Plasma insulin-like peptide 3 concentrations are acutely regulated by luteinizing hormone in pubertal Japanese Black beef bulls

Acute regulation of insulin-like peptide 3 secretion in peripheral blood by LH in pubertal Japanese Black beef bulls

Minhaj A. Hannan[a], Yuri Fukami[a], Noritoshi Kawate[a,*], Mitsuhiro Sakase[b], Moriyuki Fukushima[b], Indunil N. Pathirana[c], Erika E. Büllesbach[d], Toshio Inaba[a], and Hiromichi Tamada[a]

[a]Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisano, Osaka, Japan

[b]Northern Center of Agricultural Technology, General Technological Center of Hyogo Prefecture for Agriculture, Forest and Fishery, Wadayama, Hyogo, Japan

[c]Department of Animal Science, Faculty of Agriculture, University of Ruhuna, Kamburupitiya, Sri Lanka

[d]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina, USA

*__Corresponding author:__ N. Kawate, Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Izumisano, Osaka 598-8531, Japan. Tel.: +81-72-463-5354; Fax: +81-72-463-5354.

E-mail address: nkawate@vet.osakafu-u.ac.jp

Abstract

Insulin-like peptide 3 (INSL3) is a major secretory product of testicular Leydig cells. The mechanism of acute regulation of INSL3 secretion is still unknown. The present study was undertaken in pubertal beef bulls to: (1) determine the temporal relationship of pulsatile secretion among LH, INSL3 and testosterone; and (2) monitor acute regulation of INSL3 secretion by LH using GnRH analogue and hCG. Blood samples were collected from Japanese Black beef bulls (n=6) at 15-min intervals for 8 h. Moreover, blood samples were collected after GnRH (−0.5 h, 0 h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h) and after hCG (−0.5 h, 0 h, 2 h, 4 h, 8 h, day 1, day 2, day 4, day 8 and day 12) treatments. Concentrations of LH, INSL3 and testosterone determined by enzyme-immunoassays (EIA) indicated that secretion in the general circulation was pulsatile. The frequency of LH, INSL3 and testosterone pulses was 4.7 ± 0.9, 3.8 ± 0.2 and 1.0 ± 0.0, respectively, during the 8 h period. Seventy percent of these INSL3 pulses peaked within 1 h after a peak of an LH pulse had occurred. The mean increasing rate (peak/basal concentration) of testosterone pulses was higher ($P<0.001$) than those of INSL3 pulses. After GnRH treatment, LH concentrations increased ($P<0.01$) dramatically 1 h post-treatment and remained high ($P<0.01$) until 5 h, while an elevated ($P<0.05$) INSL3 concentration was observed at 1 h, 2 h and 6 h after treatment.

Testosterone concentrations increased (P<0.01) 1 h after the treatment and remained high till the end of sampling. After hCG treatment, an increase of INSL3 concentration was observed at 2 h, 4 h, day 2, day 4 and day 8 of treatment (P<0.05), whereas in case of testosterone, concentrations remained significantly (P<0.01) high till 8 day after treatment. The increasing rate (maximum/pre-treatment concentration) of testosterone concentrations after injecting GnRH or hCG was much higher (P<0.001) than that of INSL3. Our results demonstrate that the secretory pattern of INSL3 in the peripheral blood is pulsatile in bull and that endogenous and exogenous LH can stimulate INSL3 secretion soon after the treatment. This suggests an acute regulation of INSL3 by LH in beef bulls. Moreover, the increasing rate of INSL3 pulses are much smaller than those of testosterone pulses, and therefore INSL3 can be used as a less-fluctuating marker than testosterone to evaluate functions of testicular Leydig cells in the pubertal beef bulls.

Keywords: INSL3; LH; Testosterone; GnRH; HCG; Beef bull

1. Introduction

Insulin-like peptide 3 (INSL3) is a major secretory product of testicular Leydig cells in all mammalian species examined so far [1–2]. The two main known functions of INSL3 in the male are the endocrine regulatory effect involved in completing the trans-abdominal phase of testicular descent in mice [3, 4] and a paracrine function exhibiting an anti-apoptotic effect to protect male germ cells in rats [5]. According to studies on human, secretion of INSL3 is related to the differentiation status of testicular Leydig cells and is stimulated by the long-term trophic effects of LH [1, 6–9]. However, the process of acute regulation of INSL3 secretion is mostly unknown. Detection of INSL3 in the peripheral blood of humans [7, 10, 11], dogs [12], and cattle [13] indicate that INSL3 may have additional endocrine effects in mammalian male species. According to recent studies in our laboratory, dynamics of secretory patterns of INSL3 and testosterone in peripheral plasma are different during sexual development in male dogs [12] and beef bulls [13], although both hormones are secreted from the unique source of testicular Leydig cells. It is well documented that in many species including bull [14, 15] secretion of LH occurred in a pulsatile manner stimulating testicular Leydig cells to produce pulsatile secretion of testosterone. However, under physiological conditions the pulsatile secretory pattern of INSL3 and its relation with

71 LH has not been elucidated.

72 Endogenous LH increased by gonadotropin releasing hormone (GnRH) or human
73 chorionic gonadotropin (hCG), which possesses LH activity, caused a significant
74 increase of testosterone in the general circulation of bulls [16–19], male goats [20], and
75 rams [21]. In men testosterone concentrations in peripheral blood taken daily for 8 days
76 increased after hCG treatment while INSL3 concentrations did not change [22]. It
77 remains unknown whether endogenous and exogenous LH can acutely regulate the
78 secretion of INSL3 in domestic animals.

