Insulin-like peptide 3 (INSL3) secretion in male domestic animals and the effects of INSL3 on Leydig cells

大阪府立大学博士（獣医学）学位論文

Insulin-like peptide 3 (INSL3) secretion in male domestic animals and the effects of INSL3 on Leydig cells

（雄飼育動物におけるインスリン様ペプチド３（INSL3）分泌とライディッヒ細胞に及ぼす INSL3 の効果）

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General Introduction

Insulin-like peptide 3 (INSL3; formerly, Leydig cell insulin-like factor or relaxin-like factor) was first discovered in 1993 as a novel gene product from porcine Leydig cells (Adham et al., 1993). In 1999, it was first recognized as a circulating hormone in human blood (Büllesbach et al., 1999). INSL3 is a member of the relaxin-insulin family of peptide hormones owing to its structural similarities with the rest of the group members including relaxin, insulin and insulin-like growth factors. It is highly expressed in the Leydig cells of many mammalians including human (Ivell et al., 1997), canine (Klonisch et al., 2001), bovine (Irving-Rodgers et al., 2002) and rodent (Balvers et al., 1998; Sadeghian et al., 2005) species, and also in the ovarian counterpart to testicular Leydig cells, namely theca cells and in the cells of corpus luteum (Bamberger et al., 1999). However, circulating INSL3 concentrations are very high in males compared with that of females (Büllesbach et al., 1999; Bay et al., 2007). Thus, at present, it is considered as a specific product of testicular Leydig cells and a major circulating hormone in males (Ivell and Anand-Ivell, 2009; Bay and Andersson, 2010). INSL3 acts through a G protein-coupled receptor called relaxin family peptide receptor 2 (RXFP2; previously called LGR8) (Ivell and Bathgate, 2007). RXFP2 is expressed in Leydig cells and postmeiotic germ cells within the testis, and also in several non-gonadal tissues including gubernacular ligament in male humans (Anand-Ivell et al., 2006b), mice (Feng et al., 2007) and dogs (Arrighi et al., 2010).

INSL3 has been strongly suggested as a useful marker of Leydig cell differentiation and maturation according to the evidences of INSL3 expression in rodent testes of varying postnatal ages (Balvers et al., 1998; Mendis-Handagama et al., 2007). Moreover, Balvers et

al. (1998) demonstrated that differentiation status of Leydig cells influence the quantitative expression of INSL3, using hypogonadal (*hpg*) mice. The substantial INSL3 production by both fetal and adult-type Leydig cells contributes to most if not all circulating hormone levels in the blood, thus INSL3 can be considered as an important peripheral marker in assessing Leydig cells function and testicular status (Ivell and Bathgate, 2007). During the recent past, serum INSL3 has been emerged as a novel clinical marker of Leydig cell function in humans (Bay et al., 2005; Ermetici et al., 2009; Foresta et al., 2009). Peripheral INSL3 concentrations have been increasingly used to assess the Leydig cell function during sexual development (Ferlin et al., 2006; Wikström et al., 2006; Bay et al., 2007), ageing (Anand-Ivell et al., 2006a) and testicular status-related (both directly and indirectly) clinical conditions such as cryptorchidism (Bay et al., 2007), hypogonadotropic hypogonadism (Bay et al., 2005), obesity (Foresta et al., 2009), diabetes mellitus (Ermetici et al., 2009). INSL3 dynamics related to sexual development have been reported only in humans (reviewed by Bay and Andersson, 2010) and rodents (Boockfor et al., 2001; Anand-Ivell et al., 2009) and claimed that it reflects the functional status of Leydig cells in those species; peripheral INSL3 concentrations were relatively higher at birth, but remained very low at pre-pubertal period, and increased during puberty to reach high levels at puberty and post-puberty. Despite these important advances in INSL3 research in humans and rodents, blood INSL3 profiles in male domestic animals, including the changes during sexual development, are totally unknown. Thus, blood INSL3 dynamics in a broad age range is worth investigating in many species, including bulls and male dogs. Here, the main constraint is the lack of quantification assays for plasma INSL3 in domestic animal species.

INSL3 is essential for the process of testicular descent, and *Insl3* knockout mice showed a cryptorchid phenotype with both testes lying in an intra-abdominal position (Nef and Parada, 1999; Zimmermann et al., 1999; Sozubir et al., 2010). It has been demonstrated that INSL3 is vital for the thickening of gubernacular ligament ("swelling reaction") which facilitate the descent of testis from higher abdominal position to a lower abdominal position i.e. trans-abdominal descent (Nef and Parada, 1999). Moreover, this whole process is strictly hormonally regulated and INSL3, along with testosterone, plays a critical role (Hutson and Hasthorpe, 2005; Toppari et al., 2006; Foresta et al., 2008). The congenital failure of descent of one or of both testes is called cryptorchidism (CO), and it is a common male anomaly in many species including humans (Kaleva and Toppari, 2005; Foresta et al., 2008) and dogs (Romagnoli, 1991; Amann and Veeramachaneni, 2007; Birchard and Nappier, 2008). In dogs, incidence of CO ranges from 1.2 and 10.7% (Kawakami et al., 1984; Ruble and Hird, 1993; Nielen et al., 2001; Yates et al., 2003), and the susceptibility is higher in small pure breeds such as the Toy Poodle, Pomeranian, Miniature Dachshund and Chihuahua (Hayes et al., 1985). It has been previously reported that plasma testosterone concentrations were lower in bilateral cryptorchid dogs compared to normal dogs (Kawakami et al., 1995). Despite its (of INSL3) important role in testicular descent, the changes in INSL3 concentrations of cryptorchid dogs, if any, is yet to be investigated. In humans, Bay et al. (2007) reported that INSL3 concentrations were significantly lower in umbilical cord blood of infants with persistent cryptorchidism than in normal infants. Peripheral INSL3 and testosterone concentrations are obviously worth comparing in normal and cryptorchid dogs, with a view to assess the functional status of Leydig cells in CO. Both these hormones are from Leydig cell origin and, in humans, serum concentrations of

the above two hormones were positively correlated (Ferlin et al., 2005; Taneli et al., 2010). Recently, Ferlin et al. (2010) suggested that INSL3 and testosterone measurements provide different information on the status of Leydig cells, as the former reflects the differentiation status and general wellness while the latter reflects the steroidogenic activity.

Information is lacking regarding the successful effectors on INSL3 secretion from Leydig cells. The limited experimental data available in human males suggested that INSL3 secretion is regulated by long-term trophic effects of luteinizing hormone (LH), and at present, it is believed to be constitutively regulated. In adult men, administration of human chorionic gonadotropin (hCG) did not result in increased serum levels up to 8 days after administration (Bay et al., 2005, 2006). However, demonstrating the long term regulatory effects of LH, parallel INSL3 increase has been observed with LH during the period of pre-puberty to puberty; these INSL3 and LH levels were highly positively correlated (Ferlin et al., 2006). It should be noted that the regulatory pattern of INSL3 is very different from that of testosterone, the latter being acutely sensitive to LH (Bay and Andersson, 2010). Owing to differential patterns of regulation, the measurement of circulating concentrations of both INSL3 and testosterone in the same animal may provide an added benefit in assessing Leydig cell function *in vivo*.

Confirming the *in vivo* evidences in humans, Anand-Ivell et al. (2009) reported that hCG (0-150 ng/mL) had no acute stimulatory effect on primary rat Leydig cells for INSL3 secretion. At present, no direct *in vitro* evidences were found in literature regarding the direct effectors on INSL3 secretion from Leydig cells of any species including humans. For the regulation of INSL3 gene, scarce evidence showed that testosterone-stimulated INSL3 gene expression has been suppressed by mono-(2-ethylhexyl) phthalate (MEHP) (Laguë

and Tremblay, 2008). Phthalates, a group of estrogenic environmental chemicals, are readily available in health and beauty supplies (e.g. cosmetics and perfumes) and flexible plastics such as children's toys, medical equipments (e.g. dialysis tubing and intravenous bags) (Lovekamp-Swan and Davis, 2003; Ge et al., 2007a; Howdeshell et al., 2008). Monoesters of phthalates are biologically active and exert profound direct effects on Leydig cells (Ge et al., 2007a) and therefore, considered as reproductive toxicants. Among phthalates, MEHP and monobutyl phthalate (MBP) are the most widely studied phthalate monoesters which have been investigated for their adverse effects on reproduction.

In addition to well-elucidated role of INSL3 in testicular descent process during fetal and neonatal periods, it is presumably involved in the prevention of germ cell apoptosis (Kawamura et al., 2004) and in proliferation of osteoblasts (Ferlin et al., 2008) in adults. Although RXFP2 is expressed in a vast variety of tissues in the body, very little is known about other functions of INSL3. Regardless of the localization of RXFP2 in Leydig cells of several mammalian species i.e. humans (Anand-Ivell et al., 2006b), mice (Feng et al., 2007) and dogs (Arrighi et al., 2010), the effects of INSL3 on primary Leydig cells are also unknown. Kumagai et al. (2002) showed that INSL3 stimulates adenosine 3', 5'-cyclic monophosphate (cAMP) production in human embryonic kidney (HEK)-293T cells which have been transfected with recombinant RXFP2, and has been suggested that cAMP as an important mediator for the cellular effects of INSL3. However, previous attempts have been unsuccessful in investigating the effects of INSL3 on primary Leydig cells, considering testosterone and cAMP production as endpoint measurements (Anand-Ivell et al., 2006b).

In this thesis, the author presents experimental evidences on novel immunoassay systems which were developed to quantify bovine and canine INSL3. Establishment of

these assays enabled us to conduct a series of *in vivo* and *in vitro* studies on bovine and canine INSL3, and to compare those novel findings with a well-elucidated hormone such as testosterone. In chapter 1, dynamics of plasma INSL3 and testosterone concentrations were investigated in normal beef bulls from birth to pubertal age. In chapter 2, the effects of age and CO on changes of plasma INSL3 concentrations were examined in dogs, and were compared with those of plasma testosterone concentration changes. In chapter 3, INSL3 and testosterone secretory capacity in response to various effectors of Leydig cells was investigated in cultured testicular interstitial cells of dogs. In chapter 4, the effects of INSL3 on canine and mouse testicular interstitial cells, and on purified mouse Leydig cells were examined by measuring testosterone and cAMP as end-points.

Chapter 1

Dynamics of plasma INSL3 and testosterone from birth to pubertal age in bulls

Introduction

Radioimmunoassay (RIA) and time-resolved fluorescence immunoassay (TRFIA) were developed for determination of peripheral blood concentrations of INSL3 in rats and humans (Büllesbach et al., 1999; Boockfor et al., 2001; Bay et al., 2005; Ferlin et al., 2006; Anand-Ivell et al., 2009). Serum INSL3 concentrations increased during puberty in male rats and humans. However, it is noteworthy that changes in concentration differed from those of testosterone (Ferlin et al., 2006; Anand-Ivell et al., 2009), which is also secreted from Leydig cells and is essential for puberty. In men, serum INSL3 concentration was apparently not affected acutely by LH bioactivity, and INSL3 is clearly more sensitive in assessing the functional capacity of Leydig cells, compared with testosterone (Bay and Andersson, 2010).

For bulls at various sexual developmental stages, concentrations of INSL3 in peripheral blood are not yet known. Lack of assay systems for bovine INSL3 quantification appeared as the main constraint. Therefore, in this study, an enzyme immunoassay (EIA) was developed to quantify INSL3 in bovine plasma to investigate changes in plasma INSL3 concentrations from birth to pubertal age in beef bulls, and to compare changes in INSL3 concentrations with those of testosterone.

Materials and Methods

Bull calves

Japanese Black beef bull calves (n = 15) born in 2005 and 2007 in an experimental beef cattle station in the Northern Center of Agriculture Technology of Hyogo Prefecture in Japan were used in this study. All of these calves were normal at birth and were healthy throughout the experiment. These calves were weaned at 2 days of age and were fed milk replacer and calf starter pellets, with *ad libitum* access to hay and water. Growth rates for calves on the early weaning program did not differ from those of calves which suckled for 3 months after birth (unpublished data). Blood samples were collected on the day of birth (0 months; 0 days) and at 1 month (28 days), 2 months (56 days), and 3 months (84 days) after birth. Blood was collected (jugular vein) in heparinized vacutainers and centrifuged (1,700 × g for 20 min). Plasma was separated and stored at – 30 ^{o}C.

Bulls during puberty

Japanese Black beef bulls (n = 26, age 3 – 22 months) that were kept at the same station were also used from June 2008 to July 2009. These bulls were normal in appearance and healthy during the experiment. Bulls used in this study were fed hay and concentrate to meet or exceed Japanese Feeding Standard recommendations for beef bull calves and bulls. Blood samples were collected at 1 – 4 months intervals starting from 3 –21 months of age until a maximum age of 22 months. This was not a complete longitudinal study, although in most of the bulls blood samples were taken repeatedly. The number of blood collections varied among bulls (range, 1 to 12). Blood was collected from the jugular vein in

heparinized vacutainers and was centrifuged at 1,700 × g for 20 min. Plasma was separated and stored at –30 ^{o}C prior to conducting hormone assays.

Hormone assays

Reagents

Anti-bovine INSL3 mouse monoclonal antibody (2-8F) and synthetic bovine INSL3 have been described (Büllesbach and Schwabe, 2002). The same synthetic approach was used to produce A1-biotinylated bovine INSL3, starting with A1-biotinyl-aminohexanoyl-A chain and unmodified B chain.

ProClin 950, 3,3',5,5'-tetramethylbenzidine (TMB), bovine serum albumin (BSA) Cohn Fraction V and bovine insulin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-rabbit IgG goat polyclonal antibody and anti-mouse IgG rabbit polyclonal antibody were products of MP Biochemicals (Solon, OH, USA). Anti-testosterone-3(E)-carboxymethyloxime-BSA rabbit polyclonal antibody and horseradish peroxidase (HRP)-labeled testosterone-3-carboxymethyloxime were purchased from Cosmo Bio Co. (Tokyo, Japan), whereas HRP-labeled streptavidin was obtained from KPL Inc. (Gaithersburg, MD, USA). Human relaxin was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Human insulin-like growth factor (IGF)-1 was a gift from NIDDK, NIH (Bethesda, MD, USA).

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 min, 200 μL of acetonitrile was added, the sample vortexed, and left for 30 min at room temperature. Next, the mixture was centrifuged at 15,000 × g for 10 min at room temperature. The resulting supernatant was concentrated by vacuum centrifugation (Centrifugal Concentrator CC-105; Tomy Seiko Inc., Tokyo, Japan) for approximately 2 h (final volume, approximately 30 μL). Then, 80 μL of 0.05 M phosphate buffer (pH 7.5) was added to the concentrated supernatant, and finally, distilled water was added to bring the final volume to 125 μL.

Preliminary experiments were conducted to determine the need to extract bovine plasma prior to determination of INSL3 concentrations. Plasma samples from four steers were measured with or without the extraction procedure as described above, and values compared. To determine recovery rates of INSL3 from bovine plasma, two sets of same-concentration standards were compared as follows: 1) INSL3 standards were diluted in the assay buffer and assayed directly; and 2) standards were diluted with steer plasma, followed by the extraction procedure, and assayed. Recovery rates were determined from the assayed concentrations in the plasma, divided by the concentrations added to the plasma. To determine parallelism with the INSL3 standards curve, a plasma sample from a normal bull was serially diluted in plasma obtained from a steer (1, 2.5, 5 and 10 times dilutions) and assayed after the extraction procedure.

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 (5 µg/mL in 0.05 M sodium bicarbonate, pH 9.7) for 2 h 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 samples were kept overnight at 4 ^{o}C for blocking the wells. 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 4 ^{o}C overnight. Bovine INSL3 standards were diluted with steer plasma to create various concentrations (0.5 – 20 ng/mL). The INSL3 standards and plasma samples were extracted simultaneously using the above procedure. The next day, the wells were kept for 30 min at room temperature and 50 µL of the biotinylated bovine INSL3 was added (1.33 ng/mL in assay buffer I); the mixture was kept for 1 h at room temperature. After the reaction, the wells were drained and washed five 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 (100 ng/mL in assay buffer I) was added to the wells, which were kept for 30 min at room temperature. The wells were drained, washed three times with wash buffer, and 100 µL of substrate solution containing the TMB was added. After 30 min 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 and also with a 570 nm reference, using a

microplate absorbance spectrophotometer (xMark; Bio-Rad Laboratories Inc., Hercules, CA, USA).

The minimum detection limit of the assay was 0.5 ng/mL, and the percent binding (B/B_0 74.3 ± 4.2%, n = 6) of the concentration was lower ($P < 0.05$) than B_0 (100%). The intra- and inter-assay coefficients of variation (CV) were 19.4% (n = 8) and 14.9% (n = 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 (5 µg/mL in 0.05 M sodium bicarbonate; pH 9.7), and non specific binding sites were blocked overnight with 200 µL of 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 (0.04 – 20 ng/mL) were diluted with the assay buffer T. Then, 200 µL of standards or bovine plasma samples were extracted with 3 mL of diethylether and the dried extract was dissolved in 200 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 (1:1,500 dilution in the assay buffer T), and 50 µL of anti-testosterone rabbit polyclonal antibody (1:400,000 dilution in the assay buffer T). The mixture was then incubated for 2 h at room temperature. After the reaction, the wells were drained and washed three times with 400 µL of wash buffer. Further steps were the same as in the INSL3 EIA.

The minimum detection limit of the assay was 0.04 ng/mL. The cross-reactivities of the anti-testosterone antibody with 5α-dihydrotestosterone, 4-androstenedione, androsterone, and estradiol-17ß were 7.0, 2.0, 0.2, and <0.01%, respectively. The intra- and inter-assay CVs were 7.2% (n = 4) and 7.8% (n = 6).

Statistical analysis

For plasma concentrations of INSL3 and testosterone in the young bull calves, the effect of time (age in months) was evaluated by repeated measures ANOVA, using the Generalized Estimating Equations (GEE) procedure (IBM SPSS (PASW) Statistics 18.0; IBM Corporation, Somers, NY, USA). Ages of the bulls (during pubertal ages) were categorized into four groups: 1) pre-pubertal phase (3 – 6 months, n = 13); 2) early pubertal phase (6 – 12 months, n = 68); 3) late pubertal phase (12 – 18 months, n = 36); and 4) post-pubertal phase (18 – 22 months, n = 7), according to range of the pubertal age in bulls (Roberts, 1971). For plasma concentrations of INSL3 and testosterone of the bulls during the pubertal phase, the effect of age was evaluated by ANOVA using the Generalized Linear Model procedure (IBM SPSS Statistics 18.0). Differences in mean concentrations between two groups were analyzed using the Least Significant Difference (LSD) post hoc test of the GEE or Generalized Linear Model procedure (IBM SPSS Statistics 18.0). Best regression curves were estimated between two hormone concentrations using the Curve Estimation procedure of Regression Analysis (IBM SPSS Statistics 18.0). For all statistical analyses, $P < 0.05$ was considered significant.

Results

Establishment of an assay for INSL3 in bovine plasma

In the preliminary experiment, plasma INSL3 concentrations in steers were determined with or without the extraction procedure. Without the extraction procedure, the assay yielded higher values than the minimum detection limit (0.5 ng/mL) in two of the four plasma samples from castrated bulls. However, with the extraction procedure, all samples from steers yielded values less than the minimum detection limit. Thereafter, the extraction procedure using TFA and acetonitrile was done before the INSL3 assay.

The INSL3 standards, which were diluted in steer plasma and assayed after extraction, were compared with the same concentrations of standards diluted in the assay buffer assayed without extraction. Both standards had almost identical inhibition at the same concentrations in the range 0.5 – 20 ng/mL (Fig. 1-1). The recovery rates of the added bovine INSL3 in steer plasma (1 – 20 ng/mL) were greater than 91%. The B/B0 values of the serially diluted bovine plasma sample were almost parallel to both standards (Fig. 1-1). The bovine INSL3 assay system did not cross-react with bovine insulin, human relaxin or human IGF-1 (1 ng/mL to 1 μg/mL) (Fig. 1-2).

Plasma concentrations of INSL3 and testosterone in bull calves

There was an effect of age on plasma INSL3 concentrations in bull calves ($P < 0.001$). These concentrations increased from 0 to 1 month ($P < 0.05$), from 1 to 2 months ($P < 0.001$), and from 2 to 3 months ($P < 0.005$; Fig. 1-3A). The INSL3 concentration increased from birth to 3 months by a factor of 3.5. There was an effect of age on plasma testosterone concentrations in bull calves ($P < 0.001$); these concentrations increased ($P<0.001$) from 0

to 1 month, and from 1 to 2 months, but did not change ($P > 0.15$) from 2 to 3 months (Fig. 1-3B). Plasma testosterone concentrations increased from birth to 2 months by a factor of 14.9.

Plasma concentrations of INSL3 and testosterone in bulls during puberty

There was an effect of age group on plasma INSL3 concentrations in bulls during pubertal age ($P < 0.001$). Although INSL3 concentrations did not change from pre-pubertal to early pubertal phases ($P > 0.15$; Fig. 1-4A); there was an increase from the early to late pubertal phases ($P < 0.005$), and from the late pubertal to post-pubertal phases ($P < 0.05$). There was an effect of age group on plasma testosterone concentrations in bulls during pubertal age ($P < 0.005$); these concentrations increased ($P < 0.001$) from the pre-pubertal to early pubertal phases (Fig. 1-4B), but did not change ($P > 0.15$) from the early to late puberty phases, or from the late pubertal to post-pubertal phases.

Regression analyses among INSL3 and testosterone concentrations

The R^2 values of best regression curves between INSL3 and testosterone concentrations in bull calves, bulls around pubertal age and the full period (from birth through pubertal age) were 0.639 (n = 60, $P < 0.001$;Fig. 1-5A), 0.059 (n = 124, $P < 0.01$; Fig. 1-5B) and 0.359 (n = 184, $P < 0.001$; Fig. 1-5C), respectively.

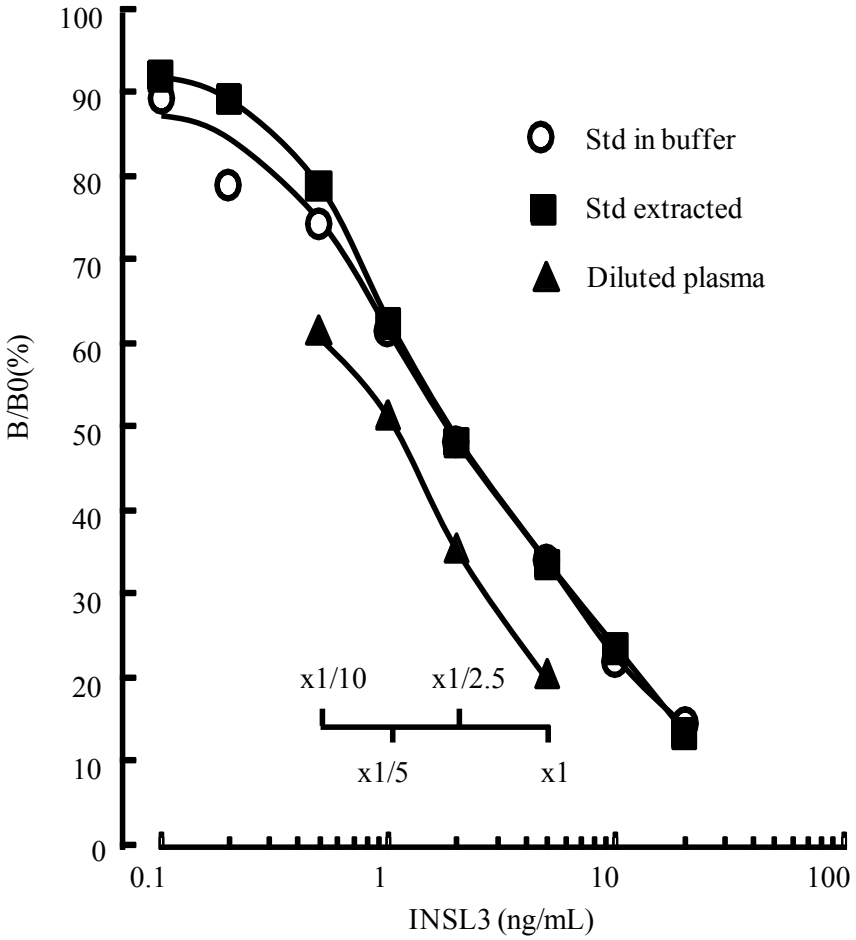

Fig. 1-1. Competition curves for INSL3 standards in assay buffer (Std in buffer), standards in plasma followed by extraction (Std extracted), and a serially diluted bull plasma sample. Data are expressed as the percent binding of each concentration (B/B0 X 100). The bull plasma sample was diluted 1, 2.5, 5, and 10 times.

