the early and late pubertal periods (Fig. 4-3A). The mean plasma LH concentrations increased significantly (P<0.05) from the early pubertal to late pubertal and from the late pubertal to post-pubertal periods (Fig. 4-3B). A significant increase (P<0.05) in the mean scrotal circumference from the early pubertal to late pubertal and from the late pubertal to post-pubertal periods was observed (Fig. 4-3B).

The correlations between scrotal circumference and the plasma concentrations of INSL3 and testosterone are shown in Fig. 4-4. The R² values of the best regression curves between scrotal circumference and INSL3, and between scrotal circumference and testosterone were 0.513 (n = 99, P<0.001) and 0.162 (n = 99, P<0.01), respectively. The correlation was thus higher between INSL3 and scrotal circumference. In addition, the R² values of the best regression curves in the plasma concentrations between INSL3 and testosterone, INSL3 and LH, and LH and testosterone were 0.246 (n = 99, P<0.001), 0.193 (n = 99, P<0.001) and 0.045 (n = 99, p = 0.226), respectively, in male goats from 10 to 52 weeks of age (data not shown).
Fig. 4-1. Changes of mean scrotal circumference and body weight from 14 to 52 weeks in male Shiba goats. Data are expressed as mean ± SEM (n = 4 and 5 for 14 to 16, and 18 to 52 weeks, respectively). P <0.001 was overall age effects.
Fig. 4-2. Changes of mean plasma concentrations of INSL3, testosterone and LH from 14 to 52 weeks in male Shiba goats. Data are expressed as mean ± SEM (n = 4 and 5 for 14 to 16, and 18 to 52 weeks, respectively). P <0.05 was overall age effects.
Fig. 4-3A. Changes in mean plasma concentrations of INSL3 (A) and testosterone (B) during and after puberty in male Shiba goats (n = 5). Blood samples were collected every two weeks from 10 to 52 weeks. Results are shown for early pubertal (10 to 22 weeks, n = 24), late pubertal (22 to 34 weeks, n = 30) and post-pubertal age (34 to 52 weeks, n = 45). Data are mean ± SEM. a–c Values without a common superscript differed significantly (P<0.05).
Fig. 4-3B. Changes in mean plasma concentrations of LH (A) as well as scrotal circumference data (B) during and after puberty in male Shiba goats (n = 5). Blood samples and scrotal circumference measurement were done every two weeks from 10 to 52 weeks. Results are shown for early pubertal (10 to 22 weeks, n = 24), late pubertal (22 to 34 weeks, n = 30) and post-pubertal age (34 to 52 weeks, n = 45). Data are mean ± SEM. a-c Values without a common superscript differed significantly (P<0.05).
Fig. 4-4. Best regression curves between scrotal circumference and plasma concentrations of INSL3 (n = 99; $R^2 = 0.513$), and scrotal circumference and plasma concentrations of testosterone (n = 99; $R^2 = 0.162$) from 10 to 52 weeks of age in male Shiba goats. An estimated equation of the best regression curve and an $R^2$ value are shown.
Discussion

A progressive increase of serum INSL3 with testicular volume during puberty was reported in boys (Johansen et al., 2014). To the best of the author’s knowledge, there is no other longitudinal report in mammals investigating the correlation between peripheral levels of INSL3 and testicular development. The present study is the first to investigate age-related changes of plasma INSL3 concentrations and its association with scrotal circumference in male goats.

The biweekly changes of scrotal circumference, body weight and testosterone in the present male Shiba goats were by and large in accordance with a previous report in the same species (Tani et al., 1992), except that the present findings suggesting a longer period of puberty. Tani et al. (1992) showed no increase of scrotal circumference after 5 months of age, which approximately coincides with the early pubertal period in the present study. The author noted an apparent moderate increase of scrotal circumference until around 9 months, which was assigned as late puberty.

The steep increasing of scrotal circumference from weeks 14 to 22 followed by a slower increase until week 34 in the present study is mostly in accord with a study in British breeds of male goats (Ahmad and Noakes, 1996). In that study, a rapid growth of scrotal circumference from 12 to 20 weeks of age followed by a period of slower growth until 25 weeks was reported. Though the author categorized pubertal stages based on the changes in scrotal circumference in the present Shiba goats, further studies should be conducted with frequent semen collection and scrotal circumference measurement throughout the pubertal development in order to more accurately determine the testicular size and age at which Shiba goats may start functional ejaculation.
The author observed here that the biweekly data of plasma INSL3 secretions implied a moderate increase throughout the study period, whereas the testosterone secretions fluctuated. There were apparently two sharp increase in testosterone secretions: the first increase occurred at around 6 months of age (the middle of puberty) and the second increase started after puberty at approximately week 42 (Fig. 4-2), confirming the results of an earlier report in the same species (Tani et al., 1992). In bulls, two testosterone peaks were reported during puberty: one at the beginning (around 6 months) and the other toward the end of puberty (10–12 months) (Rawlings et al., 1972; Amann RP, 1983; Evans et al., 1996; Rawlings et al., 2008). It was described that in bulls the first testosterone peak occurred after the pre-pubertal LH rise, which was associated with an increased number of Sertoli cells and a partial advance of spermatogenesis (Amann RP, 1983), whereas the second peak was associated with the completion of sexual maturation (Evans et al., 1996).