79 The objectives of this paper are: (1) to determine the temporal relationship of
80 pulsatile secretion among LH, INSL3 and testosterone; and (2) to monitor acute
81 regulation of INSL3 secretion by LH using GnRH analogue and hCG in pubertal beef
82 bulls.

83

84 **2. Materials and methods**

85 *2.1. Animals*

86 Japanese Black beef bulls (n=6, aged 10–19 mo) raised in an experimental beef
87 cattle station in the Northern Center of Agriculture Technology of Hyogo Prefecture in
88 Japan were used for the present study. The selected beef bulls had no apparent

abnormalities of the reproductive status and testicular presence was checked manually to confirm the presence of both testes inside the scrotum. These bulls remained normal in appearance and health during all experiments. Bulls were kept under natural light in an open shelter covered by a roof and were maintained by ad libitum hey and concentrate to meet or exceed Japanese Feeding Standard recommendations for the beef bulls.

2.2. Experiment 1

Experiment 1 was done to determine the temporal relationship among INSL3, LH and testosterone at 15-min intervals sampling for an 8 h session in beef bulls (aged, 10–11 mo; n=6). Blood sampling for all bulls was started at 10:00 AM and ended at 6:00 PM. An indwelling jugular venous catheter (Argyle™ Covidien Ltd., Dublin, Ireland) was inserted about 1 h before the beginning of sampling. No sedation was performed before inserting the intravenous catheter and during sampling. Head restraint by either a stanchion or a halter was not used, except during insertion of the intravenous catheter. The bulls were given access to water and hay at every 2 to 3 h during collection of the samples. Blood samples were collected into heparinized tubes and immediately placed in ice before centrifuging (1700 × g for 15 min at 4°C). The plasma

was decanted and stored (−30°C) until assay.

2.3. Experiment 2

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

2.4. Experiment 3

Six beef bulls that were used for experiments 1 and 2 were also used for experiment 3. This experiment was conducted about 6 mo after completion of experiment 2. A single dose of hCG (5 IU/kg, im; Veterinary Puberogen^{R}, Novartis Animal Health, Tokyo) was administered. Two pre-treatment blood samples were taken

at –0.5 h and immediate before the hCG treatment (0 h). The sampling was then continued at 2 h, 4 h, 8 h, day 1, day 2, day 4, day 8 and day 12 of the post-treatment. Blood collection and processing of plasma were done as mentioned above in experiment 2.

2.5. Hormone assays

2.5.1. INSL3 and testosterone

Plasma concentrations of INSL3 were measured using an enzyme-immunoassay (EIA). A homologous bovine plasma EIA developed and validated in our laboratory [13] was used with a minor variation using a biotinylated canine INSL3 instead of biotinylated bovine INSL3. An anti-bovine INSL3 mouse monoclonal antibody (2-8F) and synthetic bovine INSL3 [23] were used. The minimum detection limit of the INSL3 EIA was 0.31 ng/mL, and the detection was reliable in the range from 0.31 to 20 ng/mL. The intra- and inter-assay CVs were 7.5% and 13.7%, respectively. Plasma testosterone concentrations were determined by an EIA using the procedure previously described by us [13]. An anti-testosterone rabbit polyclonal antibody and horseradish peroxidase (HRP) -labeled testosterone (Cosmo Bio Co., Ltd., Tokyo) were used. The minimum detection limit was 0.07 ng/mL, and the reliable

143 detection range for testosterone EIA was 0.07 to 20 ng/mL. The intra- and inter-assay
144 CVs were 6.6% and 11.3%, respectively.
145
146 *2.5.2. LH*
147 An EIA procedure described below was used to measure LH concentrations in
148 the bovine plasma. Eight-well strips (Corning Inc. Life Sciences, Lowell, MA, USA)
149 were coated with 100 µL per well of anti-rabbit IgG mouse polyclonal antibody (MP
150 Biochemicals , Solon, OH, USA; 5 µg/mL in 0.05 M sodium bicarbonate, pH 9.7) for 2
151 h at room temperature. The wells were then drained and washed three times with 300
152 µL of 0.15 M sodium chloride. Next, 200 µL of assay buffer (0.01M PBS, pH 7.4)
153 supplemented with 2% bovine serum albumin (BSA; Cohn Fraction V, Sigma-Aldrich,
154 St. Louis, MO), and 0.02% ProClin 950 (Sigma-Aldrich) was added and kept overnight
155 at 4°C to block areas of the well that were not coated with antibody. Various
156 concentrations of bovine LH standards (AFP11743B, NIDDK, USA; 0.31 to 40 ng/mL)
157 were diluted with assay buffer. The plasma was centrifuged at 15,000 × g for 5 min at
158 4°C to sediment fibrin and other particles and then supernatant was collected and
159 diluted 2- times with assay buffer. Immediately before the assay, the wells were drained,
160 and 50 µL of standards or plasma samples followed by 50 µL of the anti-bovine LH