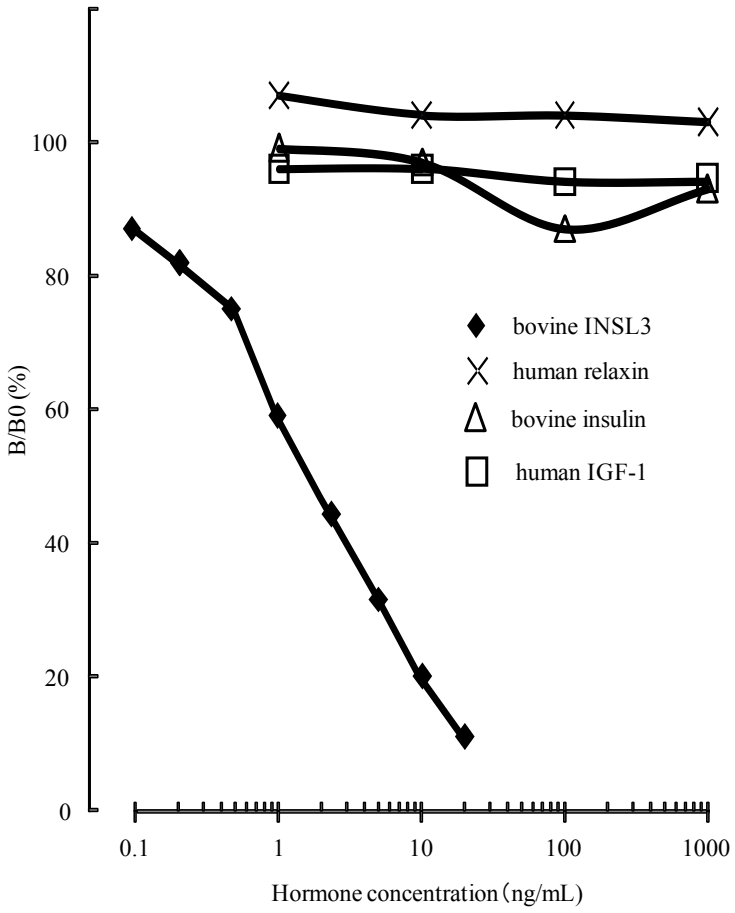

Fig. 1-2. Cross-reactivity of anti-bovine INSL3 antibody with bovine INSL3 and other structurally related peptides. Data are expressed as the percent binding of each concentration (B/B0 X 100). IGF-1, Insulin-like growth factor-1.

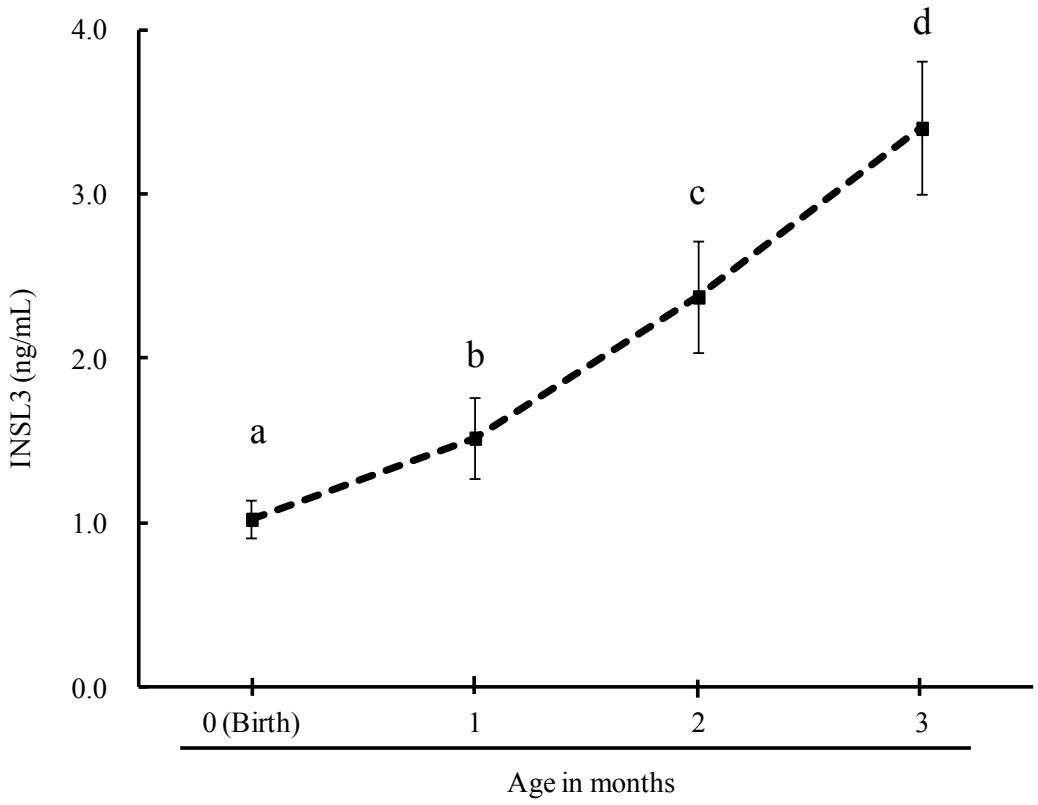

Fig. 1-3A. Mean ± SEM plasma concentrations of INSL3 in beef bull calves (n = 15).

[a-d]Values without a common superscript differed ($P < 0.05$).

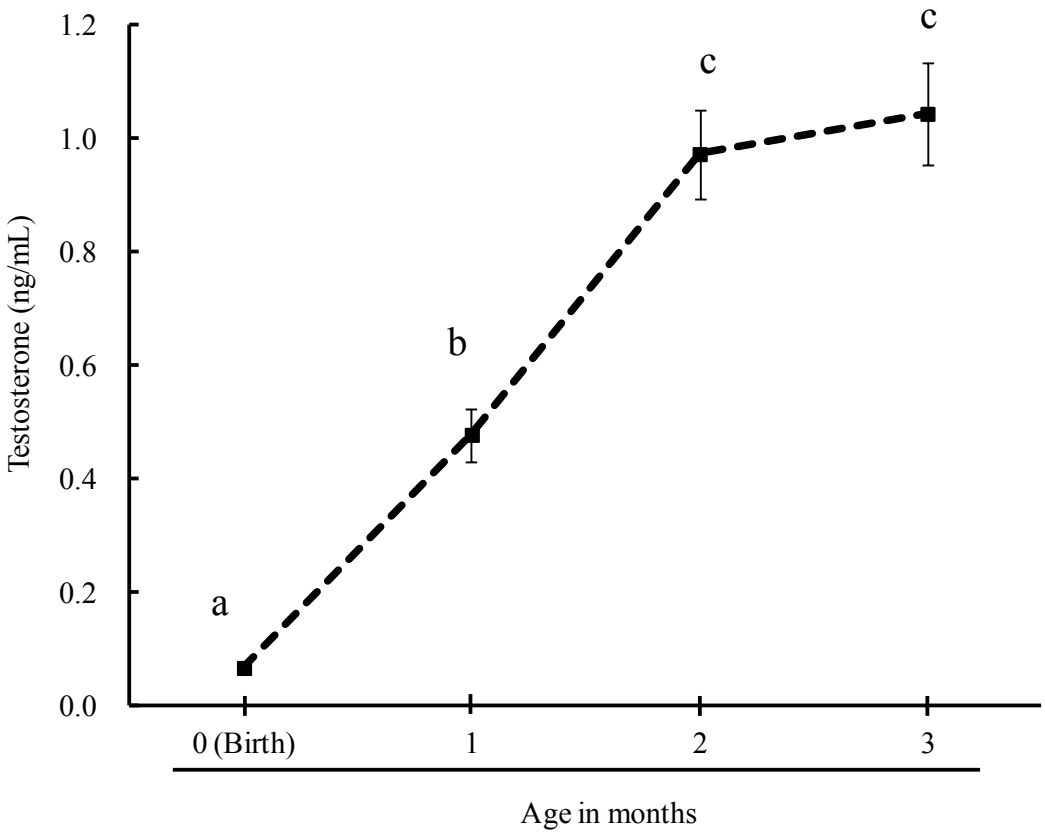

Fig. 1-3B. Mean ± SEM plasma concentrations of testosterone in beef bull calves (n = 15). [a-c] Values without a common superscript differed ($P < 0.05$).

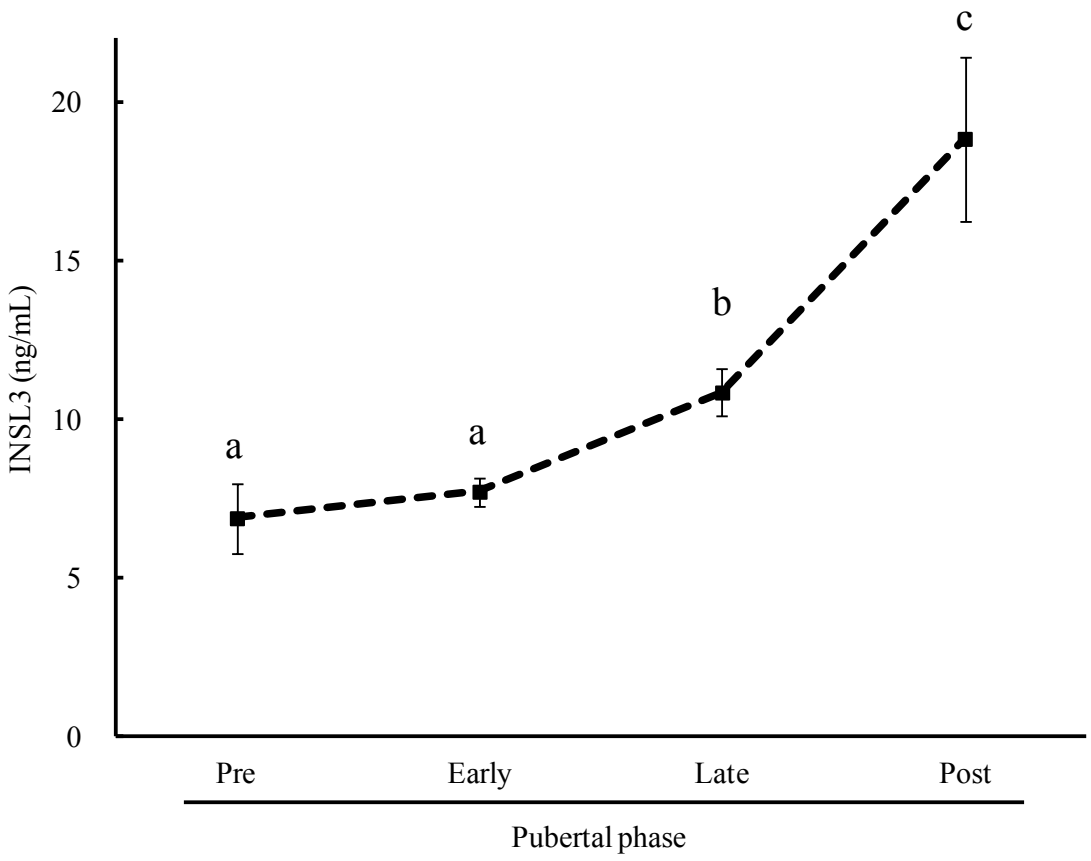

Fig. 1-4A. Mean ± SEM plasma concentrations of INSL3 in beef bulls around pubertal age. Data are for pre-pubertal (3 – 6 months, n = 13), early pubertal (6 – 12 months, n = 68), late pubertal (12 – 18 months, n = 36), and post-pubertal (18 – 22 months, n = 7) phases. [a-c] Values without a common superscript differed ($P < 0.05$).

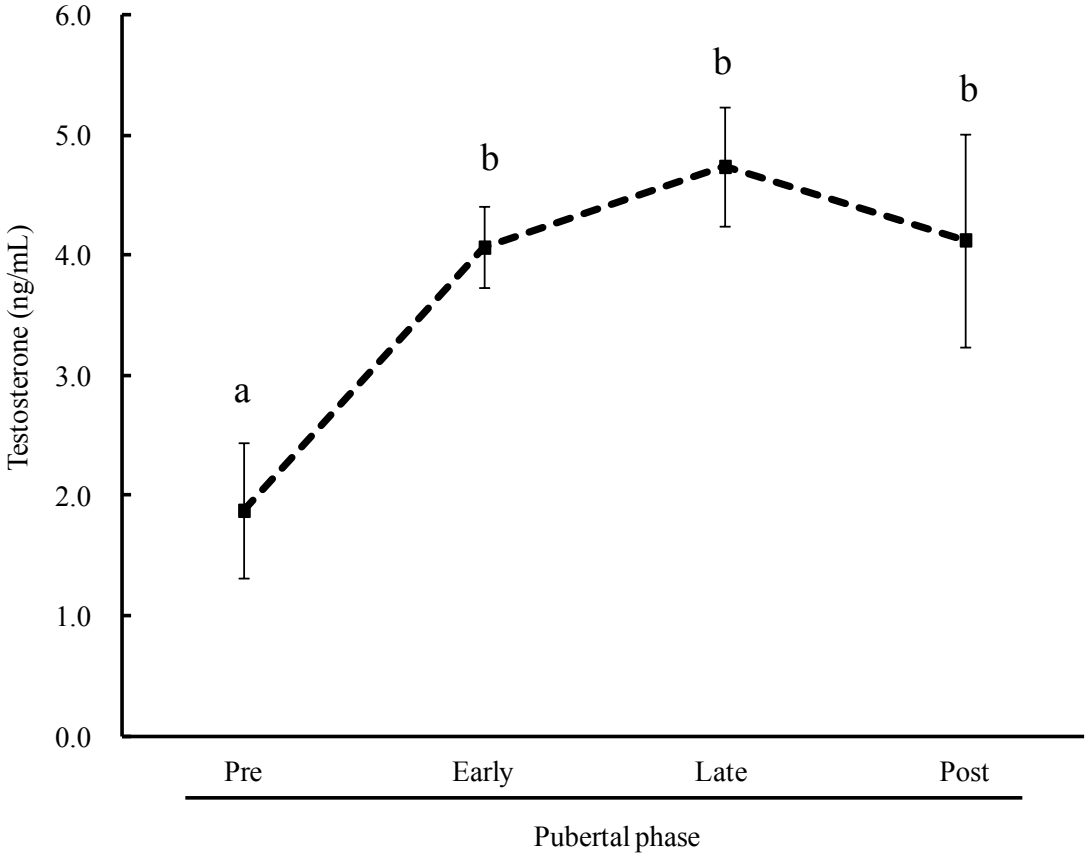

Fig. 1-4B. Mean ± SEM plasma concentrations of testosterone in beef bulls around pubertal age. Data are for pre-pubertal (3 – 6 months, n = 13), early pubertal (6 – 12 months, n = 68), late pubertal (12 – 18 months, n = 36), and post-pubertal (18 – 22 months, n = 7) phases. [a-b] Values without a common superscript differed ($P < 0.05$).

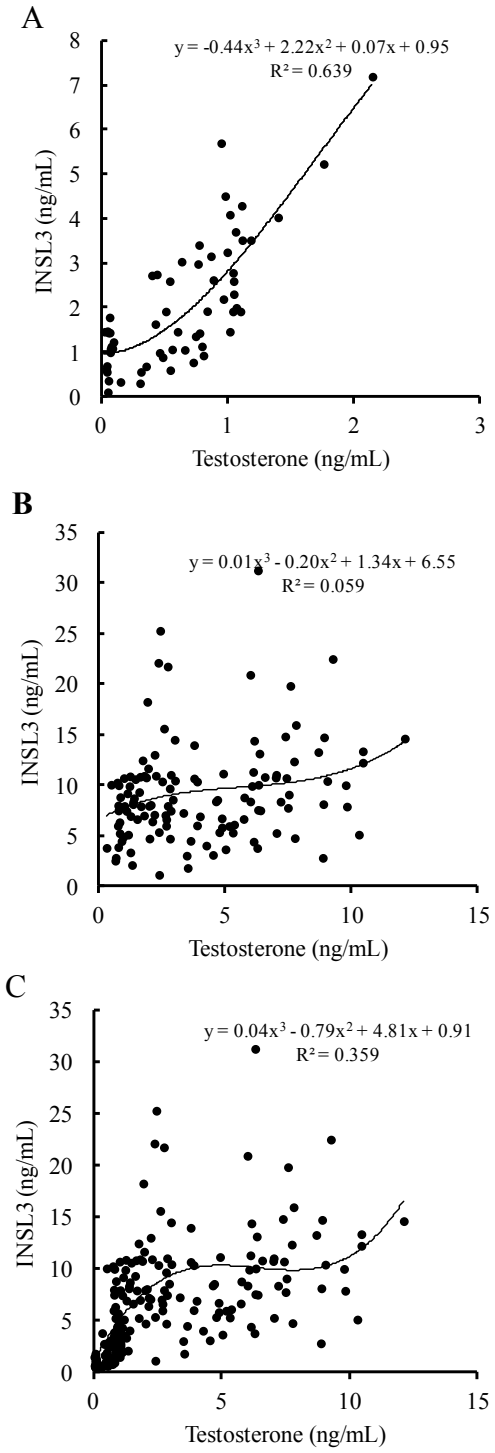

Fig. 1-5. Best regression curves between plasma concentrations of INSL3 and testosterone in bull calves (0 – 3 months, n = 60) (A), bulls around pubertal age (3 – 22 mo, n = 124) (B), and the overall period from birth through pubertal age (0 – 22 months, n = 184) (C). An estimated equation of best regression curve and an R^2 value are shown for each figure.

Discussion

Reports regarding INSL3 concentrations in peripheral blood are apparently limited to humans (Büllesbach et al., 1999; Bay et al., 2005, 2007; Ferlin et al., 2006; Anand-Ivell et al., 2006a; Wikström et al., 2006) and rodents (Boockfor et al., 2001; Anand-Ivell et al., 2009). In the present study, an EIA for INSL3 in bovine plasma was developed using biotinylated bovine INSL3 and anti-bovine INSL3 antibody, with an extraction procedure using TFA and acetonitrile prior to the assay. In this study, the anti-bovine INSL3 antibody was confirmed to be specific to INSL3, and the sensitivity of INSL3 EIA was similar to that of RIA for rats (Boockfor et al., 2001), but lower than that of TRFIA for humans (Bay et al., 2005). Recovery of the bovine INSL3 added to the steer plasma exceeded 90%, suggesting reasonable accuracy and reliable measurements of bovine plasma samples for the assay system, including the extraction step. The intra- and inter-assay coefficients of variation of this assay seemed slightly higher than those of TRFIA for humans (Bay et al., 2005).

In the present study, changes in plasma INSL3 concentrations in bulls from birth to pubertal age were elucidated using the EIA described above. Plasma INSL3 concentrations in bull calves were low (approximately 1 ng/mL) at birth and then increased continuously from birth to 3 months of age. These concentrations in bulls at the pre-pubertal phase (3 to 6 months) remained unchanged to the early pubertal phase (6 to 12 months), but started to increase again from the early to late pubertal phases (12 to 18 months) and continued to rise during the post-pubertal phase (18 to 22 months). Therefore, we inferred that INSL3 secretions increased in most of the periods from birth to post-pubertal ages in bulls. In humans, serum INSL3 concentrations in males were high for a few months after birth,

decreased sharply to very low concentrations between a few months and 1 year of age (Cabrol et al., 2011), remained low until 10 years of age, and thereafter increased during puberty (Ferlin et al., 2006; Bay et al., 2007). In rats, serum INSL3 concentrations decreased rapidly from 2 days before birth to 3 days after birth, remained low up to 10 days after birth, and then increased to pubertal age (Boockfor et al., 2001; Anand-Ivell et al., 2009). Decreased INSL3 secretion between the neonatal and pubertal periods in male humans and rats seemed absent in bulls.

The high R^2 value between plasma concentrations of INSL3 and testosterone in bulls for the overall interval from birth to post-pubertal phases indicated a similarity in release patterns for these hormones (which are both produced in Leydig cells), especially during infancy, since the R^2 value was higher for infancy than for the period around pubertal age. Conversely, around the pubertal age, it appeared that these two hormones diverged; there was a sustained increase of INSL3 concentrations during and after the pubertal age, whereas testosterone concentrations reached a maximal concentration in the early pubertal phase. The pattern of an increase in plasma INSL3 concentrations, but not testosterone concentrations, around pubertal age in the present study appeared to be consistent with the pattern of a sustained increase in Leydig cell mass per testis in bulls around the pubertal age (Wrobel, 1990). A similar sustained increase in INSL3 secretion, during and even after puberty, was reported in male humans (Ferlin et al., 2006).

In conclusion, an EIA to measure plasma INSL3 concentrations in bulls was established. Based on this assay, plasma INSL3 concentrations in beef bulls increased during the first 3 months after birth and throughout the pubertal age. There were similar dynamic patterns for

INSL3 and testosterone during the first 3 months of life, whereas there were differential patterns of release of these two hormones around the pubertal age.

Summary

Previous reports on INSL3 concentrations in peripheral blood are limited to humans and rodents. Owing to lack of availability in assay systems, concentrations of INSL3 in peripheral blood of bulls (in various development stages) have not been reported. Therefore, a novel EIA was developed for the quantification of INSL3 in bovine plasma; employing this assay, the changes in INSL3 concentrations were investigated from birth to pubertal age in beef bulls. Then, the changes in INSL3 concentrations were compared with those of testosterone.

Plasma samples were collected from beef bull calves (n = 15) at birth (0 months) and at 1, 2 and 3 months after birth. Furthermore, in beef bulls around pubertal age (n = 26; age range 3 – 22 months), plasma samples were collected at 1 – 4 months intervals. Plasma INSL3 concentrations increased ($P < 0.05$) from 0 to 1, 1 to 2, and from 2 to 3 months of age. Plasma testosterone concentrations increased ($P < 0.001$) from 0 to 1 month, and from 1 to 2 months, but did not change from 2 to 3 months. For bulls around pubertal age, plasma INSL3 concentrations did not change from the pre-pubertal phase (3 – 6 months) to the early pubertal phase (6 – 12 months), but increased ($P < 0.05$) from the early to late pubertal phases (12 – 18 months), and from the late pubertal to post-pubertal phases (18 – 22 months). Plasma testosterone concentrations increased from the pre-pubertal to early pubertal phases ($P < 0.001$), but did not change thereafter. Plasma INSL3 concentrations increased during the first 3 months of life and throughout the pubertal age in beef bulls. There were similar dynamic patterns for INSL3 and testosterone during the first 3 months of life, but patterns subsequently diverged in bulls around pubertal ages.