After categorizing the present data into three distinct groups of advancing puberty, it became clear that the secretory profiles of plasma INSL3, testosterone and LH in Shiba goats are different than the data reported for bulls (Kawate et al., 2011). Although the changes in INSL3 are similar between these species, the changes in testosterone are quite distinct. No increase in testosterone was observed from the late pubertal to the post-pubertal phase in bulls (Kawate et al., 2011), whereas in goats the author observed a clear increase in testosterone that was probably due to an increase in LH during the same period. Such an LH increase was absent in bulls. The author recently reported that plasma INSL3 is acutely regulated by LH in addition to testosterone in male goats and bulls (Hannan et al., 2015a; Hannan et al., 2016), but there are apparent differences in the
secretory profiles of testicular hormones during and after puberty between these ruminants.

In the present study, a strong positive correlation between the plasma concentrations of INSL3 and scrotal circumference existed for the overall period (10 to 52 weeks). This is in accord with previous reports in men (Ferlin et al., 2006; Johansen et al., 2014), in which increasing serum INSL3 secretions during pubertal development were shown to be concomitant with increasing testicular volume. Conversely, the weak correlation between the plasma concentrations of testosterone and scrotal circumference in the present study could be related to the high pulsatile nature of testosterone secretion. Although it was recently reported that plasma INSL3 is also pulsatile in male goats (Hannan et al., 2016) and bulls (Hannan et al., 2015a), the magnitude (peak per basal concentration) of testosterone pulses was significantly higher than that of the INSL3 pulses. It is thus most likely that testosterone concentrations are always more variable than INSL3 concentrations in single-time point blood sample measurements for the evaluation of Leydig cell status. The present findings thus support the hypothesis that plasma INSL3 would be a superior marker to evaluate testicular total Leydig cells during pubertal development in male Shiba goats.

Scrotal circumference has been used as a good indicator of puberty in bulls (Kastelic, 2014). In crossbred male goats, a marked increase in scrotal circumference indicated the onset of active spermatogenesis at 6–7 months (Bongso et al., 1982), and a scrotal circumference of 24.13 cm was shown at the time of sexual maturity at around 5–6 months in British breeds of male goats (Ahmad and Noakes, 1996). The present results thus imply that peripheral levels of INSL3 would be useful as a male fertility indicator,
although there is still no other evidence to support this. In rats, the known role of INSL3 during and after puberty is to suppress male germ cell apoptosis (Kawamura et al., 2004). In ruminants, further studies are needed to elucidate INSL3 concentrations in peripheral blood under various reproductive conditions (including normal and abnormal testicular functions and fertility status) to assess the importance of INSL3 as a male fertility indicator.

In conclusion, the author observed that in male Shiba goats, the plasma INSL3 concentrations increased continuously during and after puberty, but the testosterone secretions fluctuated during the same period. The scrotal circumference correlated with the INSL3 concentrations more strongly than with testosterone concentrations, suggesting that INSL3 is superior as a biomarker of testicular total Leydig cell volume.
Summary

Insulin-like peptide 3 (INSL3) has been used as a testis-specific biomarker for puberty in several species, but the secretory profile of INSL3 during pubertal development in small ruminants is unknown. The objectives were to study the age-related changes in the plasma concentrations of INSL3 and testosterone and their association with scrotal circumference during pubertal development in five male Shiba goats.