Chapter 2

Age-related plasma INSL3 and testosterone dynamics and effects of cryptorchidism in dogs

Introduction

In human males, blood INSL3 profile has been worked out with their age, and serum INSL3 has emerged as a novel clinical marker of Leydig cell function (Bay et al., 2005; Ermetici et al., 2009; Foresta et al., 2009). However, blood INSL3 profile in a broad age range is yet to be investigated in many species, including dogs. Circulating concentrations of INSL3, along with well-elucidated hormones such as testosterone, are worth studying in dogs to evaluate Leydig cell function *in vivo*.

The process of testicular descent is hormonally regulated, and INSL3 and testosterone are critical for the above process (Hutson and Hasthorpe, 2005; Toppari et al., 2006; Foresta et al., 2008). Peripheral plasma testosterone concentrations were lower in bilateral cryptorchid dogs than in normal dogs, according to limited information available on naturally occurring CO (Kawakami et al., 1995). In contrast, no differences have been observed for the plasma testosterone between normal and unilateral cryptorchid dogs (Mattheeuws and Comhaire, 1989; Mischke et al., 2002; Ortega-Pacheco et al., 2006). Despite its importance in the process of testicular descent, plasma INSL3 concentrations have apparently not been investigated in both normal and cryptorchid dogs.

Here, enabling further investigations of INSL3 in dogs, a TRFIA was developed for the determination of INSL3 in canine plasma. Using this assay, plasma INSL3 concentrations

have been measured to determine the changes of plasma INSL3 concentrations with age in normal male dogs, and to relate them with testosterone concentrations. Furthermore, the present study compares the hormonal concentrations among cryptorchid, normal and castrated dogs to evaluate endocrine function of Leydig cell component in retained testes.

Materials and Methods

Animals and sample collection

One hundred and thirty male dogs (normal, n = 89; unilateral cryptorchid, n = 31; bilateral cryptorchid, n = 7; castrated, n = 3) employed in this study were presented to a private animal hospital close to our university. All dogs were privately owned, and the prior consent of owners' was obtained to use the testes and blood samples for this study. The study was conducted according to the regulations of the local Institutional Animal Care and Use Committee. In normal dogs, both testes were diagnosed as being within the scrotum. Canine CO was diagnosed if one (unilateral) or both (bilateral) testes were not identified in the scrotum after 6 months of age (Johnston et al., 2001a; Pathirana et al., 2010). All dogs were apparently free of testicular tumors and other reproductive disorders. All dogs belonged to small breeds, and nearly 80% were Toy Poodles, Chihuahuas, Miniature Dachshunds, Yorkshire Terriers and Shih Tzus. The ages of animals ranged from 4 months to 14 years. The range of body weight was 0.9 to 11.8 kg (4.35 ± 0.19 kg; mean ± SEM). Blood samples were collected between 1000 and 1200 h for routine blood examinations prior to surgery (castration or cryptorchidectomy) or during other routine clinical practices in the animal hospital. A portion of heparinized blood (1 – 1.5 mL each) from collected blood samples and testes obtained after the surgery were immediately dispatched to the

laboratory on ice. The blood was centrifuged at 4,000 × g for 5 min at 4 ^{o}C in the laboratory, and separated plasma was stored at −30 ^{o}C pending hormone analyses. The total weight of both testes was recorded from 50 dogs (normal, n = 28; unilateral cryptorchid, n = 16; bilateral cryptorchid, n = 6) and included in this study.

Hormone determinations

Reagents

Anti-bovine INSL3 mouse monoclonal antibody (2-8F) has been described elsewhere (Büllesbach and Schwabe, 2002). Canine INSL3 was synthesized by basically the same procedure as previously described for bovine INSL3 (Büllesbach and Schwabe, 2002). The principle of this synthesis is the use of different cysteine protecting groups that allow the directed synthesis of the three disulfide bonds. Amino acid sequences of the synthesized canine INSL3 (A-chain: AAATNPARYCCLSGCSRQDLLTLCPH, B-chain: PAPGAREKLCGHHFVRALVRVCGGPRWSSE) were according to the cDNA coding for canine INSL3 (Truong et al., 2003). A mass/charge ratio of synthesized canine INSL3 was examined by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) using an Autoflex III linear (Bruker Daltonics, Billerica, MA, USA). Human INSL3 was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Europium-labeled human INSL3 (Eu-human INSL3) was donated by Prof. J.D.Wade (University of Melbourne, Victoria, Australia).

Bovine serum albumin (BSA) Cohn Fraction V, ProClin 950 and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO, USA). Anti-mouse IgG rabbit polyclonal antibody and anti-rabbit IgG goat polyclonal antibody were obtained

from MP Biochemicals (Solon, OH, USA). The DELFIA wash concentrate, assay buffer and enhancement solution were obtained from Perkin Elmer Life Sciences (Wallac Oy, Turku, Finland). Anti-testosterone-3(E)-carboxymethyloxime-BSA rabbit polyclonal antibody and horseradish peroxidase (HRP)-labeled testosterone-3- carboxymethyloxime were purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan).

Extraction of INSL3

Extraction of INSL3 from canine plasma was performed according to the procedure described for the extraction of bovine INSL3 in Chapter 1 of this thesis. In brief, 300 μL of 0.1% trifluoroacetic acid was added to 125 μL of canine plasma and mixed immediately by vortexing. After leaving for 30 min at room temperature, 200 μL of acetonitrile was added to the mixture and vortexed immediately. After 30 min, the mixture was centrifuged at 15,000 × g for 10 min at room temperature. The resulting supernatant was concentrated by vacuum centrifugation (Centrifugal Concentrator CC-105, Tomy Seiko Inc., Tokyo, Japan) for approximately 2 h, up to approximately 30 μL. Then 80 μL of 0.05 M phosphate buffer (pH 7.5) was added to the concentrated supernatant, and the final volume was adjusted to 125 μL using distilled water.

A series of human INSL3 standards (0.02 – 20 ng/mL) were used to quantify canine INSL3 in plasma samples. The human INSL3 standards diluted with canine plasma collected from a bitch to make up various concentrations. Both human INSL3 standards and plasma samples were extracted simultaneously.

The recovery of the extraction procedure for canine INSL3 from the plasma samples was determined through two sets of same-concentration serial dilutions (0.02 – 20 ng/mL):

(1) the canine INSL3 diluted in the assay buffer and assayed directly; (2) the canine INSL3 diluted in bitch plasma followed by the extraction procedure and assayed. The recovery rates were determined from the assayed concentrations in the plasma divided by the added concentrations to the plasma. To examine the parallelism with known concentrations of canine INSL3, plasma sample from a normal male dog was serially diluted in plasma obtained from the bitch (1, 2, 4 and 8 times dilutions) and assayed after the extraction procedure.

Time-resolved fluorescence immunoassay of canine INSL3

Microtitration plate wells (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 h at room temperature. The wells were drained and washed with 300 μL saline (0.15 M sodium chloride), and non-specific binding sites were blocked overnight at 4 ^{o}C with DELFIA assay buffer (AB-I). The wells were drained, and 50 μL of extracted human INSL3 standards or extracted samples and 50 μL of anti-bovine INSL3 mouse monoclonal antibody (1:1,000,000 dilution in AB-I) were dispensed and incubated overnight at 4 ^{o}C. The anti-bovine INSL3 antibody was the same one used in Chapter 1. Then, the wells were kept for 30 min at room temperature and 50 μL Eu-human INSL3 (2 ng/mL in AB-I) was added, and incubated for further 1 h at room temperature. The wells were washed three times with 400 μL DELFIA wash buffer. The enhancement solution (100 μL each well) was dispensed and shacked (80 rpm) for 15 min at room temperature. Finally, time-resolved fluorescence was measured by ARVO multilabel counter (Perkin Elmer, Wallac Oy, Finland).

The minimum detection limit of the assay was 0.02 ng/mL, and the percent binding (B/B_0 X 100, 92.8 ± 1.8%) at the above limit was lower ($P < 0.001$) than B_0 (100%), as observed in three consecutive standard curves. The reliable detection range was 0.02–20 ng/mL. The intra-assay CV was 5.8% (n = 2), and the inter-assay CV was 3.0% (n = 4).

Testosterone extraction

Various concentrations of testosterone standards (0.04 – 20 ng/mL) were diluted with the assay buffer (AB-II; 0.01 M phosphate buffer containing 0.15M sodium chloride, 0.1% BSA and 0.02% ProClin 950, pH 7.4) for steroid EIA. After that, 50 µL of standards or canine plasma samples were dispensed into glass tubes and mixed with 1 mL of diethyl ether by vortexing for 1 min. The tubes were centrifuged at 1,500 × g for 5 min at 4 ^{o}C to separate the upper ether phase from the lower water phase. The lower phase was frozen at −40 ^{o}C, and the upper phase was decanted into another glass tube. The ether phase was then evaporated under a N_2 gas stream at 40 ^{o}C. For testosterone EIA, the dried extracts were dissolved in 125 µL of AB-II by vigorous vortexing. The standards and plasma samples were extracted simultaneously.

Testosterone EIA

For extracted standards or extracted samples, testosterone EIA was performed according to the procedure described in Chapter 1, using the HRP-labeled testosterone and anti-testosterone antibody. The minimum detection limit of the assay was 0.04 ng/mL, and detection was reliable in the range 0.04 – 5 ng/mL. The intra-assay and inter-assay CVs were 6.0% (n = 4) and 15.3% (n = 3), respectively.

Statistical Analysis

Evaluation of INSL3 and testosterone data were performed by two-way ANOVA using generalized linear models (GENLIN) of SPSS version 18.0 (IBM Corporation, Somers, NY, USA) to assess the effects of age and the testicular status of the animal (normal vs. unilateral cryptorchid vs. bilateral cryptorchid dogs). Differences in hormone levels and paired testicular weights among various groups were compared using pairwise comparisons of the GENLIN procedure by the least significant difference (LSD) post hoc test. To evaluate the changes in the plasma hormone concentrations with the age, normal dogs (n = 89) were categorized into five age groups: (I) pre-pubertal age (4 – 6 months, n = 5) (II) pubertal age (6 – 12 months, n = 35) (III) post-pubertal age (1 – 5 years, n = 30) (IV) middle age (5 – 10 years, n = 15) (V) advanced age (10 – 14 years, n = 4). Samples obtained from the pre-pubertal and advanced ages were not included in the analysis among normal, unilateral and bilateral cryptorchid dogs, as the cryptorchid dogs were devoid of such samples. As this study was based on multiple breeds, the consistency of breed distribution among groups (based on age or testicular status) was tested by chi-square analysis (IBM SPSS Statistics 18.0). To check the breed distribution across all comparisons, four breed categories were considered according number of subjects belonging to a particular breed: (I) Toy Poodle (II) Chihuahua (III) Miniature Dachshund (IV) Other (individual breeds with few subjects i.e. less than 5% of total subjects were combined). Spearman correlation coefficient was calculated to assess the relationship between INSL3 and testosterone (IBM SPSS Statistics 18.0). Data are expressed as mean ± SEM. Differences were considered significant at $P < 0.05$.

Results

A new TRFIA was established to measure INSL3 concentrations in canine plasma. To verify cross-reactivity with the anti-bovine INSL3 antibody, canine INSL3 was newly synthesized. Integrity of synthesized INSL3 was confirmed by MALDI TOF MS, and its mass/charge ratio of 6014.80 was identical to the calculated mass/charge ratio of 6014.85 (Fig. 2-1). Anti-bovine INSL3 antibody showed an excellent cross reactivity with both canine and human INSL3 (Fig. 2-2). Curves for human INSL3 standards and varying concentrations of canine INSL3 showed a parallel drop in percent binding with increasing canine and human INSL3 concentrations (0.02 – 20 ng/mL). The B/B_0 values of the serially diluted plasma sample from a male dog were also parallel to the above curves (Fig. 2-2). The final recovery rate was 64.3 ± 6.6% for the added canine INSL3 (0.1 – 5 ng/mL) in bitch plasma.

There was a significant effect of age group on plasma INSL3 ($P < 0.01$) and testosterone ($P < 0.0001$) concentrations in dogs. As depicted in Fig. 2-3A, plasma INSL3 concentrations were increased ($P < 0.05$) from pre-pubertal age to pubertal age, and then declined ($P < 0.05$) from pubertal age to post-pubertal age. From one year onwards, i.e., in middle and advanced ages, it reached a plateau. Testosterone concentrations were increased drastically ($P < 0.0001$) from pre-pubertal age to pubertal age, and there onwards it seemed to plateau (Fig. 2-3B).

There was a significant effect of testicular status of the animal on plasma INSL3 ($P < 0.01$) and testosterone ($P < 0.001$) concentrations in dogs. Plasma INSL3 and testosterone concentrations were lower ($P < 0.0001$ for each) in bilateral cryptorchids compared with normal and unilateral dogs (Table 2-1). However, there was no statistical difference ($P >$

0.15) in plasma INSL3 and testosterone concentrations in unilateral cryptorchids compared with normal dogs. The INSL3 (range: 0.05 – 0.43 ng/mL) and testosterone (range: 0.10 – 0.94 ng/mL) concentrations in bilateral cryptorchid dogs were higher than in castrated dogs; the hormone concentration of castrated dogs were under the minimum detection limits of the assays (INSL3 < 0.02 ng/mL; testosterone < 0.04 ng/mL). Demonstrating a similar trend to the above plasma hormones, total weight of both testes was lower (P < 0.0001) in bilateral cryptorchids (1.42 ± 0.32 g) than in normal (5.57 ± 0.62 g) and unilateral cryptorchid (4.35 ± 0.59 g) dogs. Paired testes weights between normal and unilateral cryptorchid dogs were not statistically different (P > 0.15).

Upon considering all plasma samples excluding the samples collected from castrated dogs, canine INSL3 and testosterone concentrations were positively correlated (r = 0.35, P < 0.0001; n = 127). In the present study, breed distribution among groups was consistent across all comparisons with the exception between pre-pubertal age and middle age. In the middle age, fewer Toy Poodles were included than in the pre-pubertal age.

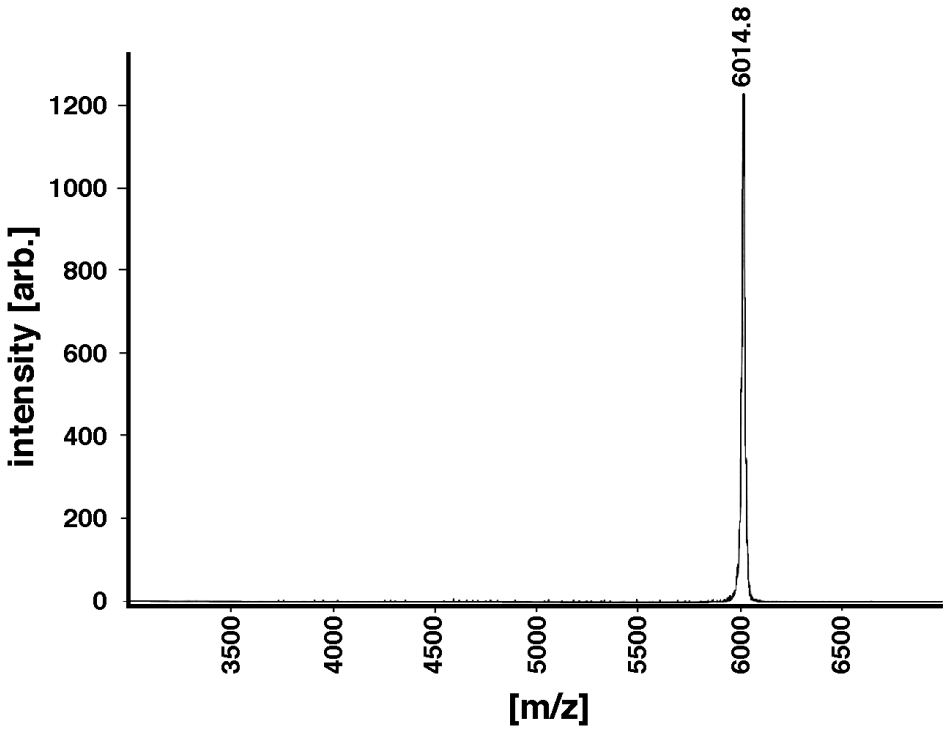

Fig. 2-1. Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS) of synthesized canine INSL3. The single peak indicates a mass/charge ratio of the canine INSL3 (6014.80).

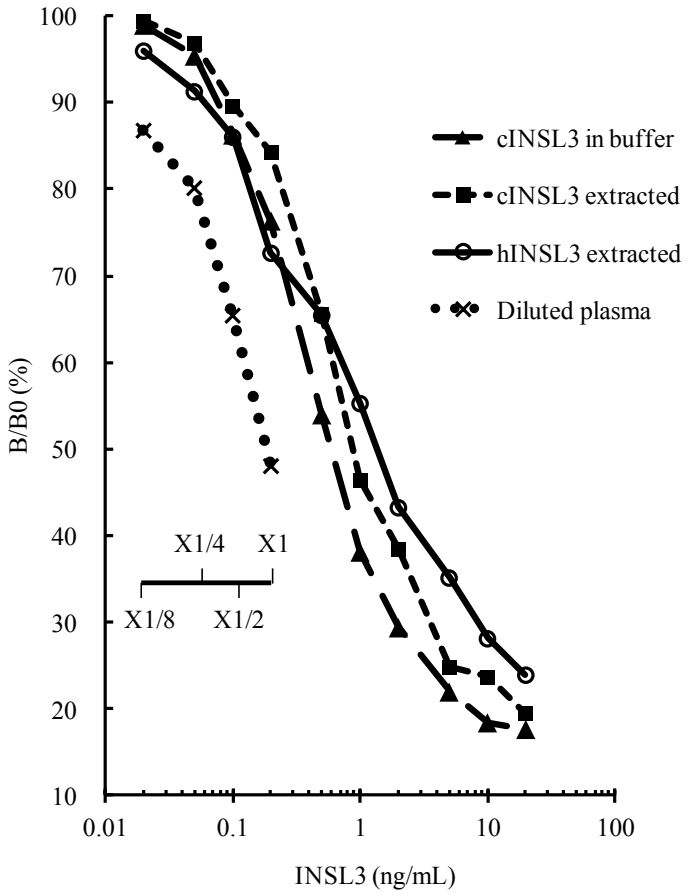

Fig. 2-2. Competition curves for cINSL3 diluted in assay buffer (cINSL3 in buffer), the cINSL3 diluted in plasma followed by extraction (cINSL3 extracted), the hINSL3 standards in plasma followed by extraction (hINSL3 extracted) and a serially diluted (2 to 8 fold) plasma sample from a normal male dog (Diluted plasma). Data are expressed as the percent binding (B/B_0 X 100) at each INSL3 concentration. cINSL3, canine INSL3; hINSL3, human INSL3.

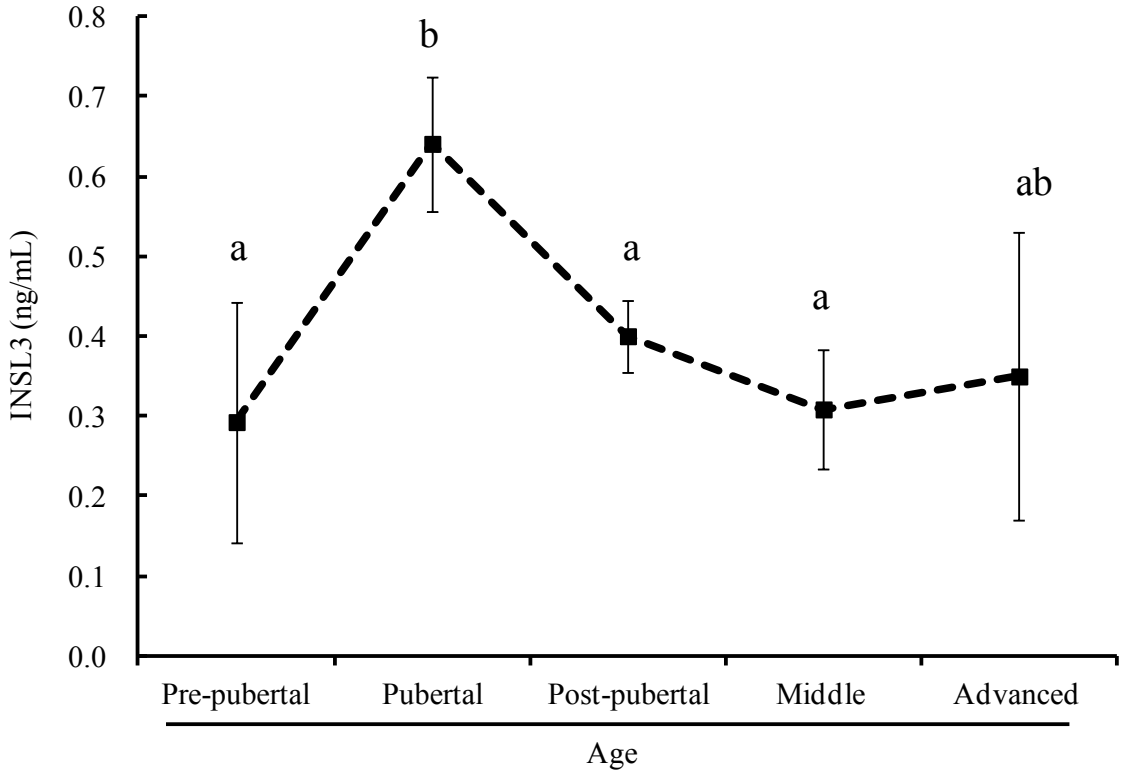

Fig. 2-3A. Changes in plasma INSL3 concentrations in various age groups of normal dogs. Results are shown for pre-pubertal age (4 – 6 months, n = 5), pubertal age (6 – 12 months, n = 35), post-pubertal age (1 – 5 years, n = 30), middle age (5 – 10 years, n = 15) and advanced age (10 – 14 years, n = 4). Data are expressed as the mean ± SEM.

[a,b] Values with differing superscripts differ significantly between groups ($P < 0.05$).