Blood samples and scrotal circumference measurement were taken every 2 weeks from weeks 10 to 52 of each goat's lifespan. Based on the changes in scrotal circumference, data were grouped into early pubertal (10–22 weeks), late pubertal (22–34 weeks) and post-pubertal (34–52 weeks) categories. The plasma concentrations of testosterone and LH were measured by EIAs, and a time-resolved fluorescence immunoassay (TRFIA) was used to measure plasma INSL3. The biweekly sampling showed that the plasma INSL3 secretions maintained a moderate increase during and after puberty, whereas the plasma testosterone secretions fluctuated over the same period. The comparison of the three age categories revealed a significant increase (P<0.01) in the mean plasma INSL3 concentrations during the late and post-pubertal periods compared to the early pubertal period. There was no difference in the mean plasma testosterone concentrations between the early and late pubertal periods, but a significant increase (P<0.01) was observed during the post-pubertal period compared to early and late pubertal periods. The mean plasma LH concentrations and scrotal circumference data increased significantly (P<0.05) from the early pubertal to late pubertal and from the late pubertal to post-pubertal periods. The $R^2$ value of the best regression curves between scrotal circumference and INSL3 (0.513; P<0.001) was higher than that between
scrotal circumference and testosterone (0.162; P<0.01) from 10 to 52 weeks of age.

In conclusion, in male goats, plasma concentrations of INSL3 increased continuously during and after puberty, whereas testosterone secretions were fluctuated. The scrotal circumference was more highly correlated with the INSL3 concentrations than with testosterone, implying that INSL3 is superior as a biomarker of testicular total Leydig cell volume.
General Discussion

As a marker of Leydig cell function, insulin-like peptide 3 (INSL3) has been emerged as a lucrative field of study in human reproduction (Bay et al., 2005; Ermetici et al., 2009; Foresta et al., 2009; Taneli et al., 2010; Cabrol et al., 2011). The extensive research on INSL3 in domestic male animals (Kawate et al., 2011; Pathirana et al., 2012) has also been undergoing in the author’s laboratory after developing immunoassay systems to quantify this peptide hormone. The age-related changes of INSL3 have been studied in humans (Anand-Ivell et al., 2006a), rodents (Anand-Ivell et al., 2009), cattle (Kawate et al., 2011), and male dogs (Pathirana et al., 2012); the secretory patterns of this hormone have been shown variable during sexual development among species. However, none of the above study has checked INSL3 secretion with frequent blood sampling. It is necessary to collect 10 to 15-min intervals sampling in order to depict the clear secretory profile of a hormone. In this thesis, the author has investigated secretory profile of INSL3 with 15-min intervals sampling in multiple male ruminants.

The clear information regarding the regulation of INSL3 secretion is still lacking in mammalian species. Only in humans, it was suggested that INSL3 secretion is stimulated by the long-term trophic effects of luteinizing hormone (LH) and dependent on the status of Leydig cell differentiation (Ivell et al., 1997; Bay et al., 2005; Foresta et al., 2004; Ferlin et al., 2006; Wikström et al., 2006). Moreover, it was shown that administration of human chorionic gonadotropin (hCG) stimulates testosterone secretion, but not INSL3, in human blood (Bay et al., 2006), implying that INSL3 is insensitive to acute LH stimulation. Here, the author has studied deeply to unveil the regulatory agent of INSL3 secretion with gonadotropin releasing hormone (GnRH), hCG and GnRH antagonist
treatment in male ruminants.

In chapter 1, the temporal relationship of pulsatile secretion among LH, INSL3 and testosterone, and the regulation of INSL3 secretion by LH using GnRH analogue and hCG was studied in pubertal Japanese Black beef bulls. The results suggested that the nature of releasing INSL3 from the testicular Leydig cells into the general circulation was pulsatile and 70% of INSL3 pulses peaked within 1 hour after the peak of an LH pulse, indicating that in most cases INSL3 pulses were associated with LH pulses. Moreover, the increase of INSL3 and testosterone concentrations within 1 to 2-hour after GnRH and hCG treatments, provided another information that LH acutely regulated the secretion of INSL3 in bulls. Previously, a significant increase of testosterone in the general circulation of bulls was reported after those treatments (D’Occhio and Aspden, 1996; Mongkonpunya et al., 1975; Sundby et al., 1975; Murase et al., 1990). The increase of INSL3 concentrations during normal pulsatile secretion and after stimulation by GnRH and hCG was much lower than that of testosterone in bulls.

A similar kind of study as mentioned above in chapter 1 is also done in male goats in chapter 2 for investigating whether or not the pulsatile secretory pattern of INSL3 and its acute regulation by LH are similar in small ruminants as shown for bulls. The results demonstrated that the pulsatile secretory patterns of LH, INSL3 and testosterone at 15-minute intervals for an 8-hour session were mostly in accordance with those in bulls as shown in chapter 1. Seventy one percent of INSL3 pulses peaked within 1 hour after a peak of an LH pulse. The significant increase of INSL3 release was observed just 30 minutes after GnRH and hCG treatment, confirming its acute regulation by LH. However, a 9.3-fold higher mean plasma concentrations of INSL3 in the male goat was observed.
than that shown in bulls. This was probably because of observed 14.4-times higher scrotal circumference per body weight ratio in male goats than in bulls.

In chapter 3, a long-acting GnRH antagonist, degarelix acetate, was administered in order to confirm the results of chapters 1 and 2, particularly acute regulation of INSL3 by LH. Here, the effect of LH suppression on INSL3 secretion and scrotal circumference was studied. The mean plasma concentrations of INSL3, based on mean concentrations of 15-minute intervals sampling, was lower on post-treatment Day 3, but according to a daily sampling, a significantly lower concentration was observed already 1 day after the GnRH antagonist treatment. The pulse frequency and amplitude of INSL3 were also decreased within 3 days after treatment indicating that secretion of this hormone was acutely regulated by LH. The reduction of plasma INSL3 secretion was in agreement with reported lower serum INSL3 concentrations after gonadotropins deprivation in men (Foresta et al., 2004; Bay et al., 2006). The long-term sampling revealed that the suppression of plasma concentrations of INSL3 and testosterone persisted for 8.5 weeks and both hormonal concentrations returned to pre-treatment levels at week 9. In male dogs, a single dose of another long-acting GnRH antagonist, acyline, decreased serum concentrations of testosterone for Day 9 after administration, and thereafter on Day 14 concentrations gradually began to increase and a clear rebound above baseline could also be seen at the end of the follow-up period of the study on Day 29 (Gobello, 2012; García Romero et al., 2009). A quick and long-lasting suppression of testosterone and INSL3 after a single injection, with subsequent recovery of the hormonal concentrations, implies a potential application of this antagonist by means of reversible long-term chemical castration in male goats. In addition to acute hormonal suppression, a reduction in scrotal
circumference was observed from week 4 to at least week 10 after treatment, suggesting that the reduction in testicular size was subsequent to peripheral hormonal suppression. It was reported in rats that degarelix acetate caused a remarkable decrement of testicular weight on Day 45 which was largely restored to pretreatment values on Day 102 (Broqua et al., 2002).

In chapter 4, the age-related changes of plasma concentrations of INSL3 and testosterone, and their association with scrotal circumference during pubertal development (10 to 52 weeks of age) was studied in male Shiba goats. Plasma INSL3 secretions maintained a moderate increase during and after puberty, whereas plasma testosterone secretions were fluctuated. There were apparently two sharp increase/peak in testosterone secretions, first at around 6 months (middle of puberty) and the second one started after puberty at about week 42, confirming the results of earlier report in the same species (Tani et al., 1992). Previously, in bulls two testosterone peaks were reported during puberty, one at the beginning (around 6 months) and the other toward the end of puberty (10-12 months) (Rawlings et al., 1972; Amann RP, 1983; Evans et al., 1996; Rawlings et al., 2008).

A strong positive correlation between plasma concentrations of INSL3 and scrotal circumference in present study is in accordance with reported studies in men (Ferlin et al., 2006; Johansen et al., 2014). On the other hand, a weak correlation between plasma concentrations of testosterone and scrotal circumference in the present study could relate to the high pulsatile nature of testosterone secretion. Therefore, it is believed that plasma INSL3 would be a superior marker to evaluate testicular total Leydig cell volume during pubertal development in male Shiba goats.
Taken together, the outcomes of the studies presented in this thesis have clearly depicted the secretory profile of INSL3 and its temporal association with LH and testosterone during normal physiology in the peripheral blood of male ruminants. And, the findings have unveiled that LH is the controlling agent of INSL3 secretion. It is also shown that INSL3 is a better correlator with scrotal circumference than testosterone in evaluating testicular Leydig cell’s status in small ruminants.
Conclusions

1. The secretion of INSL3 in the general circulation of bulls and male goats occurred in a pulsatile manner.
2. INSL3 secretion was acutely regulated by LH in bulls and male goats.
3. The increase of plasma INSL3 concentrations during normal pulsatile secretion and after LH stimulation was much lower than that for testosterone in bulls and male goats.
4. The absolute concentration of plasma INSL3 in male goats was much higher than that in bulls.
5. The acute regulation of INSL3 by LH was further confirmed by a long-acting GnRH antagonist (degarelix acetate) treatment in male goats.
6. The GnRH antagonist can be used for reversible long-term chemical castration in male goats.
7. Scrotal circumference was more strongly correlated with plasma INSL3 concentrations than with plasma testosterone concentrations during pubertal development in goats. Thus, INSL3 is suggested superior as a biomarker of testicular total Leydig cell volume in male goats.
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