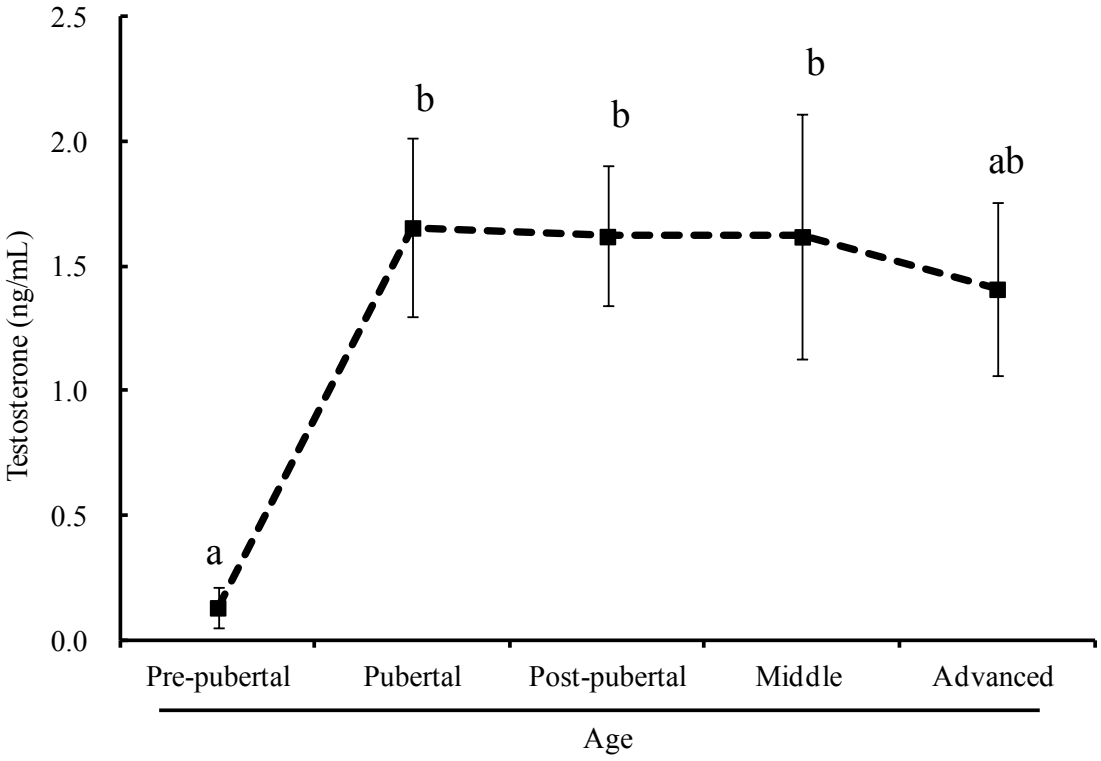

Fig. 2-3B. Changes in plasma testosterone concentrations in various age groups of normal dogs. Results are shown for pre-pubertal age (4 – 6 months, n = 5), pubertal age (6 – 12 months, n = 35), post-pubertal age (1 – 5 years, n = 30), middle age (5 – 10 years, n = 15) and advanced age (10 – 14 years, n = 4). Data are expressed as the mean ± SEM. [a,b] Values with differing superscripts differ significantly between groups ($P < 0.0001$).

Table 2-1. Plasma INSL3 and testosterone concentrations in normal, UCO, BCO and castrated dogs

	Normal (n = 80)	UCO (n = 31)	BCO (n = 7)	Castrated (n = 3)
INSL3	0.49 ± 0.04	0.55 ± 0.08	0.21 ± 0.05*	UD
Testosterone	1.64 ± 0.21	1.57 ± 0.32	0.37 ± 0.10*	UD

Data are expressed as the mean ± SEM. * $P < 0.0001$ compared with normal and unilateral cryptorchid dogs. UD, undetectable; hormone concentration was below the minimum detection limit (INSL3 < 0.02 ng/mL; testosterone < 0.04 ng/mL) of the assay. Normal, normal dogs; UCO, unilateral cryptorchid dogs; BCO, bilateral cryptorchid dogs; Castrated; castrated dogs

Discussion

The TRFIA developed herein provides a sensitive and reliable method to quantify INSL3 in canine plasma. The binding of canine INSL3 to anti-bovine INSL3 antibody was proved by using canine INSL3 synthesized in this study, and the binding was compared with human INSL3. To the best of our knowledge, this is the first study to assess circulating INSL3 concentrations in normal and cryptorchid dogs, and measure both INSL3 and testosterone in each plasma sample with a view to examine the effect of CO on endocrine function of Leydig cell component.

In the present study, plasma INSL3 and testosterone secretory patterns were determined in a broad age range of normal dogs. Plasma concentrations of both hormones were significantly increased from pre-pubertal age to pubertal age. The above increment in plasma INSL3 was transient, and returned to its original level by the next age group (1 – 5 years). The transient surge of circulating INSL3 may be attributed to its paracrine function in maintaining spermatogenesis through the suppression of germ cell apoptosis (Kawamura et al., 2004). In dogs, active spermatogenesis has been first recognized in a similar time-course (i.e. around 8 months of age) to that of transient INSL3 surge (Kawakami et al., 1991), and generally reaches the sexual maturity within 6 to 12 months of age (Johnston et al., 2001b). However, the exact reason for the transient surge of INSL3 is unknown and remains to be elucidated. In contrast to INSL3, increased plasma testosterone concentrations remain almost constant up to 10 years of age. The present findings on plasma testosterone concentrations were consistent with previous reports (Inaba et al., 1988; Mialot et al., 1988; Kawakami et al., 1995), and the testosterone secretory pattern is

closely associated with persistent influence of trophic effects of high plasma LH concentrations (Inaba et al., 1988; Kawakami et al., 1995).

Plasma INSL3 and testosterone concentrations were significantly lower in bilateral cryptorchids than in normal and unilateral cryptorchid dogs. Consistent with our testosterone data, it has been previously reported that plasma testosterone concentrations were lower in bilateral cryptorchid dogs than in normal dogs (Kawakami et al., 1995), whereas no differences were reported between normal and unilateral cryptorchid dogs (Mattheeuws and Comhaire, 1989; Mischke et al., 2002; Ortega-Pacheco et al., 2006). The observed lower hormone concentrations in the present study demonstrate impaired endocrine functions of Leydig cell component per paired retained testes in bilateral cryptorchid dogs. The possible reasons for the reduced INSL3 and testosterone secretions in bilateral cryptorchid dogs may be: (1) reduced total Leydig cell count per paired testes; (2) impaired endocrine functions per each Leydig cell in the retained testes; or (3) both (1) and (2). In addition, the weights per paired testes were significantly lower in bilateral cryptorchids than in normal and unilateral cryptorchid dogs. Similarly, Mattheeuws and Comhaire (1989) and Ortega-Pacheco et al. (2006) reported that weight of the retained testis is significantly reduced compared with scrotal testis. The reduced weight of retained testis seemed to be associated with decreased hourly production of testosterone from retained testis than from scrotal testis in dogs (Eik-Nes, 1966). However, quantitative and qualitative differences in Leydig cell populations between retained and scrotal canine testes remains to be determined.

In bilateral cryptorchid dogs, plasma INSL3 and testosterone concentrations were readily detectable, and higher than in castrated dogs whose plasma hormone concentrations

were under the detection limit of the assays. The above hormone measurements can be used as a diagnostic tool to predict the presence of bilaterally retained testes and to distinguish the bilateral CO from anorchism in dogs. It should be noted that the palpation and ultrasonography have limited success in diagnosing the bilateral CO; the smaller size and large number of possible locations of the retained testes decrease the likelihood of diagnosis by the above methods (Johnston et al., 2001a). As previously reported in humans (Foresta et al., 2004; Bay et al., 2005), our findings demonstrated that Leydig cell-derived INSL3 is the major source of circulating INSL3 in dogs, as evidenced by undetectable concentrations of the hormone in plasma from castrated dogs.

In the current study, a positive correlation was observed between canine INSL3 and testosterone concentrations. The comparison of hormone measurements among normal, unilateral and bilateral cryptorchid dogs resulted in similar dynamic patterns for both INSL3 and testosterone. Based on published data, Ferlin et al. (2010) suggested that INSL3 and testosterone measurements provide different information on the status of Leydig cells, as the former reflects the differentiation status and general wellness while the latter reflects the steroidogenic activity. Moreover, contrary to pulsatile secretory pattern of testosterone which is acutely regulated by LH, INSL3 is secreted in constitutive manner and it is not acutely influenced by LH (Bay and Andersson, 2010). Thus, for single-blood sample measurements, INSL3 may be more reliable than testosterone in evaluating Leydig cell function.

In conclusion, a new TRFIA system was developed to measure INSL3 in canine plasma, and circulating INSL3 concentrations were determined in male dogs using the above assay. The present findings elucidated a transient surge in INSL3 at pubertal age, but it was not

observed for testosterone. Lower plasma INSL3 and testosterone concentrations in bilateral cryptorchid dogs suggest impaired endocrine functions of Leydig cell component per paired retained testes. Furthermore, this study indicates the diagnostic value of plasma concentrations of both hormones for predicting the presence of bilaterally retained testes in dogs.

Summary

Along with testosterone, serum INSL3 has emerged as a novel clinical marker of Leydig cell function in humans. *In vivo* assessment of Leydig cell function is clinically important in male subjects of domestic animals including dogs. However, circulating INSL3 concentrations are totally unknown in dogs. Therefore, blood INSL3 profile should be determined in dogs using a reliable assay system. On the other hand, dogs suffer a high incidence of CO; it is important to evaluate the function of Leydig component in retained testes of cryptorchid dogs *in vivo*. In this study, age-related plasma INSL3 dynamics in dogs were investigated across a wide age range using a novel TRFIA, and compared with plasma testosterone concentrations. Furthermore, hormone concentrations were compared among cryptorchid, normal and castrated dogs to evaluate endocrine function of Leydig cell component in retained testes.

Blood samples were taken from normal male dogs from pre-pubertal age to advanced age (4 months – 14 years, n = 89) and from unilateral cryptorchid (n = 31), bilateral cryptorchid (n = 7) and castrated dogs (n = 3). Canine plasma INSL3 was measured by using a newly developed TRFIA. The minimum detection limit of the INSL3 assay was 0.02 ng/mL and the detection range was 0.02 – 20 ng/mL. Plasma INSL3 concentrations increased ($P < 0.05$) from pre-pubertal age (4 – 6 months) to pubertal age (6 – 12 months), and then declined ($P < 0.05$) from pubertal age to post-pubertal age (1 – 5 years), reaching a plateau. Plasma testosterone concentrations increased ($P < 0.0001$) drastically from pre-pubertal age to pubertal age and seemed to plateau. INSL3 and testosterone concentrations were lower ($P < 0.0001$ for each) in bilateral cryptorchid dogs than in normal and unilateral cryptorchid dogs. The INSL3 (range: 0.05 – 0.43 ng/mL) and testosterone (range: 0.10 –

0.94 ng/mL) concentrations were readily detected in bilateral cryptorchids, but not in castrated dogs (INSL3 < 0.02 ng/mL; testosterone < 0.04 ng/mL). In conclusion, plasma INSL3 concentrations in male dogs measured by newly developed TRFIA elucidated a transient surge at pubertal age, whereas testosterone did not. Lower plasma INSL3 and testosterone concentrations in bilateral cryptorchid dogs suggest impaired endocrine functions of Leydig cell component per paired retained testes. Peripheral plasma INSL3 and testosterone concentrations indicate a diagnostic value in predicting the presence of bilaterally retained testes in male dogs.

Chapter 3

INSL3 and testosterone secretory responses to various effectors in cultured testicular interstitial cells of dogs

Section 1

INSL3 and testosterone secretory responses to human chorionic gonadotropin in cultured interstitial cells from scrotal and retained testes

Introduction

LH and hCG (which has LH activity) are generally considered as main regulators of Leydig cell function. Although both INSL3 and testosterone are of Leydig cell origin, the two hormones are regulated very differently in humans under *in vivo* conditions; INSL3 is constitutively regulated whereas testosterone is acutely sensitive to LH (Bay et al., 2005, 2006). In primary rat Leydig cells, INSL3 secretion was not regulated by hCG (Anand-Ivell et al., 2009). No *in vitro* evidences are available on the effect of hCG on INSL3 secretion from testes of humans and domestic animals including dogs.

It has been shown that CO can alter the Leydig cell activity in rodents (Bergh et al., 1985; Murphy and O'Shaughnessy, 1991; Shikone et al., 1994). Thus, differences in secretory capacity of INSL3 and testosterone between Leydig cells of scrotal and retained testes are plausible. In response to hCG, reduced testosterone release has been demonstrated in retained testes *in vitro*, compared with scrotal testis of mice (Tahri-Joutei and Pointis, 1989; Mendis-Handagama et al., 1990; Murphy and O'Shaughnessy, 1991).

Although CO is a common anomaly in dogs (Romagnoli, 1991; Amann and Veeramachaneni, 2007; Birchard and Nappier, 2008), information is lacking on the *in vitro* testosterone secretory response to various concentrations of LH or hCG in testes deriving from dogs with spontaneous CO. Moreover, a comparison of *in vitro* INSL3 secretion between interstitial cells of scrotal and retained testes is yet to be done in humans and domestic animals.

The present study compares the INSL3 and testosterone secretory responses of cultured interstitial cells to various concentrations of hCG in scrotal testes of small-breed dogs. Moreover, these hormonal responses were compared between the scrotal and retained testes with a view to evaluate Leydig cell function in CO.

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F-12 (DME/F-12), ProClin 950 and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) Fraction V, insulin-transferrin-selenium 100 x solution (ITS), penicillin-streptomycin mixture and trypan blue stain were obtained from Gibco Laboratories (Grass Island, NY, USA). Fetal bovine serum (FBS) was obtained from Biological industries (Haemek, Israel). Dispase II was purchased from Sanko Junyaku Co., Ltd (Tokyo, Japan). hCG (Puberogen 5000 U) was obtained from Novartis Animal Health (Tokyo, Japan). A bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Scientific (Rockford, IL, USA). Anti rabbit IgG goat polyclonal antibody and anti mouse IgG rabbit polyclonal antibody were products of MP Biochemicals (Solon, OH,

USA). Anti testosterone-3(E)-carboxymethyloxime-BSA rabbit polyclonal antibody and testosterone-3-carboxymethyloxime-horseradish peroxidase were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Peroxidase-labeled streptavidin was obtained from KPL Inc. (Gaithersburg, MD, USA). Biotinylated human insulin-like factor 3 was purchased from Phoenix pharmaceuticals, Inc. (Burlingame, CA, USA). Anti bovine INSL3 mouse monoclonal antibody (2-8F) (Büllesbach and Schwabe, 2002) was kindly provided by Dr. E. E. Büllesbach (Medical University of South Carolina, Charleston, CA, USA). Anti human type 2 3β-hydroxysteroid dehydrogenase (3β-HSD) rabbit polyclonal antibody (R1484) was donated by Dr. J. I. Mason (University of Edinburgh, Edinburgh, Scotland, UK).

Animals and sample collection

All testicular tissues were obtained from dogs referred to the veterinary clinic of Osaka Prefecture University and other animal clinics outside the University, following routine castration or treatment for CO. All dogs were privately owned, and the prior consent of owners' was obtained to use testicular samples for this study. Testes from normal dogs (5 – 36 months; n = 9) and cryptorchid dogs (8 – 36 months; n = 10) were used in this study. In normal dogs, both testes were diagnosed as being within the scrotum prior to surgery. Canine CO was diagnosed if one or both testes were not identified in the scrotum after 6 months of age (Johnston et al., 2001a; Pathirana et al., 2010). In cryptorchid dogs, number (unilateral; n = 5 or bilateral; n = 5) and location (abdominal; n = 6 or inguinal; n = 4) of retained testes were recorded. All dogs were of small breeds, and more than 75% were Toy Poodles, Chihuahuas or Miniature Dachshunds. In the normal dogs, only one out of the two scrotal testes was used in preparing testicular interstitial cells. In unilateral CO, only the

retained testis was used in this study. In bilateral CO both retained testes were combined, so as to increase the yield of testicular cells. Following surgery, testes were dispatched to the laboratory in sterile normal saline (0.9% sodium chloride) at room temperature.

Preparation of testicular interstitial cells

The testes were rinsed briefly in sterile normal saline and were placed in DME/F-12 medium containing 10% FBS, 1.2 g/L sodium bicarbonate, 50 units/ml penicillin and 50 μg/ml streptomycin (M-FBS). Each testis was perfused with approximately 2 – 3 mL of M-FBS until the vessels were devoid of red blood cells, as described by Klinefelter et al. (1993) except that a 5mL disposable syringe with a 27G needle was used instead of perfusion apparatus. The testes were decapsulated, and 0.5 – 1.2 g of testicular tissue from each testis was minced into 2 – 3 mm pieces. The minced tissues were washed once in 10 mL of M-FBS, and then dispersed in 5 ml M-FBS containing 2,000 PU/mL dispase II at 37 ^{o}C for 60 – 90 min in a bio-shaker (BR-15LF, Taitec Corp., Saitama, Japan) operating at 120 cycles per min. The dispersion medium was then diluted with 25 mL M-FBS. The suspension was decanted through 0.5 mm stainless steel wire mesh in order to remove the seminiferous tubules, and this was followed by further filtering through 0.25 mm (approximately) silicone gauze (TREX, Fuji Systems Corp., Tokyo, Japan). The interstitial cell suspension was centrifuged at 230 × g for 10 min at 25 ^{o}C. The resulting pellet was washed once by centrifuging using 15 mL M-FBS, and was resuspended in DME/F-12 supplemented with 1.2 g/L sodium bicarbonate, 1X ITS, 0.1% BSA, 50 Units/mL penicillin and 50 μg/mL streptomycin (M-Sup). Cell viability was determined by the trypan blue dye exclusion method, and was greater than 88% in all samples.

The presence of canine Leydig cells in interstitial cell suspensions was confirmed by 3β-hydroxysteroid dehydrogenase (3β-HSD) immunohistochemical staining. For 3β-HSD staining of interstitial cell suspensions, air-dried smears, fixation and permeabilization were undertaken as described by Harlow and Lane (1988). For blocking and staining of smears, Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's instructions.

Culture and treatments

The cells were cultured for 18 h at 37 ^{o}C in a humidified atmosphere of 5% CO_2 and 95% air. Approximately 50,000 live cells were distributed into each well of 96-well non-treated culture plates (Iwaki Glass, Tokyo, Japan). The cells were treated with different dosages of hCG (0, 0.01, 0.1, 1.0 and 10 IU/mL) in 0.2 mL M-Sup per well. After incubation, the non-adhered suspension of cells and medium was collected by repeated gentle pipetting. Culture media and cell pellets were separated by centrifuging at 14,000 × g for 5 min at 4 ^{o}C, and were stored at –30 ^{o}C. The culture media were used for EIA of INSL3 and testosterone. The cell pellets were used to determine the cell protein content, using a BCA protein assay kit according to the manufacturer's instructions.

Within each experiment, three replicate wells were used per treatment. Separate culture experiments were performed for the testicular cell suspensions prepared from retained and scrotal testes.

Partial purification of canine INSL3 from testicular tissue extract

To see whether the anti-bovine INSL3 antibody recognizes the canine INSL3, we undertook partial purification from canine testicular tissue extract, using a centrifugal ultrafiltration device. Minced canine testicular tissue (1 g of tissue per 5 ml of ice-cold 0.1% trifluoroacetic acid) was homogenized for 1 min with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in an ice bath. The homogenate was centrifuged at 14,000 × g for 10 min at 4 ^{o}C. Acetonitrile was added to the collected supernatant (3:5, v/v) and the mixture was maintained at room temperature for 30 min. The supernatant was collected after centrifuging at 14,000 × g for 10 min at 25 ^{o}C, and was dried using a centrifugal concentrator (Tomy, Tokyo, Japan). The pellet was dissolved in distilled water and loaded on a YM-30K Microcon filtration device (molecular weight cut-off at 30K; Millipore, Bedford, MA, USA). The assembly was centrifuged at 14,000 × g for 20 min at 25 ^{o}C. The filtrate was loaded on a YM-3K Microcon filtration device (molecular weight cut-off at 3K) and centrifuged at the above conditions for 80 min. The retentate (the fraction between 3K and 30K) is expected to contain canine INSL3, since the molecular weight of INSL3 is between 7K and 8K; this and the filtrate (the fraction less than 3K, which is expected not to contain INSL3) were collected separately according to the Microcon user guide. We examined the effects of the retentate and filtrate, after ultrafiltration, on the binding of biotinylated human INSL3 to the anti-bovine INSL3 antibody, for this we used using the EIA system specified below.

Hormone Assays

The concentrations of INSL3 and testosterone were analyzed in the same culture medium. A new EIA procedure was established to measure the concentrations of INSL3. Strip wells were coated with 100 μL of anti-mouse IgG (5 μg/mL in 0.05 M sodium bicarbonate; pH 9.7), and non-specific binding sites were blocked overnight with 2% BSA, 0.02% ProClin 950 in 0.01 M PBS, pH 7.4 (assay buffer). Next, 50 μL of each standard or sample medium and 50 μL of anti bovine INSL3 (1:1,000,000 dilution in assay buffer) were dispensed and incubated for 2h at room temperature. After that, 50 μL of biotinylated human INSL3 peptide (1 ng/mL in assay buffer) was added and incubated for a further 1h. The wells were washed three times with saline and incubated for 30 min with horseradish peroxidase-labeled streptavidin (100 ng/mL in assay buffer). Next, the wells were washed three times with saline containing 0.05% tween-20, and incubated with 100 μL substrate solution containing TMB for 30 min at room temperature. The reaction was stopped by adding 100 μL of 2 M sulfuric acid, and optical density was measured at 450 nm with a 570 nm reference using a xMark microplate absorbance spectrophotometer (Bio-Rad, Hercules, CA, USA).

In the present INSL3 EIA procedure, the minimum detection limit of the assay was 0.025 ng/mL, and the percent binding (B/B_0, 94.3 ± 0.9%) at this limit was significantly less ($P < 0.05$) than B_0, as observed in five consecutive standard curves. The reliable detection range for this assay was 0.025 – 5 ng/mL (Fig. 3-1-1). The intra-assay CV was 8.6% (n = 6), and the inter-assay CV was 10.7% (n = 4).

The EIA to measure testosterone concentrations in the spent medium was established, based upon the procedure for progesterone EIA described previously (Kawate et al., 1997,

2000). Microtiter strip wells (Costar, Corning, NY, USA) were coated with 100 µL of anti-rabbit IgG (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 (PBS). Next, 50 µl of each standard or sample medium and 50 µL of anti testosterone-3(E)-carboxymethyloxime-BSA and 50 µL of horseradish peroxidase labeled testosterone-3(E)-carboxymethyloxime were incubated for 2 h at room temperature. The wells were washed three times with saline (0.15 M sodium chloride), and were incubated for another 30 min with 100 µL substrate solution. The remaining steps were the same as for INSL3 EIA. The minimum detection limit of the assay was 0.01 ng/mL, and detection was reliable in the range 0.01 – 5 ng/mL. The intra-assay CV was 11.8% (n = 6), and the inter-assay CV was 9.6% (n = 6).

Statistical analysis

The INSL3 and testosterone concentrations measured in the spent medium were divided by the protein content of the cells in each culture well so as to normalize the variation of cell numbers among wells. The normalized INSL3 and testosterone data are expressed as percentages (mean ± SEM) of each concurrently tested unstimulated control (0 hCG), to iron out variability among the clinical samples. For INSL3 and testosterone data expressed as percentages, the effects of treatment (0 – 10 IU/mL hCG), of testicular status (scrotal vs. retained), and the treatment by testicular status interaction were all evaluated by ANOVA, using the generalized estimating equations (GEE) procedure of a generalized linear model (IBM SPSS Statistics 18.0; IBM Corporation, Somers, NY, USA). For both sets of hormonal data expressed as percentages, the effect of number (unilateral vs. bilateral) or of

position (abdominal vs. inguinal) of the retained testis was also evaluated by the GEE. The two means were compared using pairwise comparison of the GEE by the least significant difference (LSD) post hoc test (IBM SPSS Statistics 18.0). Statistical significance was set at $P < 0.05$.

Results

There was an effect of treatment on INSL3 ($P < 0.01$) and testosterone ($P < 0.0001$) concentrations in spent medium. Fig. 3-1-2 shows the percentage stimulation of INSL3 *in vitro* by different hCG concentrations in the interstitial cells of scrotal testes. In the interstitial cells of scrotal testes, INSL3 secretion was stimulated ($P < 0.01$) at 10 IU/mL hCG (158.4% compared with the control). However, there was no significant stimulation of INSL3 secretion at 0.1 IU/mL hCG. Fig. 3-1-3 shows the percentage stimulation of testosterone *in vitro* by differing hCG concentrations in the cells of scrotal testes. In contrast to INSL3, testosterone was clearly stimulated by 0.01 IU/mL hCG or higher concentrations (0.1, 1 and 10 IU/mL) in a dose-dependent manner ($P < 0.01$). The testosterone stimulation percentage in interstitial cells of scrotal testes ranged from 682.1% to 1949.4% for the given hCG concentrations.

Upon comparing the percent stimulations of testosterone and INSL3 (as measured in the same culture medium) in interstitial cells of scrotal testes, we found that percent stimulation of INSL3 was 6.8-times and 12.3-times less than that of testosterone at 0.1 and 10 IU/mL hCG, respectively (Fig. 3-1-4).

There was an effect of testicular status (scrotal vs. retained) on INSL3 ($P < 0.05$) and testosterone ($P < 0.0001$) concentrations in spent medium. In retained testes, INSL3

secretion was not stimulated at either of the hCG concentrations tested. However, the incremental rate of INSL3 secretion was significantly less ($P < 0.05$) in the cells of retained testes than in the interstitial cells of scrotal testes at 10 IU/mL hCG, and there was a borderline significant reduction ($P = 0.07$) when the same comparison was made at 0.1 IU/mL hCG (Fig. 3-1-5). For testosterone, dose-dependent stimulation of testosterone was observed in the interstitial cells of retained testes, but the incremental rate of testosterone secretion was reduced ($P < 0.05$) in the cells of retained testes relative to the interstitial cells of scrotal testes at 0.1, 1 and 10 IU/mL hCG (Fig. 3-1-6). The testosterone stimulation percentage in interstitial cells of retained testes ranged from 306.5% and 780.2% for the given hCG concentrations. Testosterone stimulatory capacity in interstitial cells of retained testes, relative to scrotal testes, was reduced to more than a half at each hCG concentration.

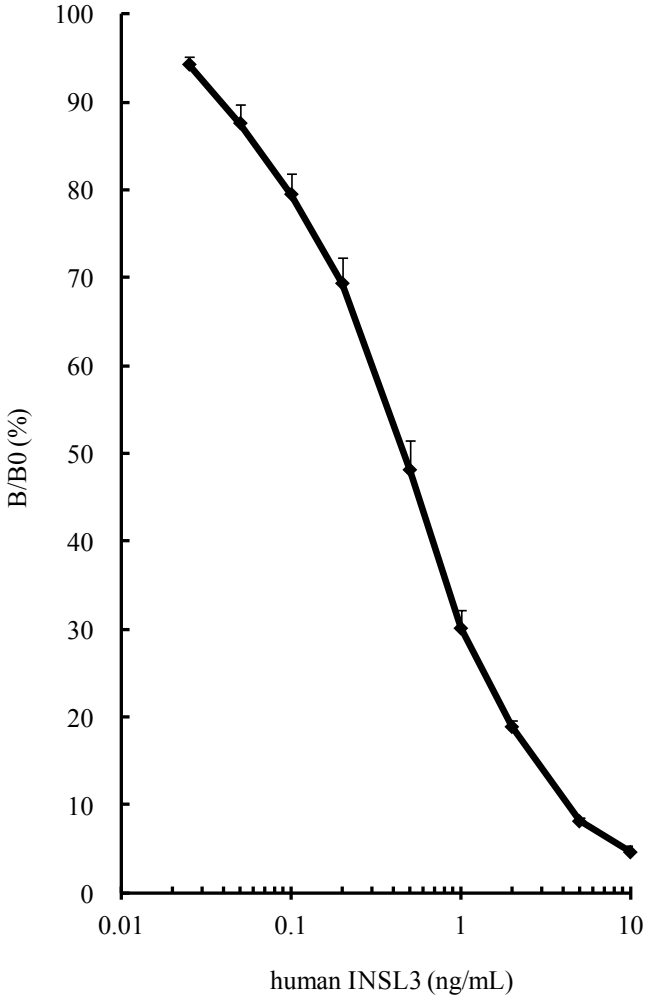

Fig. 3-1-1. Competition curve of the enzyme immunoassay for INSL3 showing the reliable detection range, 0.025 – 5 ng/mL. Mean percentage B/B_0 ±SE of 5 curves. Standards for human INSL3 ranged from 0.025 to 25 ng/mL.

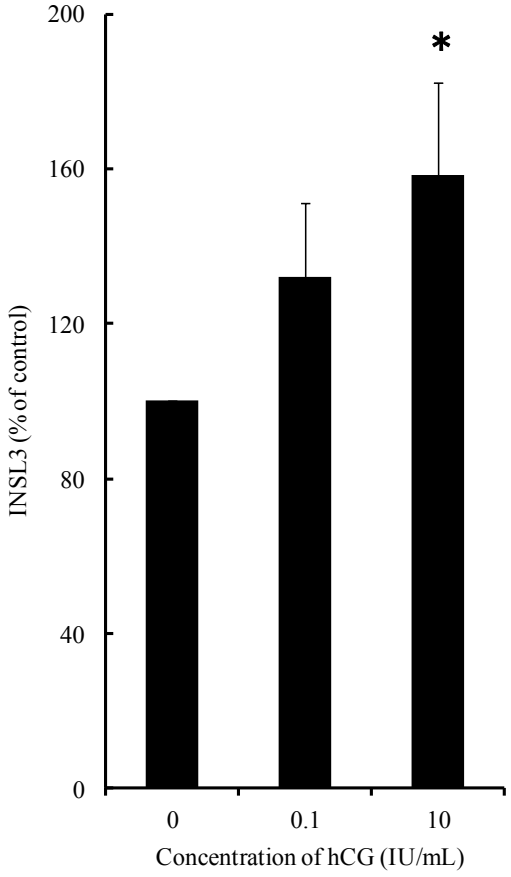

Fig. 3-1-2. Percentage stimulation of INSL3 in response to hCG *in vitro* by interstitial cells of scrotal testes (n = 9). Values are expressed as mean percentage stimulation of the corresponding control (0 hCG) ± SEM. **P* < 0.01 compared with the control (0 hCG).

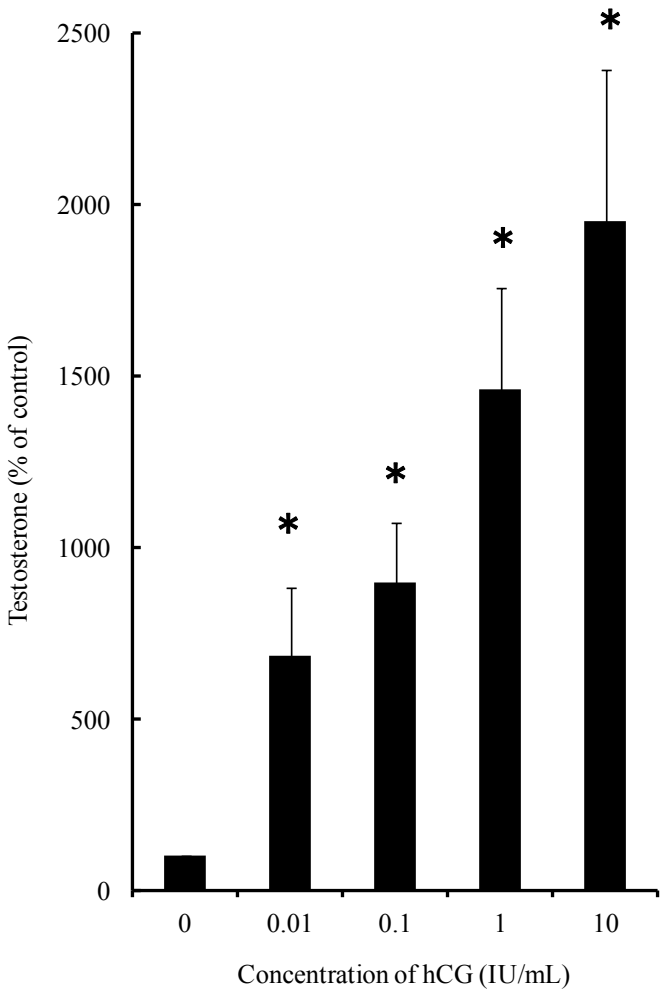

Fig. 3-1-3. Percentage stimulation of testosterone in response to hCG *in vitro* by the interstitial cells of scrotal testes (n = 9). Values are expressed as mean percentage stimulation of corresponding controls (0 hCG) ± SEM. **P* < 0.01 compared with the controls (0 hCG).

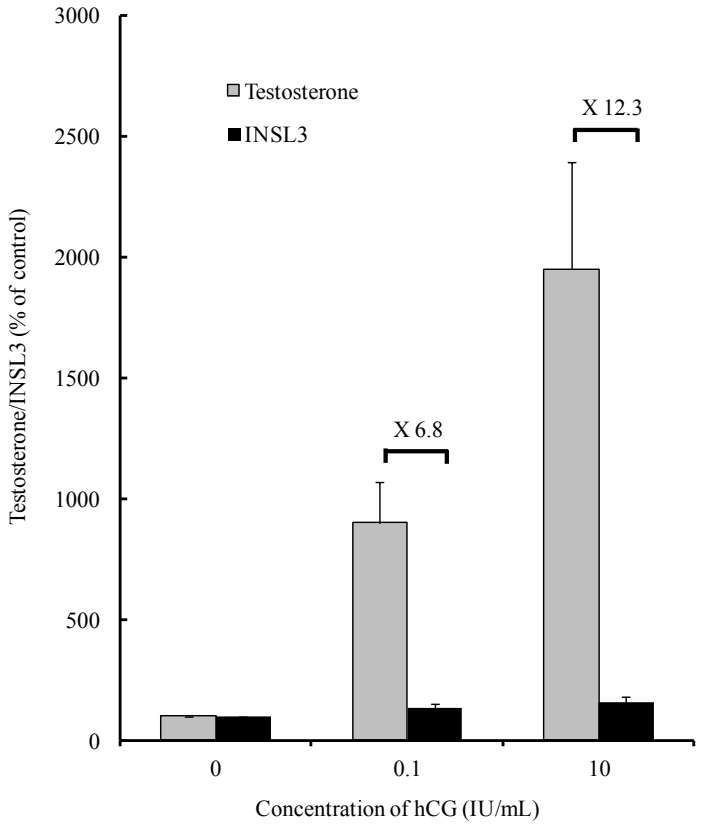

Fig. 3-1-4. Comparison of percent stimulations of testosterone and INSL3 at each hCG concentration in scrotal testes (n = 9). Values are expressed as mean percentage stimulation of corresponding controls (0 hCG) ± SEM (n = 9).

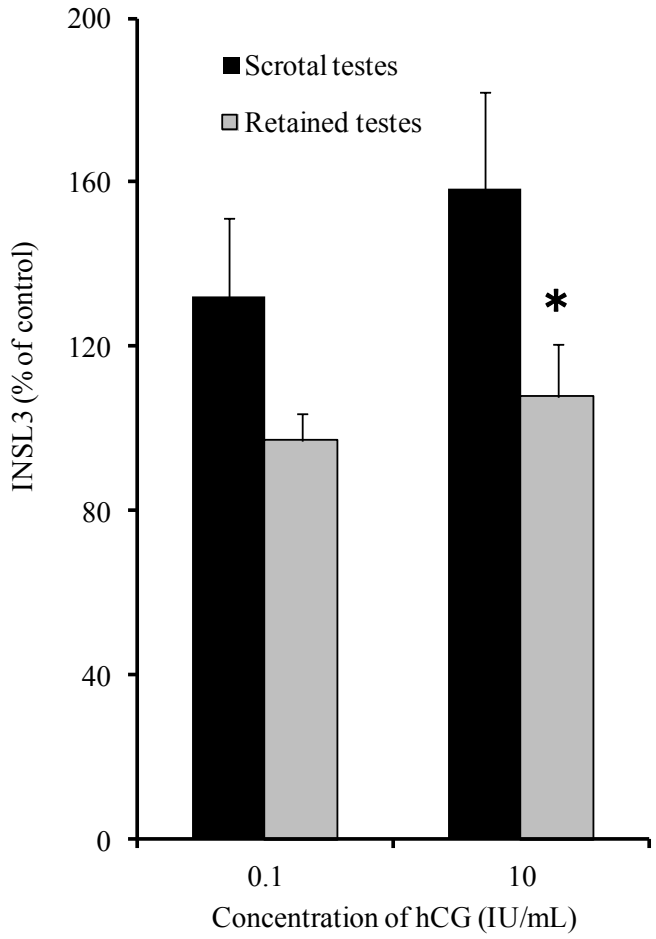

Fig. 3-1-5. Comparison of percent stimulations of INSL3 at each hCG concentration between scrotal (n = 9) and retained (n = 10) testes. Values are expressed as mean percentage stimulation of corresponding controls (0 hCG) ± SEM. *$P < 0.05$ compared with the scrotal testes.

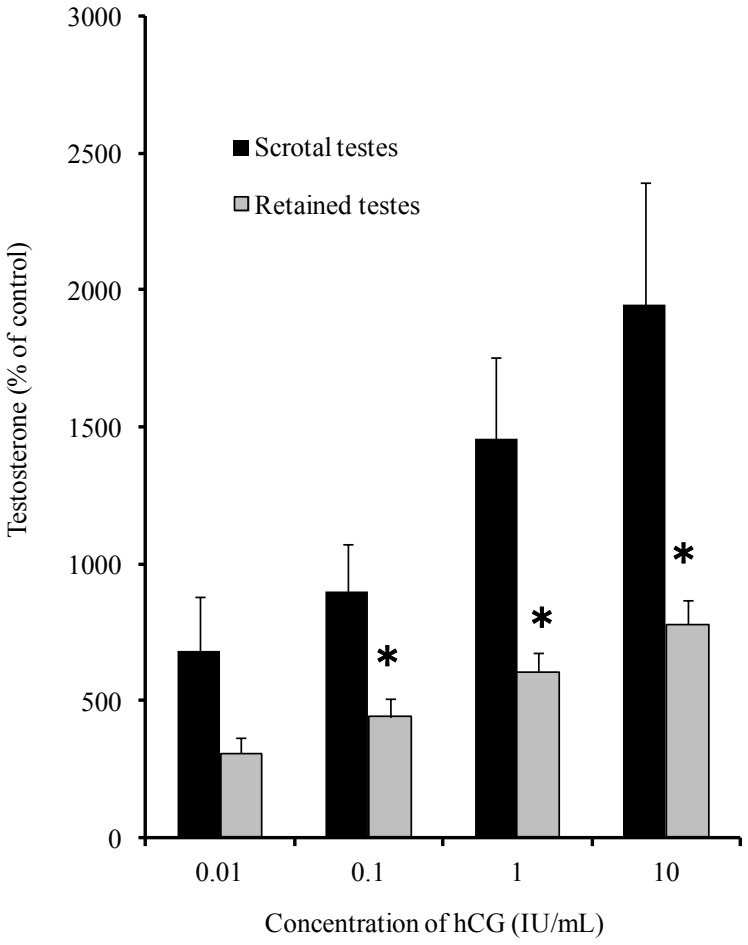

Fig. 3-1-6. Comparison of percent stimulations of testosterone at each hCG concentration between scrotal (n = 9) and retained (n = 10) testes. Values are expressed as mean percentage stimulation of corresponding controls (0 hCG) ± SEM. *$P < 0.05$ compared with the scrotal testes.

Discussion

We compared the secretory testosterone and INSL3 responses of cultured interstitial cells to different dosages of hCG in scrotal testes and retained testes of small-breed dogs. The present results demonstrated that physiological concentrations of LH (0.01 and 0.1 IU/mL hCG; equivalent to approximately 0.8 ng/mL and 8 ng/mL, respectively) may acutely stimulate testosterone release in a dose-dependent manner, but not the INSL3 release, in the interstitial cells of scrotal testes in dogs. This is the first *in vitro* confirmation of the different secretory patterns of INSL3 and testosterone using primary testicular interstitial cells of a domestic animal species. Based on human serum INSL3 measurements, Bay et al. (2005, 2006) showed that INSL3 is constitutively regulated, but testosterone is acutely sensitive to LH.

Very high concentrations of hCG (10 IU/mL; equivalent to approximately 800 ng/mL) significantly stimulated the INSL3 secretion in the testicular interstitial cells of scrotal testes of dogs. This effect seems to be pharmacological, since the normal range of LH levels in peripheral blood in male dogs is 2 – 10 ng/mL (Inaba et al., 1988, 1994; Günzel-Apel et al., 1994). In a previous study using primary rat Leydig cells and the MA-10 mouse tumor Leydig cell line, INSL3 secretion was not influenced by the addition of 150 ng/mL hCG (Anand-Ivell et al., 2009). However, the present study differs from the previous study in looking at mammalian species and in the purity of cell preparations and concentrations of hCG.

For testosterone, dose-response stimulation to hCG was observed in the cells of both scrotal and retained testes. However, interstitial cells of retained testes were clearly hyporesponsive to hCG, compared to the cells of scrotal testes. A significantly lower

incremental rate was observed in the testosterone response at higher concentrations of hCG (0.1, 1 and 10 IU/mL) in the interstitial cells of retained testes than scrotal testes. These results suggest that the LH-induced testosterone secretory response is lower in the cells of retained testes than scrotal testes. In previous *in vitro* studies on experimental cryptorchidism in mice, a significantly lower testosterone secretion was observed in retained testes than in scrotal testes (Tahri-Joutei and Pointis, 1989; Mendis-Handagama et al., 1990; Murphy and O'Shaughnessy, 1991). The reduced testosterone responsiveness to hCG in the interstitial cells of retained canine testes may be a result of the reduced numbers of LH receptors on Leydig cells, or of impaired signal transduction after binding of LH. In experimental cryptorchidism, a decline in the number of available LH receptors has been observed in retained testes of the rat (de Kretser et al., 1979; Risbridger et al., 1981; Bergh et al., 1985; Shikone et al., 1994) and the ram (Barenton et al., 1982). Consistent with these findings, elevated levels of serum LH and low-to-normal serum testosterone in cryptorchid boys suggest poor responsiveness of Leydig cells in cryptorchidism (Suomi et al., 2006; Toppari et al., 2007).

The present data also provide the first evidence for a reduced INSL3 response to high levels of hCG (10 IU/mL) of interstitial cells in retained testes, relative to scrotal testes of dogs; this impaired responsiveness could be due to reduced numbers of LH receptors or impaired signal transduction by LH, as described above for the testosterone response. An alternative cause of reduced INSL3 response to hCG in retained testes may be the lower testosterone response to hCG (8 times increase to 0 IU/mL) than in scrotal testes (20 times increase). INSL3 transcription is stimulated by testosterone in primary rat Leydig cells and the MA-10 mouse tumor Leydig cell line (Laguë and Tremblay, 2008). Further

investigation would be necessary to determine the effect of testosterone on INSL3 release from canine Leydig cells.

Testosterone and INSL3 are the major regulatory hormones of testicular descent (Hutson and Hasthrope, 2005; Toppari et al., 2006; Foresta et.al. 2008). In dogs, hypothalamo-pituitary-testis axis function begins before initiation of the inguinoscrotal phase of testicular descent (Klonisch et al., 2004); a normal hypothalamo-pituitary-testis axis is necessary for normal testicular descent (Toppari and Kaleva, 1999; Toppari et al., 2007). If there is reduced testosterone and INSL3 responsiveness to hCG in fetal Leydig cells, as we observed in adult interstitial cells, this can be suggested as one of the possible causes of cryptorchidism in dogs.

Our results demonstrated that the physiological concentrations of LH do not acutely stimulate INSL3 release in canine testicular interstitial cells; in contrast, testosterone response was dramatically increased. However, very high concentrations of LH may acutely stimulate INSL3 release in scrotal testis of dogs, but not in retained testis. Furthermore, the LH-induced secretory testosterone and INSL3 responses are lower in the interstitial cells of retained testes than in scrotal testes of dogs.

Summary

LH and hCG (which has LH activity) are generally considered as main regulators of Leydig cell function. Both INSL3 and testosterone are from Leydig cell origin, however, no *in vitro* evidences are available to compare the INSL3 and testosterone secretory response to hCG in testes of any domestic animal species including dogs.

Although CO is a common anomaly in dogs, information is lacking on the *in vitro* testosterone secretory response to various concentrations of LH or hCG in testes deriving from dogs with spontaneous CO. Moreover, a comparison of *in vitro* INSL3 secretion between interstitial cells of scrotal and retained testes is yet to be done in humans and domestic animals.

Differences in secretory capacity of INSL3 and testosterone between interstitial cells of scrotal and retained testes are plausible. Thus, levels of INSL3 and testosterone secretions in response to various doses of hCG in cultured interstitial cells were compared between retained and scrotal testes in small-breed dogs. The testicular tissues were dispersed in Dulbecco's Modified Eagle Medium with Ham's nutrient mixture containing 2,000 PU/mL dispase II and 10% fetal bovine serum. Fifty-thousand cells were plated with differing concentrations (0 – 10 IU/mL) of hCG for 18 h in multiwell-plates. INSL3 and testosterone in the same spent medium were measured by EIA. A new EIA with a reliable detection range of 0.025 – 5 ng/mL was developed in order to measure canine INSL3 in culture medium.

In the cells of scrotal testis, INSL3 secretion was stimulated ($P < 0.01$) at the highest hCG concentration (10 IU/mL) relative to unstimulated controls, but remained unchanged at lower hCG concentration (0.1 IU/mL). In contrast, testosterone was clearly stimulated by

0.01 IU/mL hCG or higher concentrations (0.1, 1 and 10 IU/mL) in a dose-dependent manner ($P < 0.01$). In the cells of scrotal testes, percent stimulation of INSL3 was 6.8-times and 12.3-times less than that of testosterone at 0.1 and 10 IU/mL hCG, respectively. These results suggest that physiological concentrations of LH may acutely stimulate testosterone release, but not the INSL3 release, in scrotal testes of dogs.

In the cells of retained testes, the incremental rate of INSL3 at 10 IU/mL hCG was lower ($P < 0.05$) than that of scrotal testes. The incremental rate of testosterone secretion was lower ($P < 0.05$) at 0.1, 1 and 10 IU/mL hCG in the cells of retained testes than that of scrotal testes. In dogs, LH-induced secretory INSL3 and testosterone responses may be lower in the interstitial cells of retained testes than of scrotal testes.

Chapter 3

INSL3 and testosterone secretory responses to various effectors in cultured testicular interstitial cells of dogs

Section 2

Effects of estradiol-17β, monobutyl phthalate and mono-(2-ethylhexyl) phthalate on the secretion of INSL3 and testosterone by cultured testicular interstitial cells

Introduction

Deterioration of male reproductive health of humans and many animal species has occurred in recent decades; estrogenic environmental chemicals (xenoestrogens, e.g. phthalates), are suspected to be causative agents (Toppari et al., 1996; Skakkebaek et al., 2001; Sharpe and Skakkebaek, 2003). As companion animals, dogs share the living environment of their owners, and the risk for the long-term exposure to xenoestrogens is generally similar to humans. However, previous reports regarding the effects of xenoestrogens (including phthalates) on canine testicular function are lacking. In impaired testicular function, an association of decreased production of INSL3 and androgen by Leydig cells has been suggested in humans (Toppari et al., 2010); these two hormones has recently been used as markers of Leydig cell function (Bay et al., 2005; Ermetici et al., 2009; Foresta et al., 2009).

Phthalates inhibited testosterone production in adult rat Leydig cells (Svechnikov et al., 2008) and reduced serum testosterone concentrations in adult rats (Agarwal et al., 1986);

therefore, adult Leydig cells were a potential target of xenoestrogens. Moreover, it was previously demonstrated that experimental exposure to estrogens reduced plasma testosterone concentrations in normal dogs (Kawakami et al., 2001). Despite its importance, direct effects of estrogenic compounds on Leydig cell function have apparently not been reported in this species.

A genetic susceptibility to estrogenic environmental compounds has been suggested in cryptorchid men (Yoshida et al., 2005); owing to high incidence of CO in dogs, perhaps cryptorchid dogs have a genetic susceptibility to xenoestrogens. Both INSL3 and testosterone are being most critical hormones in the testicular descent (Hutson and Hasthorpe, 2005; Toppari et al., 2006; Foresta et al., 2008), therefore the endocrine consequence of a genetic susceptibility to CO may be elicited via altered secretions of testosterone and INSL3 from Leydig cells of cryptorchid animals.

The objectives of the present study were to examine the *in vitro* effects of estradiol-17β (the main natural estrogen) and two phthalate monoesters, mono butyl phthalate (MBP) and mono-(2-ethylhexyl) phthalate (MEHP), on INSL3 and testosterone secretions in cultured testicular interstitial cells of scrotal and retained canine testes.

Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F-12 (DME/F-12) and estradiol-17β were obtained from Sigma (St. Louis, MO, USA). MBP was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). MEHP was a product of Wako

Pure Chemical Industries, Ltd (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Biological Industries (Haemek, Israel). Bovine serum albumin (BSA) Fraction V, insulin-transferrin-selenium 100 X solution (ITS), penicillin-streptomycin mixture, and trypan blue stain were products of Gibco Laboratories (Grand Island, NY, USA). Dispase II was purchased from Sanko Junyaku Co., Ltd (Tokyo, Japan). Human chorionic gonadotropin (Puberogen 5000 U) was obtained from Novartis Animal Health (Tokyo, Japan). Bicinchoninic acid (BCA) protein assay kit and Vectastain ABC kit were purchased from Thermo Scientific (Rockford, IL, USA) and Vector Laboratories (Burlingame, CA, USA), respectively.

Animals and sample collection

Testes were collected from 26 normal and 16 cryptorchid (unilateral or bilateral) dogs. In normal dogs, only one of the two scrotal testes was used for preparation of testicular interstitial cells, whereas in unilateral CO, only the retained testis was used. In bilateral CO, both retained testes were combined to increase the yield of testicular cells. The ages of the dogs ranged from 6 to 36 months. All dogs belonged to small breeds (>80% were Toy Poodles, Chihuahuas, and Miniature Dachshunds). All testes were obtained from animals presented to the veterinary clinic of the Osaka Prefecture University and the other animal clinics outside the University, as described in Section 1 of Chapter 3. After surgery, testes were transported to the laboratory in sterile physiological saline (0.9% sodium chloride) at room temperature.

Cell isolation and culture

Interstitial cells were isolated from canine testes exactly as the procedure described in Section 1 of Chapter 3. Cell viability was >85% as determined by the trypan blue dye exclusion method. The presence of canine Leydig cells in interstitial cell suspensions was confirmed by 3β-HSD immunohistochemical staining as described in previous section of this chapter.

The cells were cultured in 0.2 mL M-Sup for 18 h at 37 ^{o}C in a humidified atmosphere of 5% CO_2 and 95% air. For testosterone measurements, approximately 50,000 live cells were distributed into each well of 96-well non-treated culture plates (Iwaki Glass, Tokyo, Japan). For INSL3 measurements, the cell number was increased to 200,000 live cells per well, to achieve higher INSL3 concentrations in spent medium.

Treatments

Cells were treated with various concentrations of estradiol-17β (0, 10, and 100 ng/mL; equivalent to 0, 36.7 and 367 nM, respectively), MBP (0, 0.8, and 8 mM), or MEHP (0, 0.2, and 0.8 mM). In the above treatments, the highest possible concentrations of estradiol-17β, MBP and MEHP were determined without affecting cell viability (after 18 h of culture) and the specificity of the testosterone assay. Cell cultures for assessment of testosterone production were carried out in the presence and absence of 0.1 IU/mL hCG, whereas those destined for determination of INSL3 were done only in the absence of hCG, as INSL3 secretion was not significantly stimulated by 0.1 IU/mL hCG in the same cell culture system (Section 1 of Chapter 3). Estadiol-17β was dissolved in ethanol, whereas MEHP and MBP were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of

ethanol and DMSO did not exceed 0.2% in any exposure medium. In all experiments, control cells (without the addition of estradiol-17β, MBP, or MEHP) were cultured in medium containing the same concentration of solvent as in the corresponding exposure medium. After incubation, the non-adhered suspension of cells and medium was collected by repeated gentle pipetting. Culture media and cell pellets were separated by centrifugation (14,000 × g, 5 min, 4 ^{o}C) and stored at –30 ^{o}C until hormone assays were performed. Culture media were used for EIA of INSL3 and testosterone, whereas cell pellets were used for determination of cell protein content. Cellular protein content was determined with a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL USA), according to the manufacturer's instructions.

For each experiment, three replicate wells were used per treatment. Separate culture experiments were performed for the testicular cell suspensions prepared from retained and scrotal testes.

INSL3 and testosterone measurements

Concentrations of INSL3 and testosterone in culture media were measured by EIA, and both INSL3 and testosterone assays were performed essentially as in previous section of this chapter. The minimum detection limit of INSL3 EIA was 0.025 ng/mL, and the detection was reliable in the range from 0.025 to 5 ng/mL. The intra- and inter-assay CVs were 8.2 (n = 6) and 14.0% (n = 4), respectively. For testosterone EIA, the minimum detection limit was 0.01 ng/mL, and the reliable detection range for testosterone EIA was 0.01 to 5 ng/mL. The intra- and inter-assay CVs were 11.6 (n = 6) and 9.2% (n = 8), respectively.

Statistical analysis

For INSL3 and testosterone concentrations, the variation of cell numbers among culture-wells was normalized by dividing the hormone concentrations in spent medium from the total cell protein content in the same culture-well. The normalized INSL3 and testosterone data were expressed as percentages (mean ± SEM) of each concurrently tested unexposed control, to minimize the effects of variation among the clinical samples. Basal and hCG-induced testosterone secretions were analyzed separately. INSL3 and testosterone data were evaluated by 2-way ANOVA using generalized linear models (GENLIN) of SPSS version 18.0 (IBM Corporation, Somers, NY, USA) to assess the effects of testicular status (scrotal vs. retained), treatment (estradiol-17β, 0 – 100 ng; MBP, 0 – 8 mM; MEHP, 0 – 0.8 mM) and the testicular status by treatment interaction. Comparisons between two means were performed using pairwise comparisons of the GENLIN with least significant difference (LSD) post hoc test (IBM SPSS Statistics 18.0). For all analyses, $P < 0.05$ was considered significant.

Results

For INSL3 secretion, there were treatment effects for MBP and MEHP ($P<0.001$), but the effect of testicular status and the treatment by testicular status interaction were not significant. The INSL3 data of scrotal (n = 4) and retained (n = 4) testes were combined and analyzed as a single group (n = 8), in the absence of differences ($P > 0.15$) between scrotal and retained testes in INSL3 secretions. The percentage secretion of INSL3 in response to MBP and MEHP in cultured canine testicular interstitial cells is shown (Fig. 3-2-1). Secretion of INSL3 was inhibited ($P < 0.01$) by 8 mM MBP and 0.8 mM MEHP. In

preliminary experiments, there was no significant effect of estradiol-17β on INSL3 secretion.

For testosterone secretion, there was a treatment effect of estradiol-17β ($P < 0.01$), but neither the effect of testicular status nor the treatment by testicular status interaction were significant. Testosterone data of scrotal (n = 11) and of retained testes (n = 7) were combined and analyzed as a single group (n = 18), since testosterone secretions were not different ($P > 0.15$) between scrotal and retained testes. In canine testicular interstitial cells, basal testosterone secretion was increased ($P < 0.01$) by addition of 100 ng/mL estradiol-17β (Fig. 3-2-2). However, estradiol-17β did not have any significant effect on hCG-induced testosterone secretion in cultured testicular interstitial cells.

There were treatment effects of MBP and MEHP on basal and hCG-induced testosterone secretions ($P < 0.001$ for each), but the effect of testicular status and the treatment by testicular status interaction were not significant. The above testosterone data of scrotal (n = 11) and retained (n = 5) testes were combined and analyzed as a single group (n = 16), due to the absence of differences ($P > 0.15$) in testosterone secretions between scrotal and retained testes. The percentage secretion of basal testosterone in response to MBP and MEHP in cultured canine testicular interstitial cells is shown (Fig. 3-2-3). Basal testosterone secretion was stimulated ($P < 0.01$) by both dosages of MEHP (0.2 and 0.8 mM). However, basal testosterone secretion was not influenced by the addition of MBP in canine testicular interstitial cells *in vitro*. As shown (Fig. 3-2-4), 8 mM MBP inhibited ($P < 0.01$) the hCG-induced testosterone concentration in cultured canine testicular interstitial cells. Treatment with 0.8 mM MEHP tended ($P = 0.056$) to inhibit hCG-induced testosterone secretion.

None of the above tested doses affected the viability of cultured canine testicular interstitial cells, based on the trypan blue dye exclusion method and total cellular protein content.

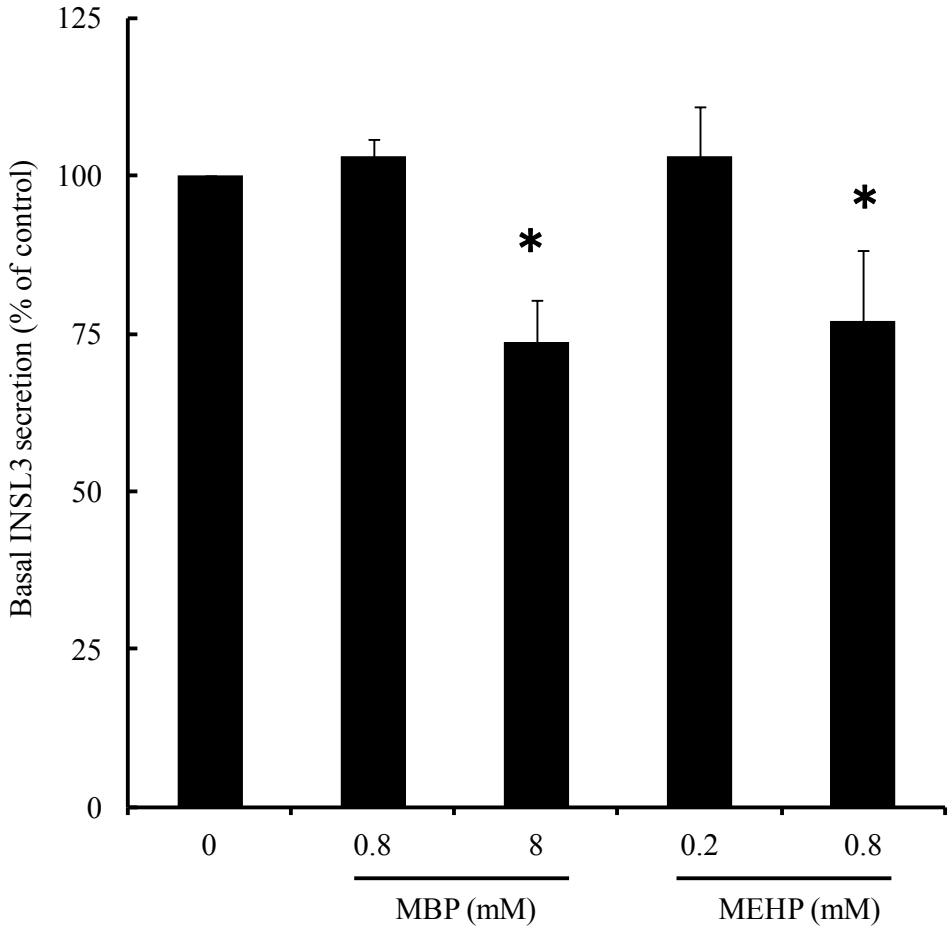

Fig. 3-2-1. Effects of monobutyl phthalate (MBP) and mono-(2-ethylhexyl) phthalate (MEHP) on percentage stimulation of basal INSL3 secretion in canine testicular interstitial cells *in vitro*. Values are expressed as mean percentage stimulation of the control (0 MBP or MEHP) ± SEM (n = 8). *$P < 0.01$ compared with the control (0 MBP or MEHP).

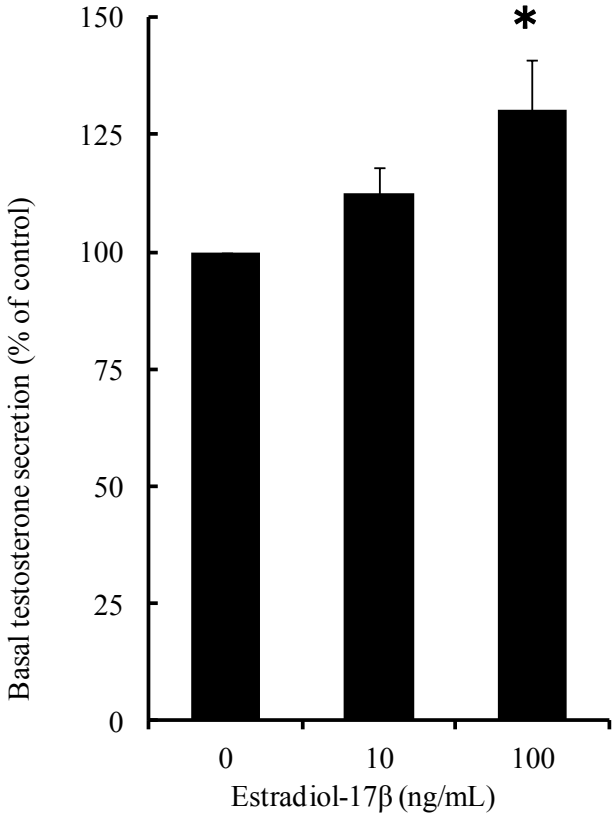

Fig. 3-2-2. Effects of estradiol-17β on percentage stimulation of basal INSL3 secretion in canine testicular interstitial cells *in vitro*. Values are expressed as mean percentage stimulation of the control (0 estradiol-17β) ± SEM (n = 18). *$P < 0.01$ compared with the control (0 estradiol-17β).

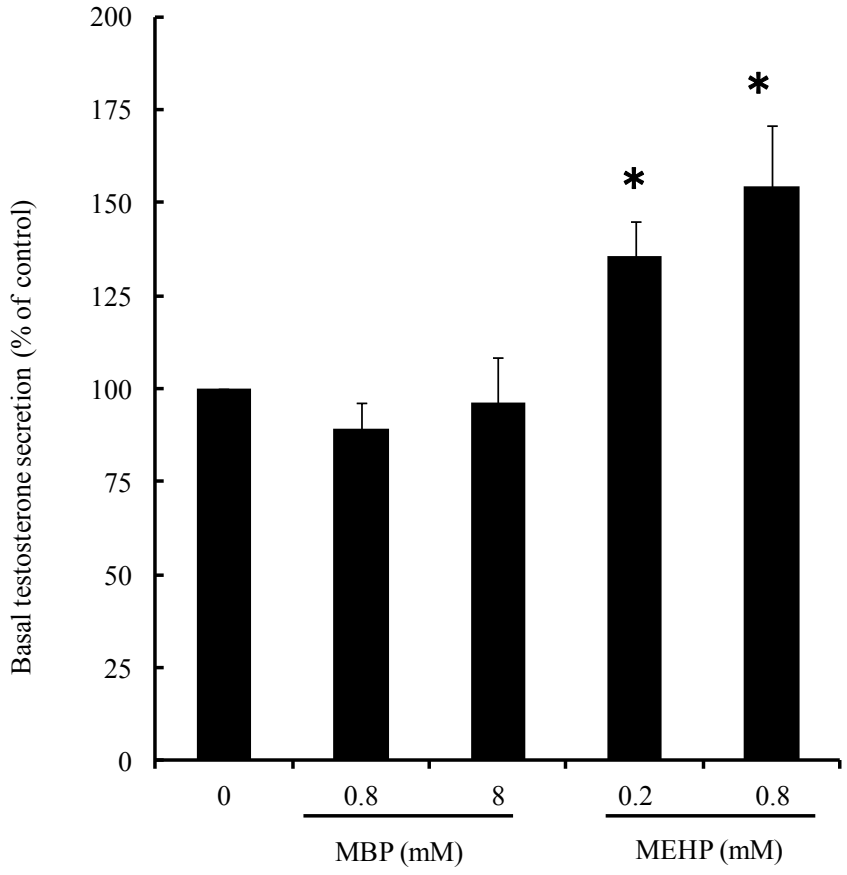

Fig. 3-2-3. Effects of monobutyl phthalate (MBP) and mono-(2-ethylhexyl) phthalate (MEHP) on percentage stimulation of basal testosterone secretion in canine testicular interstitial cells *in vitro*. Values are expressed as mean percentage stimulation of the control (0 MBP or MEHP) ± SEM (n = 16). *$P < 0.01$ compared with the control (0 MBP or MEHP).

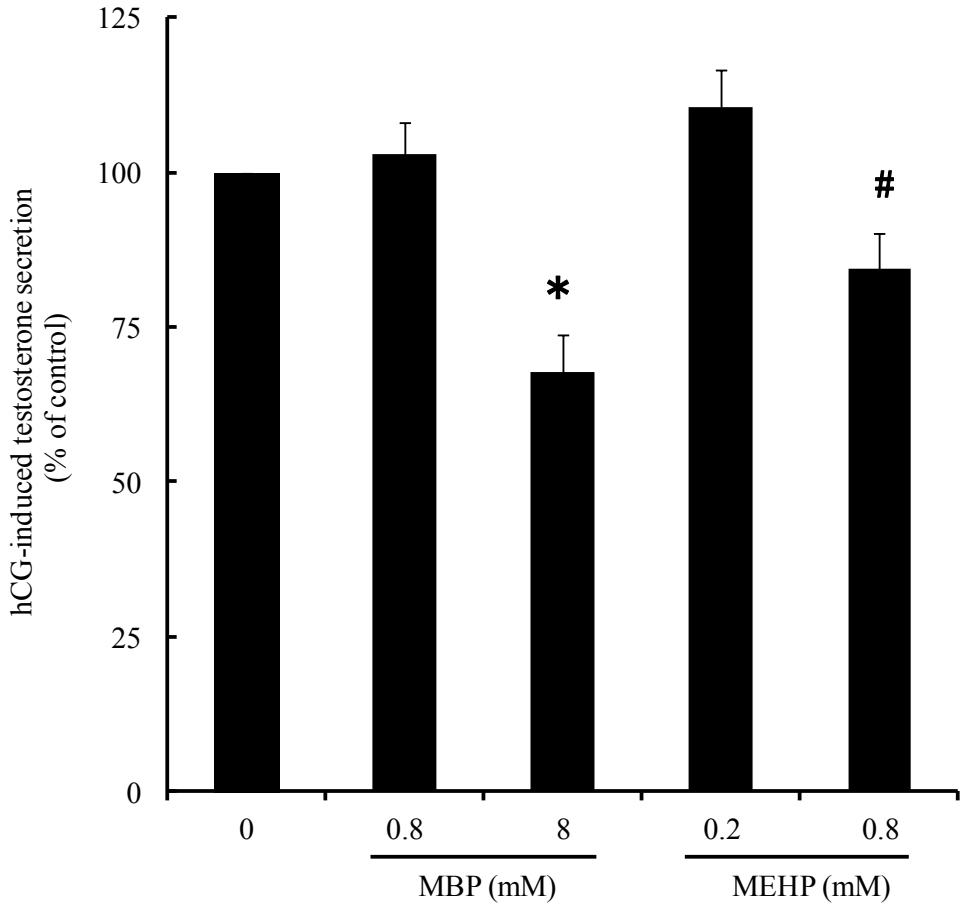

Fig. 3-2-4. Effects of monobutyl phthalate (MBP) and mono-(2-ethylhexyl) phthalate (MEHP) on percentage stimulation of hCG-induced testosterone secretion in canine testicular interstitial cells *in vitro*. Values are expressed as mean percentage stimulation of the 0.1 IU/mL hCG-induced control (0 MBP or MEHP) ± SEM (n = 16). *$P < 0.01$, # P = 0.056 compared with the control (0 MBP or MEHP).

Discussion

The findings presented here are the first *in vitro* evidence for the effects of estradiol-17β, MBP, and MEHP on secretion of testosterone and INSL3 in testicular interstitial cells of scrotal and retained testes in dogs. Under the culture conditions examined, secretion of testosterone and INSL3 were not significantly different between testicular interstitial cells of scrotal and retained testes. Therefore, the hormonal data obtained from both groups (scrotal and retained) were combined and analyzed, with a view to study the effects of the above compounds on canine testicular interstitial cells. Although both MBP and MEHP had significant inhibitory effects on hCG-induced testosterone and basal INSL3 secretions of canine testicular interstitial cells, estradiol-17β and MEHP significantly increased basal testosterone release.

Canine testicular samples were used to obtain interstitial cells that included an essential Leydig cell component. If these *in vitro* findings are related to *in vivo* conditions, inhibitory effects of phthalate on Leydig cells may be more vital than its stimulatory effects. Testes of sexually mature dogs are under the persistent influence of trophic effects of high plasma LH concentrations (Inaba et al., 1988, 1994; Kawakami et al., 1995). Previous *in vitro* studies of ours (Section 1 of Chapter 3) and others (Mushtaq et al., 1996) showed that hCG acutely stimulated testosterone secretion in canine testicular interstitial cells. Therefore, phthalate may exert inhibitory effects on testosterone secretion *in vivo* conditions in mature dogs. For INSL3, the observed phthalate inhibitory effects on basal INSL3 secretion can be predicted even under the influence of LH *in vivo*, as INSL3 is not acutely regulated by physiological dosages of hCG in humans (Bay et al., 2006) or in canine testicular interstitial cells (Section 1 of Chapter 3).

Humans are exposed daily to phthalates through ingestion, inhalation and dermal exposure (Lovekamp-Swan and Davis, 2003). As companion animals, dogs share the living environment of their owners, and the risk for the long-term exposure to multiple phthalate is generally similar to humans. Using an *in vitro* model, we demonstrated herein the direct effects of phthalate on canine testicular interstitial cells, and we inferred that environmental phthalate exposure may alter the function of canine Leydig cells *in vivo,* resulting in decreased concentrations of INSL3 and testosterone. Main et al. (2006), using a group of boys exposed to phthalates via breast milk, reported that human Leydig cell function was vulnerable to perinatal phthalate exposure. In rats, low serum testosterone concentrations were reported in experimental exposure to DEHP (diester form of MEHP available in the environment) (Agarwal et al., 1986). Peripheral concentrations of both INSL3 and testosterone are increasingly used as markers of Leydig cell function in humans (Bay et al., 2005; Ermetici et al., 2009; Foresta et al., 2009). Further investigations are needed to determine the *in vivo* effects of environmental phthalate exposure on canine Leydig cell function by comprehensive assessment of peripheral concentrations of INSL3 and testosterone in dogs challenged with phthalates.

In the present study, both estradiol-17β and MEHP increased basal testosterone secretion in canine testicular interstitial cells. Similar phthalate effects can be anticipated in prepubertal male dogs, as their testes lack a substantial LH influence. Plasma LH concentrations are low during the prepubertal period of dogs (Inaba et al., 1988; Kawakami et al., 1995). Exposure to environmental phthalates may increase testosterone secretion of prepubertal male dogs, thereby hastening puberty. Experimental low-dose DEHP exposure advanced the onset of puberty in rats (Ge at al., 2007b).

In conclusion, estradiol-17β and certain phthalate monoesters had direct effects on secretions of INSL3 and testosterone by canine testicular interstitial cells. Furthermore, there were no significant differences between scrotal and retained testes. These results may explain, at least in part, the effects of phthalate exposure on canine Leydig cell function *in vivo*.

Summary

It has recently been claimed that estrogenic environmental chemicals (xenoestrogens, e.g. phthalates) are responsible for the deterioration of male reproductive health. Interstitial cell compartment (which essentially includes Leydig cells) of the testes is an integral component of the optimal endocrine activity in males. INSL3 and testosterone, two hormones from Leydig cell origin, can be employed to assess the endocrine function of testicular interstitial component in response to xenoestrogens such as phthalates.

The objective was to determine the effects of estradiol-17β, monobutyl phthalate (MBP) and mono-(2-ethylhexyl) phthalate (MEHP) on testosterone and INSL3 secretions in cultured testicular interstitial cells isolated (enzymatic dispersion) from scrotal and retained testes of small-breed dogs. Suspension cultures were treated with estradiol-17β (0, 10, and 100 ng/mL), MBP (0, 0.8, and 8 mM) or MEHP (0, 0.2, and 0.8 mM) for 18 h, in the presence or absence of 0.1 IU/mL hCG. INSL3 (basal) and Testosterone (both basal and hCG-induced) concentrations were measured in spent medium. Effects of estradiol-17β, MBP, and MEHP on testosterone and INSL3 secretions were not affected ($P > 0.15$) by cell source (scrotal versus retained testis); therefore, data were combined, and analyzed as percentage relative to the control. In testicular interstitial cells, basal INSL3 secretion was inhibited ($P < 0.01$) by 8 mM MBP and 0.8 mM MEHP. Basal testosterone secretion was increased ($P < 0.01$) by 100 ng/ml estradiol-17β. Among phthalates, 0.2 and 0.8 mM MEHP stimulated ($P < 0.01$) basal testosterone secretion. However, hCG-induced testosterone secretion was inhibited ($P < 0.01$) by 8 mM MBP, and tended to be inhibited ($P = 0.056$) by 0.8 mM MEHP. Therefore, it was inferred that certain phthalate monoesters

and estradiol-17β had direct effects on secretions of INSL3 and testosterone in canine testicular interstitial cells, with no significant difference between scrotal and retained testes.

Chapter 4

Effects of INSL3 on canine and mouse testicular interstitial cells, and on purified mouse Leydig cells

Introduction

The effect of INSL3 on the gubernacular cells during fetal and neonatal period is elucidated, but little is known about the role of INSL3 in developing and adult males. It has been demonstrated that RXFP2 is expressed in germ cells (Kawamura et al., 2004) and osteoblasts (Ferlin et al., 2008), and potential roles of INSL3 on these two cell types were demonstrated; INSL3 has been involved in the prevention of germ cell apoptosis (Kawamura et al., 2004) and in proliferation of osteoblasts (Ferlin et al., 2008). However, regardless of the localization of RXFP2 in Leydig cells of several mammalian species i.e. humans (Anand-Ivell et al., 2006b), mice (Feng et al., 2007) and dogs (Arrighi et al., 2010), the effects of INSL3 on Leydig cells are totally unknown (Anand-Ivell et al., 2006b).

The present study was designed to examine the *in vitro* effects of INSL3 on canine and mouse testicular interstitial cells taking testosterone measurement as an end-point. To test whether the above effects are exerted directly on Leydig cells, a series of experiments was conducted using purified mouse Leydig cells, aiming both testosterone and cAMP measurements.

Materials and Methods

Reagents

Synthetic procedures of bovine INSL3 (Büllesbach and Schwabe, 2002) and canine INSL3 (Chapter 2 of this thesis) have been previously described. Bovine INSL3 B-chain dimer was synthesized based upon the amino acid sequence of B-chain of bovine INSL3 (Büllesbach and Schwabe, 2002). Human INSL3 and mouse INSL3 were purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Anti-rabbit IgG goat polyclonal antibody was a product of MP Biochemicals (Solon, OH, USA). Anti-testosterone-3(E)-carboxymethyloxime-BSA rabbit polyclonal antibody and testosterone-3-carboxymethyloxime-horseradish peroxidase were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F-12 (DME/F-12), adenosine 3', 5'-cyclic monophosphate (cAMP) sodium salt monohydrate, 3,3',5,5'-tetramethylbenzidine (TMB) and isobutylmethylxanthine (IBMX) were obtained from Sigma (St. Louis, MO, USA). Anti-cAMP antibody and cAMP-peroxidase conjugate were products of GenScript Inc. (Piscataway, NJ, USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories Inc (South Logan, UT, USA). Bovine serum albumin (BSA) Fraction V, insulin-transferrin-selenium 100 X solution (ITS), penicillin-streptomycin mixture, and trypan blue stain were products of Gibco Laboratories (Grand Island, NY, USA). Dispase II was purchased from Sanko Junyaku Co., Ltd (Tokyo, Japan). SQ 22536 was purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA, USA). Percoll was obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Scientific

(Rockford, IL, USA). Human INSL3 antagonist was a gift from Dr. Erika E. Büllesbach (Medical University of South Carolina, Charleston, SC, USA).

Animals

Canine testes were obtained from clinically healthy dogs (n = 14) referred to an animal clinic outside the University, following routine castration. All dogs were privately owned, and the prior consent of owners' was obtained to use the testicular samples for this study. All dogs were of small breeds, and the ages ranged from 7 to 36 months. Both testes were diagnosed as being within the scrotum prior to surgery. Following surgery, testes were dispatched to the laboratory in sterile normal saline at room temperature.

For mice experiments, 8-week-old ICR male mice were purchased from Japan SLC Inc (Shizuoka, Japan) and Japan CLEA Co (Tokyo, Japan). Animals were sacrificed by cervical dislocation, after purchase. Testes were removed and immediately placed on sterile normal saline (0.9% NaCl). The mouse experiments were approved by the local Institutional Animal Care and Use Committee.

Isolation of canine and mouse testicular interstitial cells

Canine testicular interstitial cells were isolated essentially as described in Section 1 of Chapter 3 of this thesis. Cell viability was >90% as determined by the trypan blue dye exclusion method. For the isolation of mouse testicular interstitial cells, non-enzymatic disaggregation of mice testes was performed as previously (Schumacher et al., 1978), with some modifications. After washing with normal saline, testes were placed in DME/F-12 medium containing 10% FBS, 1.2 g/L sodium bicarbonate, 50 IU/mL penicillin, and 50

μg/mL streptomycin (M-FBS). Decapsulated testicular tissues were minced into 2 – 3 mm pieces. Minced tissues were dispersed in 25 mL M-FBS at 37 ^{o}C for 10 min in a bio-shaker (BR-15LF, Taitec Corp., Saitama, Japan) operating at 120 cycles/min. Then the dispersion medium was decanted through 0.5 mm stainless steel wire mesh to remove seminiferous tubules, followed by another filtering step using ~0.25 mm silicone gauze (TREX, Fuji Systems Corp., Tokyo, Japan). The interstitial cell suspension was centrifuged at 230 × g for 10 min at 25 ^{o}C. The resulting pellet was washed once in M-FBS (15 mL) and resuspended in DME/F-12 supplemented with 1.2 g/L sodium bicarbonate, 1 X ITS, 0.1% BSA, 50 IU/mL penicillin, and 50 μg/mL streptomycin (M-Sup). Cell viability was >80% as determined by the trypan blue dye exclusion method.

Isolation of purified mouse Leydig cells

Testicular interstitial cell suspensions were obtained by using the procedure described for canine testicular interstitial cells, with modifications. Four testes (0.5 – 0.6 g) were rinsed briefly in sterile normal saline and placed in DME/F-12 medium containing 10% FBS, 1.2 g/L sodium bicarbonate, 50 IU/mL penicillin, and 50 μg/mL streptomycin (M-FBS). Decapsulated whole testes were dispersed in 5 mL M-FBS containing 1,000 PU/mL dispase II at 37 ^{o}C for 35 min in a bio-shaker (BR-15LF, Taitec Corp., Saitama, Japan), operating at 120 cycles per min. The dispersion medium was diluted with 20 mL M-FBS. Then undispersed tissues and seminiferous tubules were allowed to settle for 2 min, and supernatant was filtered through 0.25 mm (approximately) silicone gauze (TREX, Fuji Systems Corp., Tokyo, Japan). The interstitial cell suspension was centrifuged at 230 × g

for 10 min at 4 ^{o}C. The resulting pellet was washed once by centrifuging using 25 mL M-FBS, and was resuspended in 3 mL normal saline.

For the sedimented tissues (which resulted after the filtration of supernatant), the dissociation step was repeated for another 35 min using 5 mL of new dispersion medium. At the completion of second dissociation step, both testicular interstitial cell suspensions (each suspension in 3 mL normal saline) were pooled, and filtered through 70 μm nylon cell strainer (BD Falcon, Bedford, MA, USA).

Purified mouse Leydig cells were isolated from the above interstitial cells suspensions by using discontinuous Percoll gradients as described by Mayerhofer et al. (1992), with modifications. The gradient consisted of three layers (each 3 mL) with densities of 1.05, 1.06, and 1.08 g/mL. To achieve each density, a stock isotonic Percoll solution (9 parts of 100% Percoll + 1 part of 1.5 M NaCl; density, 1.123 g/mL) was diluted with varying volumes of 0.15 M NaCl (density, 1.0046 g/mL). Interstitial cell suspensions in 3 mL normal saline were loaded onto a discontinuous gradient of Percoll. After centrifugation at 1100 × g for 25 min, the purified Leydig cells accumulating on top of the 1.08 g/mL layer were aspirated, washed (230 × g for 5 min at 4 ^{o}C) in DME/F-12 supplemented with 1.2 g/L sodium bicarbonate, 1X ITS, 0.1% BSA, 50 Units/mL penicillin and 50 μg/mL streptomycin (M-Sup). The resultant pellet is resuspended in 0.2 mL M-Sup. The yields of Leydig cells were in the range of 1.25 – 2.25 × 10^5 cells per animal, and the purity was >80% with 3β-HSD histochemical staining. Staining of Leydig cells for 3β-HSD was performed exactly as previously described (Payne and Sha, 1993).

Culture and treatments

The cells were cultured in 96-well non-treated culture plates (Iwaki Glass, Tokyo, Japan) at 37 ^{o}C in a humidified atmosphere of 5% CO_2 and 95% air. All the treatments were performed in medium containing 0.2 mL M-Sup per well. For each experiment, three or more replicate wells were used per treatment. Testicular interstitial cells (200,000 cells for canine and 25,000 cells for mouse) were cultured in the presence or absence of INSL3 from different species (mouse, human, canine or bovine; 0 – 100 ng/mL). For purified Leydig cells, different cell densities (2,500, 5,000, 10,000 and 20,000 per well) were tested in the presence or absence of 100 ng/mL bovine INSL3. Furthermore, Leydig cells (2,500 cells per well) were cultured in the presence or absence of INSL3 from different species (mouse, human, canine or bovine; 0 – 100 ng/mL).

For the determination of the effect of INSL3 on cAMP production in Leydig cells, purified Leydig cells (2,500 cells per well) were cultured with or without 100 ng/mL bovine INSL3 in the presence of 0.25 mM IBMX.

To determine the effect of INSL3 antagonist for the INSL3 action on Leydig cells, cells (2,500 cells per well) were treated with 100 ng/mL bovine INSL3 in the presence or absence of INSL3 antagonists (bovine INSL3 B-chain dimer or human INSL3 antagonist, 100 ng/mL). The control wells were cultured without both agonist and antagonist.

To determine the effect of SQ 22536 (a specific adenylate cyclase inhibitor) for the INSL3 action on Leydig cells, cells (2,500 cells per well) were treated with 100 ng/mL bovine INSL3 in the presence or absence of 1 μM SQ 22536. The control wells were cultured without both bovine INSL3 and SQ 22536.

INSL3 was dissolved in distilled water, whereas IBMX and SQ 22536 were dissolved in dimethyl sulfoxide (DMSO). The final concentrations of DMSO did not exceed 0.2% in any exposure medium. In all experiments control cells were cultured in medium containing the same concentration of solvent as in the corresponding exposure medium. Cell cultures aimed at testosterone measurements were incubated for 18 h, whereas incubations were maintained for 1 h, 3 h and 18 h aiming cAMP measurements. After incubation, the non-adhered suspension of cells and medium was collected by repeated gentle pipetting. Culture media and cell pellets were separated by centrifuging at 14,000 × g for 5 min at 4 ^{o}C, and were stored at –80 ^{o}C and –30 ^{o}C for cAMP and testosterone measurements, respectively. The culture media were used for EIA of testosterone and cAMP. The pellets of interstitial cell suspensions were used to determine the cell protein content, using a BCA protein assay kit according to the manufacturer's instructions.

Testosterone assay

Concentrations of testosterone in culture media were measured by EIA, and the assay was performed essentially as previously (Section 1 of Chapter 3). Anti-testosterone-3(E)-carboxymethyloxime-BSA rabbit polyclonal antibody (Cosmo Bio Co., Ltd., Tokyo, Japan) was used. The minimum detection limit was 0.01 ng/mL, and the reliable detection range for testosterone EIA was 0.01 to 5 ng/mL. The intra- and interassay CVs were 11.7 (n = 6) and 12.0% (n = 8), respectively.

cAMP assay

Concentration of cAMP in culture media was measured by EIA, and the assay was performed as described by Horton et al. (1992), with modifications. Microtiter strip wells (Costar, Corning, NY, USA) were coated with 100 µL of anti-rabbit IgG (5 µg/mL in 0.05 M sodium bicarbonate; pH 9.7), and non-specific binding sites were blocked overnight with 200 µL of 1% BSA, 0.02% ProClin 950 in 0.01 M phosphate buffer containing 0.15 M sodium chloride (PBS). Next, 50 µL of each standard or sample medium and 50 µL of anti-cAMP antibody in assay buffer (0.05 M acetate buffer, pH 6.0 containing 0.02% BSA and 0.02% ProClin 950) were incubated overnight at 4 ^{o}C. Then the wells were kept for 30 min at room temperature and 50 µL of horseradish peroxidase labeled cAMP (in assay buffer) was added, and incubated for further 1 h at room temperature. The wells were washed four times with saline (0.15mM sodium chloride) containing 0.05% Tween 20, and incubated with 100 µL substrate solution containing TMB for 30 min at room temperature. The reaction was stopped by adding 100 µL of 2M sulfuric acid, and optical density was measured at 450 nm with a 570 nm reference using an xMark microplate absorbance spectrophotometer (Bio-Rad, Hercules, CA, USA). The cross-reactivities for cAMP, adenosine monophosphate, guanosine 3', 5'-cyclic monophosphate and adenosine triphosphate were 100, 0.01, 0.03 and <0.0001%, respectively. The minimum detection limit of the assay was 2 pmol/mL, and detection was reliable in the range 2 – 1000 pmol/mL. The intra-assay CV was 10.6% (n= 6 – 8), and the inter-assay CV was 8.8% (n = 5).

Statistical analysis

For testosterone concentrations in testicular interstitial cell culture, the variation of cell numbers among culture-wells was normalized by dividing the hormone concentrations in spent medium from the total cell protein content in the same culture-well. For purified Leydig cell culture, testosterone and cAMP concentrations were determined for a milliliter in spent medium. Then, testosterone data were expressed as percentages (mean ± SEM) of each concurrently tested unexposed control, to minimize the effects of variation among the different experiments. Testosterone data were evaluated by 2-way ANOVA using generalized linear models (GENLIN) of SPSS version 18.0 (IBM Corporation, Somers, NY, USA) to assess the effects of the treatments. Comparisons between two means were performed using pairwise comparisons of the GENLIN with least significant difference (LSD) post hoc test (IBM SPSS Statistics 18.0). Statistical significance was set at $P < 0.05$.

Results

There was a treatment effect by INSL3 (from different species) on testosterone secretory response in spent medium of canine and mouse testicular interstitial cells ($P < 0.0001$ for each). Fig. 4-1 shows a more than twofold stimulation ($P < 0.0001$) of testosterone in response to bovine and canine INSL3 (100 ng/mL) in canine testicular interstitial cells. In mouse testicular interstitial cells (Fig. 4-2), from different INSL3 species tested, testosterone secretion was stimulated ($P < 0.0001$) more than twofold by 100 ng/mL bovine INSL3.

In purified Leydig cells, there was an effect of cell density ($P < 0.0001$) on testosterone secretory response to bovine INSL3 (100 ng/mL). The lowest Leydig cell density (2500

cells per well) showed a nearly twofold testosterone stimulation; this was higher than ($P < 0.05$) all the other tested cell densities (Fig. 4-3). Moreover, there was a treatment effect ($P < 0.0001$) by INSL3 (from different species) on testosterone secretory response in spent medium of mouse Leydig cells. INSL3 from all species tested i.e. human, mouse, canine and bovine, stimulated ($P < 0.0001$) the testosterone secretion by Leydig cells, and maximum stimulation (nearly twofold) was observed with 100 ng/mL bovine INSL3 (Fig. 4-4). Furthermore, bovine INSL3 (100 ng/mL) stimulated ($P < 0.0001$) cAMP production from primary Leydig cells maximally at 1 h, and remained significantly elevated even at 18 h (Fig. 4-5). SQ 22536 (1 μM), the specific adenylate cyclase inhibitor reduced ($P < 0.0001$) the bovine INSL3-stimulated testosterone secretion from Leydig cells (Fig. 4-6). The addition of INSL3 antagonists (both bovine and human) into culture medium markedly reduced ($P < 0.0001$) the stimulatory effect of bovine INSL3 on testosterone secretion by Leydig cells (Fig. 4-7).

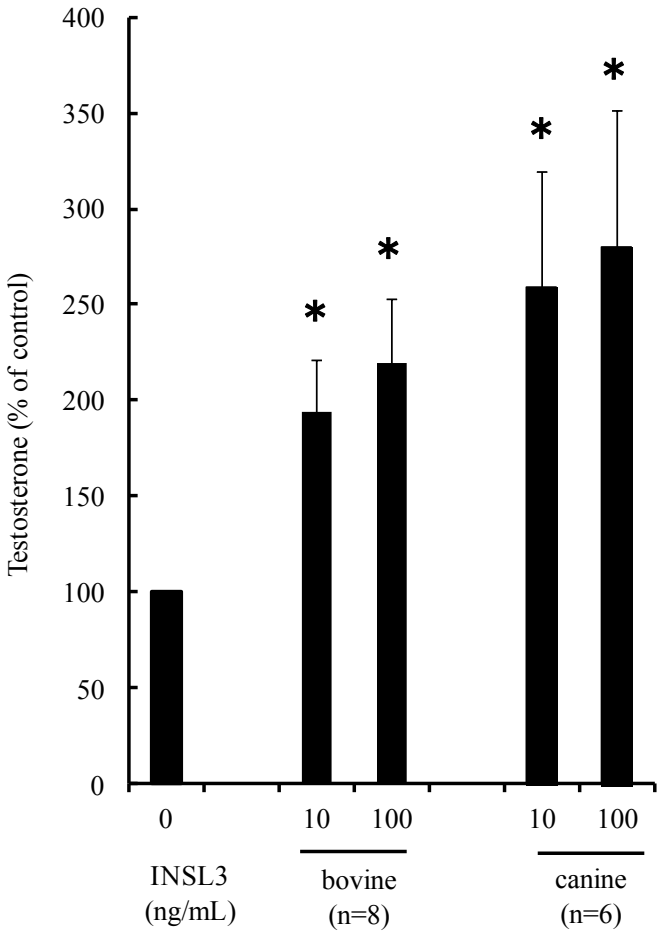

Fig. 4-1. Effects of INSL3 (from different species) on percent stimulation of testosterone in canine testicular interstitial cells. Values are expressed as mean percentage stimulation of the control (0 INSL3) ± SEM. * $P < 0.0001$ compared with control (0 INSL3).

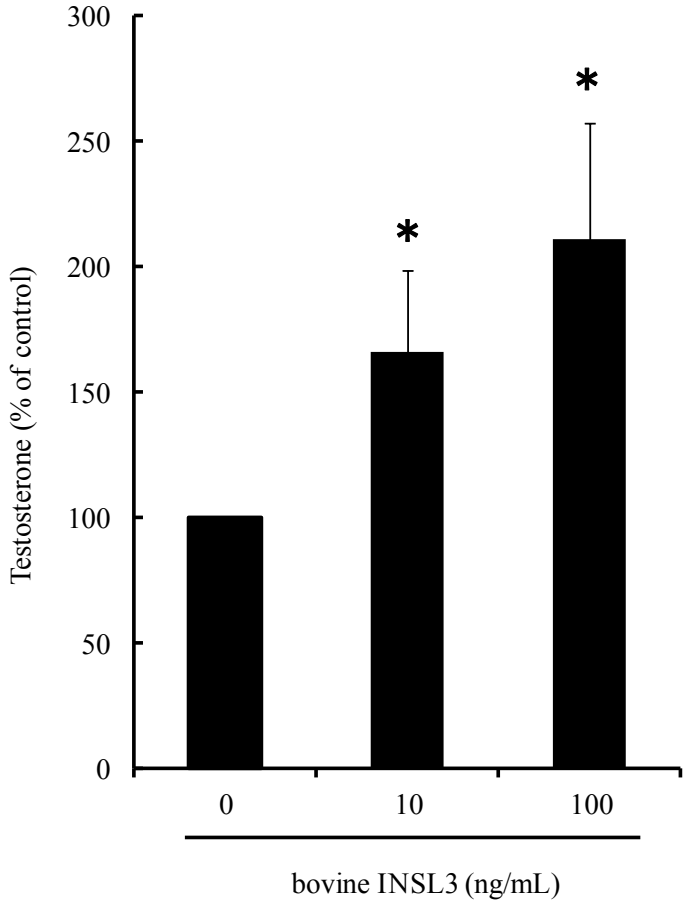

Fig. 4-2. Effects of bovine INSL3 on percent stimulation of testosterone in mouse testicular interstitial cells. Values are expressed as mean percentage stimulation of the control (0 INSL3) ± SEM (n = 5). * $P < 0.0001$ compared with control (0 INSL3).

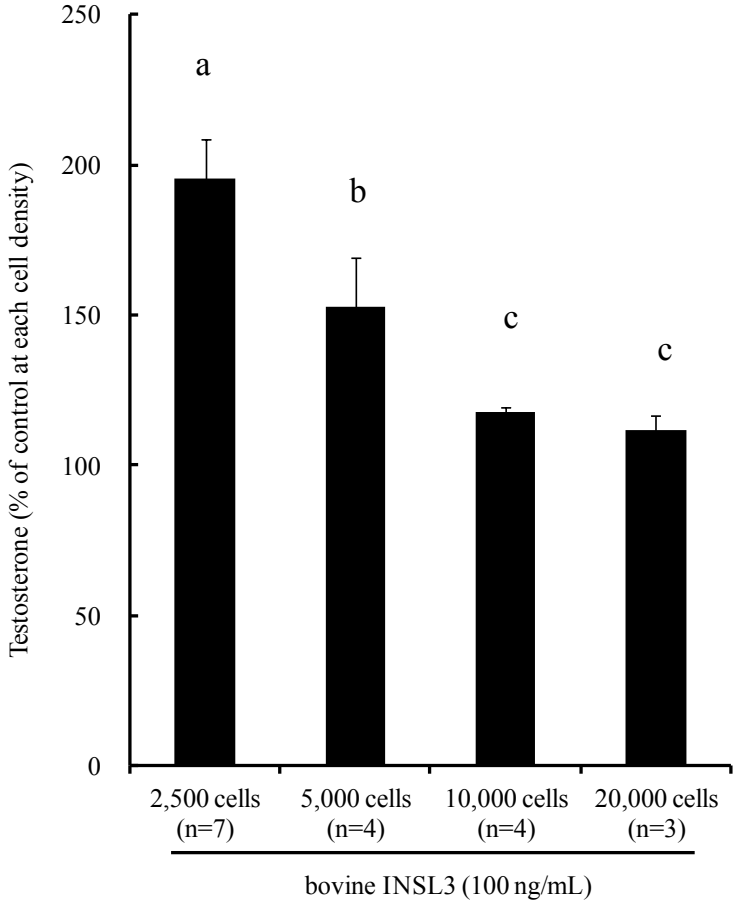

Fig. 4-3. Comparison of the effect of bovine INSL3 (100 ng/mL) on percent stimulation of testosterone at different cell densities (given cell number per well in 96-well plates) of mouse Leydig cells. Values are expressed as mean percentage stimulation of the control (0 INSL3) ± SEM. $^{a-c}$ Different superscripts indicate the significant difference ($P < 0.05$).

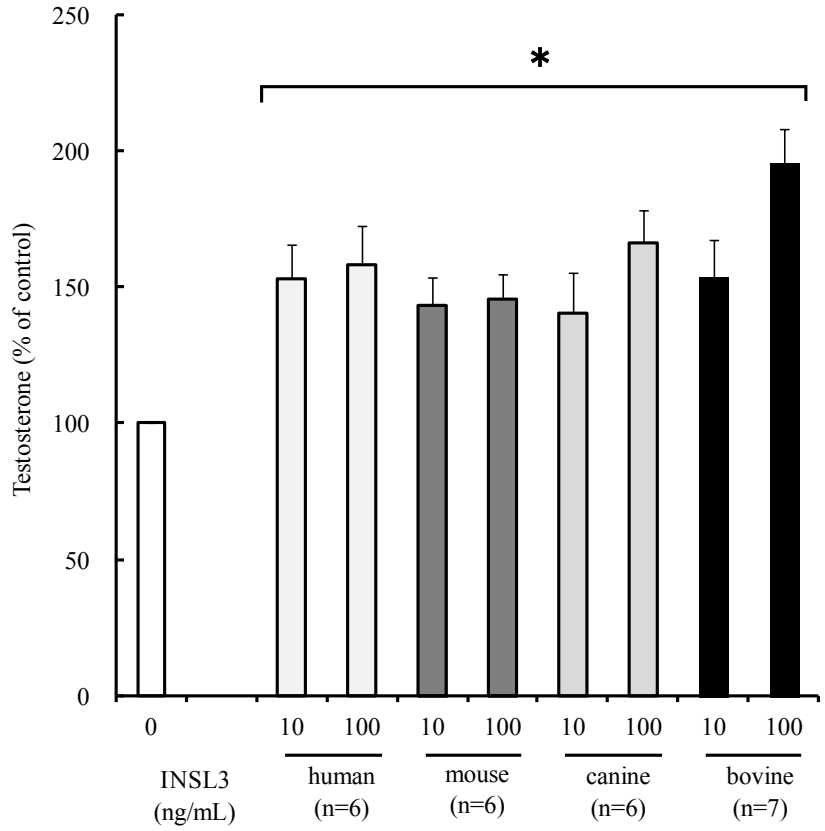

Fig. 4-4. Effects of INSL3 (from different species) on percent stimulation of testosterone in mouse Leydig cells (2,500 cells per well in 96-well plates). Values are expressed as mean percentage stimulation of the control (0 INSL3) ± SEM. * $P < 0.0001$ compared with control (0 INSL3).

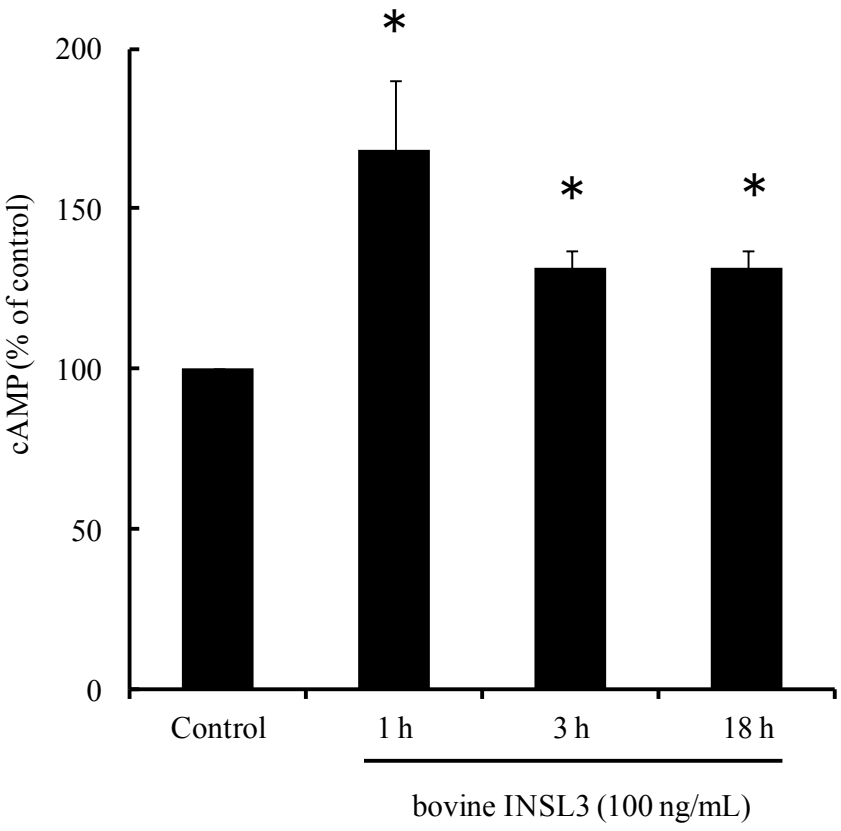

Fig. 4-5. Effects of bovine INSL3 on percent stimulation of cAMP in mouse Leydig cells (2,500 cells per well in 96-well plates) at 1 h, 3 h and 18 h of culture. Values are expressed as mean percentage stimulation of the control (0 INSL3) ± SEM (n = 4). * $P < 0.0001$ compared with control (0 INSL3).

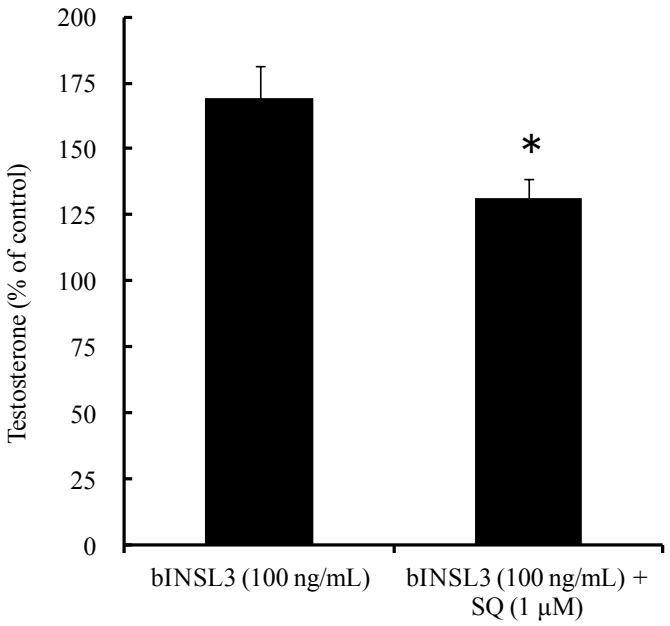

Fig. 4-6. Effect of SQ 22536 (adenylate cyclase inhibitor) on bINSL3-stimulated testosterone secretion in mouse Leydig cells (2,500 cells per well in 96-well plates). Values are expressed as mean percentage stimulation of the control (0 INSL3) ± SEM (n = 3). * $P < 0.0001$ compared with 100 ng/mL bovine INSL3. bINSL3, bovine INSL3; SQ, SQ 22536.

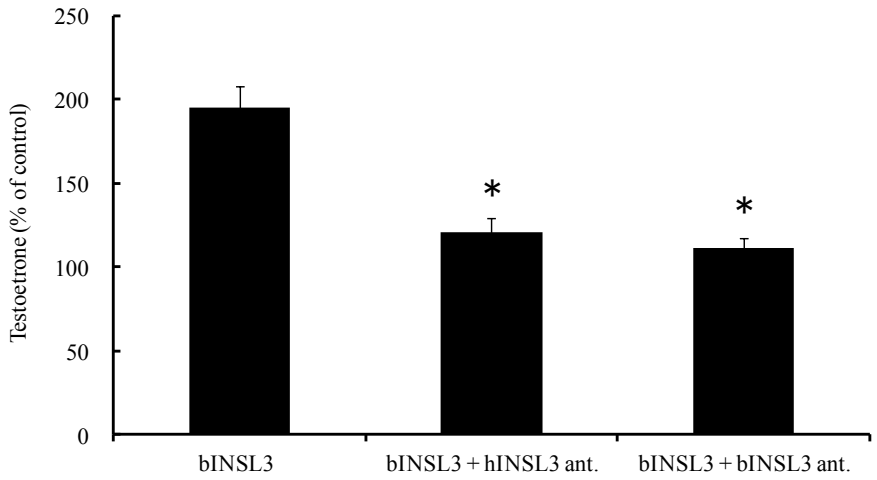

Fig. 4-7. Effects of INSL3 antagonists (bovine and human; 100 ng/mL) on bINSL3 (100 ng/mL)-stimulated testosterone secretion in mouse Leydig cells (2,500 cells per well in 96-well plates). Values are expressed as mean percentage stimulation of the control (0 INSL3) ± SEM (n = 7). * $P < 0.0001$ compared with 100 ng/mL bovine INSL3. bINSL3, bovine INSL3; bINSL3 ant., bovine INSL3 B-chain dimer; hINSL3 ant., human INSL3 antagonist (XA11-B10).

Discussion

The present data provide the first evidence for INSL3 stimulation of testosterone secretion from canine and mouse testicular interstitial cells, and also from the purified mouse Leydig cells. The evidence presented further demonstrated that stimulation of testosterone is exerted via the activation of cAMP in mouse Leydig cells. Moreover, the addition of INSL3 antagonists clearly reduced the effect of INSL3 on Leydig cells. This would suggest a novel autocrine role of INSL3 in Leydig cells. In our purified Leydig cell culture system, the maximum secretory response of testosterone was observed for the lowest cell density (2,500 cells per well) after 18 h incubation. Cultured Leydig cells being a source of INSL3 in medium, it is of prime importance to reduce the concentration of self-secreted INSL3 to obtain the maximum stimulation of testosterone by exogenously added INSL3. Therefore, varying Leydig cell densities were tested in the presence of bovine INSL3 (the most potent INSL3 species among different INSL3 species tested) with a view to obtain optimum culture conditions. As reported previously, human INSL3 had no significant effect on mouse Leydig cells for testosterone and cAMP production (Anand-Ivell et al., 2006b). Such failure could have been resulted due to high Leydig cell density (50,000 cells per well) and short incubation period (45 min) in their culture experiments.

Using an *in vitro* model, the author demonstrated herein the direct stimulatory effects of INSL3 on testosterone secretory response by canine interstitial cells, and these effects were further confirmed using purified Leydig cells of mouse. However, in the present study, the suggested autocrine roles of INSL3 in Leydig cells for the regulation of testosterone secretion are not elucidated under *in vivo* conditions. The current *in vitro* study showed that stimulatory effects of INSL3 on testosterone secretion are at most two- to three-fold

increases, and this increment is likely smaller than those of LH effects shown in Chapter 3. Thus, the stimulatory effects of INSL3 on testosterone secretion would be active *in vivo* conditions, if any, when LH concentrations are low or at basal level, for example, interval between LH pulses after pubertal age, or pre-pubertal, neonatal or even fetal age. In fact, plasma LH concentrations are significantly low in pre-pubertal dogs (Inaba et al., 1988; Kawakami et al., 1995). In contrast, the findings of Chapter 2 of this thesis demonstrated that circulating INSL3 concentrations of pre-pubertal and post-pubertal dogs are equal. Thus, the suggested autocrine function of INSL3 in Leydig cells is most likely to occur in pre-pubertal period of dogs. Also, it seems that fetal Leydig cells are receptive to INSL3, because RXFP2 has been immunolocalized in fetal Leydig cells of dogs (Arrighi et al., 2010). However, autocrine roles of INSL3 in Leydig cells at fetal or neonatal age are yet to be clarified.

The current findings demonstrated, for the first time, that cAMP is a second messenger of INSL3/RXFP2 signaling system in primary culture of mouse Leydig cells. Moreover, the adenylate cyclase inhibitor, SQ 22536 partially reduced the bovine INSL3-stimulated testosterone secretion from mouse Leydig cells. These results suggest that the cAMP pathway is involved in INSL3-stimulated testosterone secretion in Leydig cells. Based on previously reported findings on other cell types, cAMP has been identified as an important mediator in INSL3/RXFP2 signaling (Ivell and Bathgate, 2007; Halls et al., 2007). Kumagai et al. (2002) showed that INSL3 stimulates cAMP production in primary rat gubernacular cells and in HEK-293T cells which have been transfected with recombinant RXFP2. In their experiments, maximum cAMP stimulation has been observed at 30 nM (equivalent to approximately 180 ng/mL) INSL3 in two different cell types, and this is

reasonably close to the INSL3 concentration tested (100 ng/mL) on Leydig cells in the present study. In HEK-293T cells stably transfected with RXFP2, the cAMP response involved $G\alpha_s$ and was modulated by inhibition mediated by $G\alpha_{oB}$ and release of inhibitory G-$\beta\gamma$ subunits (Halls et al., 2006). The specific types of G-protein subunits involved in INSL3-induced cAMP increase in primary Leydig cells remain to be determined.

Taken together, these findings demonstrated that INSL3 has direct stimulatory effects on testosterone secretion from Leydig cells via the activation of cAMP pathway; this suggests a novel autocrine role of INSL3 in Leydig cells.

Summary

The role of INSL3 during fetal and neonatal period is well elucidated, but very little is known about the role of INSL3 in developing and adult males. Recently, it was shown that INSL3 is potentially involved in the prevention of germ cell apoptosis and in proliferation of osteoblasts in adults. However, regardless of the localization of RXFP2 in Leydig cells of several mammalian species, i.e., humans, mice and dogs, the effects of INSL3 on Leydig cells are totally unknown.

In vitro effects of INSL3 on canine and mouse testicular interstitial cells were examined taking testosterone measurement as an end-point. To test whether these effects are exerted directly on Leydig cells, a series of experiments was conducted using purified mouse Leydig cells, aiming both testosterone and cAMP measurements. Purified Leydig cells were obtained from mouse testicular interstitial cells after centrifugation on a 3-step discontinuous gradient (specific gravities: 1.05, 1.06 and 1.08) of Percoll. The testicular interstitial cells or Leydig cells were plated in the presence or absence of INSL3 from different species (mouse, human, canine or bovine; 0–100 ng/mL) for 18 h in multiwell-plates (96 wells). The effects of bovine INSL3 (100 ng/mL) on testosterone secretion by Leydig cells were tested in the presence or absence of SQ 22536 (1 μM) or INSL3 antagonist (bovine and human; 100 ng/mL). Canine and mouse testicular interstitial cells were plated in densities of 200,000 and 25,000 cells per well, respectively. For Leydig cells, different cell densities (2500, 5,000, 10,000 or 20,000 cells per well) were tested. Testosterone and cAMP in spent medium was measured by EIA. In canine testicular interstitial cells, INSL3 stimulated ($P < 0.0001$) the testosterone secretion compared with unstimulated control; canine and bovine INSL3 (100 ng/mL) showed more than twofold

stimulation. In mouse testicular interstitial cells, maximum stimulation (more than twofold) of testosterone secretion was observed with 100 ng/mL bovine INSL3 ($P < 0.0001$). In purified mouse Leydig cells, INSL3 stimulated ($P < 0.05$) testosterone secretion, and the maximum stimulation (nearly twofold) was observed with 100 ng/mL bovine INSL3 at the lowest Leydig cell density (2,500 cells per well). Interestingly, bovine INSL3 (100 ng/mL) stimulated ($P < 0.0001$) cAMP production from primary Leydig cells maximally at 1 h, and remained significantly elevated even at 18 h. SQ 22536, the specific adenylate cyclase inhibitor reduced ($P < 0.0001$) the bovine INSL3-stimulated testosterone secretion from Leydig cells. The addition of INSL3 antagonists (both bovine and human) into culture medium markedly reduced ($P < 0.0001$) the stimulatory effect of bovine INSL3 on testosterone secretion by Leydig cells. The observed stimulatory effects of INSL3 on testosterone secretion from testicular interstitial cells and Leydig cells are exerted via the activation of cAMP, and this would suggest a new autocrine function of INSL3 in males.

General Discussion

During the recent past INSL3 received a great deal of attention as a marker of Leydig cell function in humans (Bay et al., 2005; Ermetici et al., 2009; Foresta et al., 2009; Taneli et al., 2010; Cabrol et al., 2011). According to the present published evidences, INSL3 is considered as a major circulating hormone in adult males (Ivell and Anand-Ivell, 2009). These extensive human researches have been based on reliable and sensitive immunoassay assay systems (commercial or non-commercial), and the availability of such assays widened human INSL3 research during the last decade. Before the commencement of the present series of studies, there were no published quantification methods for INSL3 (in both plasma and culture medium) in domestic animal species. As a consequence, nothing is known regarding *in vivo* and *in vitro* INSL3 secretion in male domestic animals. Therefore, immunoassay systems presented here for the quantification of bovine and canine INSL3, and the INSL3-related findings based on these assays provide valuable insights to INSL3 research in domestic animals. Moreover, the author presented first evidence for the effects of INSL3 on canine and mouse interstitial cells (including purified mouse Leydig cells) to secrete testosterone, suggesting a novel autocrine function of INSL3.

In the first chapter of this study, the author has described a new, reliable EIA procedure for the measurement of plasma INSL3 in cattle. Employing this assay, plasma INSL3 dynamics were assessed in sexually developing bulls from birth to 22 months of age, and were compared with those of testosterone. In beef bulls, plasma INSL3 concentrations increased during the first 3 months after birth and throughout the pubertal age. Dynamic patterns for INSL3 and testosterone were similar during the first 3 months of life, but these

patterns for the release of the two hormones were diverged around the pubertal age. Previously, age-related circulating INSL3 concentrations in males were limited to humans (reviewed by Bay and Andersson (2010)) and rodents (Boockfor et al., 2001; Anand-Ivell et al., 2009).

The second chapter of this study has described a newly developed sensitive TRFIA which can be used for the quantification of INSL3 in dogs. Circulating INSL3 concentrations in male dogs of a broad age range (4 months to 14 years) were determined by using this assay; at pubertal age, a transient surge was observed in INSL3 concentrations, but not for testosterone. Similarly, a transient INSL3 surge has been observed in rats at 40 days of age (Anand-Ivell et al., 2009). In contrast, in humans, INSL3 concentrations are very low in pre-pubertal period and increased gradually during puberty to reach its maximum level in adulthood (Wikstrom et al., 2006; Ivell and Anand-Ivell, 2009).

Lower plasma INSL3 and testosterone concentrations in bilateral cryptorchid dogs suggest impaired endocrine functions of Leydig cell component in paired retained testes. Using the present assays, hormone concentrations were readily detectable in bilateral cryptorchid dogs, but undetectable in castrated dogs; these hormone measurements may provide a diagnostic tool to differentiate bilateral cryptorchidism from anorchism. It should be noted that the palpation and ultrasonography have limited success in diagnosing the bilateral cryptorchidism in dogs (Johnston et al., 2001a).

In the third chapter of the current study, the author attempted to study the INSL3 and testosterone *in vitro* secretory responses to a few potential effectors of testicular interstitial cells of dogs. Using interstitial cells from clinical testicular samples of normal dogs, we firstly demonstrated that physiological concentrations of hCG (0.01 and 0.1 IU/mL hCG;

equivalent to approximately 0.8 ng/mL and 8 ng/mL, respectively) do not acutely stimulate INSL3 release *in vitro*, whereas testosterone response was dramatically increased. It was further showed that very high concentrations of hCG (10 IU/mL; equivalent to approximately 800 ng/mL) acutely stimulate INSL3 release from interstitial cells of scrotal testis of dogs. Based on numerous reports in humans *in vivo*, it has been suggested that INSL3 secretion is constitutive and mediated via long-term trophic effects of LH (Ivell and Anand-Ivell, 2009; Bay and Andersson, 2010). From the findings of the same study, author demonstrated the hyporesponsiveness of INSL3 and testosterone secretions to hCG (for INSL3, supraphysiological doses of hCG) in the interstitial cells of retained testes from cryptorchid dogs than that of scrotal testes from normal dogs.

Using canine testicular interstitial cells as an *in vitro* model, we reported the direct effects of phthalate monoesters on INSL3 and testosterone secretions. It is reasonable to infer that environmental phthalate exposure may alter the function of Leydig cells *in vivo*. As human's favored companions, dogs share the living environment with their owners, and also the risk of exposure to multiple phthalates which are considered as potential reproductive toxicants.

In the final chapter of this study, for the first time, the author has presented the experimental data to prove the *in vitro* effects of INSL3 on the secretion of testosterone in canine and mouse testicular interstitial cells and in purified mouse Leydig cells, and the effect is exerted through cAMP pathway. Indeed, this may suggest a novel autocrine role of INSL3 in Leydig cells, and such role may be active during fetal and pre-pubertal phases of life where trophic effects of LH on Leydig cells are low. Although LH concentrations are very low in pre-pubertal ages (Inaba et al., 1988; Kawakami et al., 1995), plasma INSL3

concentrations in pre-pubertal dogs are similar to those in post-pubertal age. As RXFP2 is expressed in fetal cells of dogs (Arrighi et al., 2010) it can be reasonably speculated that INSL3 may play an indirect role (additionally to its direct role via gubernaculum) in testicular descent through the stimulation of testosterone secretion from Leydig cells of fetal testis.

Taken together, it should be noted that the findings presented in this thesis contributed significantly to the advancement of INSL3 research; first evidences have been presented on INSL3 secretion in male domestic animals that were obtained through the establishment of novel immunoassays for the measurement of bovine and canine INSL3, and also direct effects of INSL3 on Leydig cells, suggesting a novel autocrine role of INSL3.

Conclusions

1. Immunoassay systems were developed to quantify plasma INSL3 in cattle and dogs. According to the data obtained, it is inferred that testis-derived INSL3 is the major source of circulating INSL3 in bulls and male dogs.
2. In normal bulls, plasma INSL3 concentrations were continuously increased from birth to pubertal age and this pattern differed from that of testosterone around pubertal ages.
3. In normal male dogs, plasma INSL3 concentrations showed a transient surge at pubertal age, but testosterone did not.
4. Lower plasma INSL3 concentrations in bilateral cryptorchid dogs than normal dogs suggest impaired endocrine functions of Leydig cell component in paired retained testes. Furthermore, the higher INSL3 concentrations in bilateral cryptorchid than castrated dogs indicate the diagnostic value for INSL3 to predict the presence of retained testes.
5. Physiological concentrations of LH do not acutely stimulate INSL3 release in canine testicular interstitial cells whereas testosterone response was dramatically increased.
6. LH-induced INSL3 and testosterone secretory responses are lower in the interstitial cells of retained testes than that of scrotal testes in dogs.
7. Phthalate, an environmental xenoestrogen inhibit INSL3 secretion from canine testicular interstitial cells.
8. INSL3 stimulates testosterone secretion from mouse Leydig cells through the activation of cAMP pathway.
9. The present studies provide new knowledge on secretion of INSL3 in comparison to testosterone in bulls and male dogs, and also the effect of INSL3 on Leydig cells.

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