antibody (Immunodiagnostik AG, Bensheim, Germany; 1: 50,000 dilution in assay buffer) were added and incubated for 2 h while shaking (180 rpm). Thereafter, 50 µL of the biotinylated bovine LH was added (1: 50,000 dilution in assay buffer) and incubated for 1 h. The LH (AFP11743B) was biotinylated with EZ-Link NHS-PEG$_4$-Biotin (Thermo Fisher Scientific, Waltham, MA USA). After the reaction, the wells were drained and washed three times with 300 µL of washing buffer (0.15 M sodium chloride containing 0.05% Tween 20). Then, 100 µL of the HRP-labeled streptavidin (KPL, Gaithersburg, MD; 100 ng/mL in assay buffer) was added to the wells and incubated for 30 min. The wells were then again washed three times with saline containing 0.05% Tween 20 and incubated for another 30 min at room temperature with 100 µL substrate solution containing 3,3`,5,5`-tetramethylbenzidine (TMB; St. Louis, MO, USA). The reaction was stopped by adding 100 µL of 2 M sulfuric acid, and the optical density was measured at 450 nm using an xMark microplate absorbance spectrophotometer (Bio-Rad Laboratories). The assay detection range was from 0.31 to 40 ng/mL. The intra- and inter-assay coefficients of variation were 4.0% and 10.7%, respectively.

2.6. Data analyses

Pulses of LH, INSL3 and testosterone concentrations in plasma samples at

15-min intervals during 8 h were detected with Pulse XP software kindly provided by Prof. Michael L. Johnson, University of Virginia [24]. Basal concentrations of INSL3 and testosterone pulses were also determined with the Pulse XP software. Pre-treatment values at time 0 h were included in the data analysis while data of –0.5 h were excluded. The increasing rate (peak/basal concentration) of INSL3 and testosterone pulses was calculated from 15-min interval sampling. In addition, the increasing rate (maximum/pre-treatment concentration) of INSL3 and testosterone concentrations after administration of GnRH and hCG was calculated. Evaluation of LH, INSL3 and testosterone data were performed by a two-way analysis of variance (ANOVA) using the Generalized Estimating Equations (GEE) procedure of SPSS version 22 software (IBM, Somers, NY) to assess the effects of GnRH and hCG treatments. Differences in hormone concentrations were compared using pairwise comparisons of the GEE procedure by the least significant difference (LSD) post hoc test. Data are expressed as mean ± SEM. Differences were considered significant at $P<0.05$.

3. Results

3.1. Pulsatile interrelationships among plasma concentrations of INSL3, LH and testosterone at 15-min interval for 8 hours

We studied the secretory pattern of INSL3 and compared its secretion with LH and testosterone. Sampling was spaced in 15-min intervals over an 8 h session. Data analysis using the Pulse XP software showed that apart from the known pulsatile secretion of LH and testosterone, the secretion of INSL3 in the general circulation of beef bulls was also pulsatile. Fig. 1 shows the hormone profiles and detected LH, INSL3 and testosterone pulses for two representative beef bulls. In six beef bulls, during the 8 h period, a total of 28, 23 and 6 pulses occurred for LH, INSL3 and testosterone, respectively. Of the 23 INSL3 pulses, 16 (69.6%) pulses peaked within 1 h period after a peak of an LH pulse. Five bulls showed that testosterone levels started increasing within 30 min from a peak of an LH pulse. In case of the remaining bull (Fig. 1B), we were unable to detect the beginning of the testosterone pulse. In this case the testosterone pulse might have started before sampling and therefore we were unable to detect the LH pulse that induced the testosterone pulse. The frequency of LH, INSL3 and testosterone pulses during an 8 h period was 4.7 ± 0.9, 3.8 ± 0.2 and 1.0 ± 0.0, respectively. The mean increasing rate (peak/basal concentration) of testosterone pulses (12.9 ± 2.0 fold, n=6) was significantly higher ($P<0.001$) than those of INSL3 pulses (1.5 ± 0.1 fold, n=23).

215 *3.2. Effect of GnRH treatment on LH, INSL3 and testosterone secretion*

216 A single dose of a GnRH analogue was administered to stimulate LH secretion to
217 determine how increased plasma LH concentration facilitated the secretion of INSL3
218 from the testicular Leydig cells. Mean plasma LH concentrations increased ($P<0.01$)
219 dramatically 1 h after treatment and reached a maximum concentration at 2 h (Fig. 2A).
220 Thereafter, the concentration slowly decreased but remained significantly high ($P<0.01$)
221 up to 5 h post GnRH treatment but approached basal LH levels at 6 h.

222 Mean plasma INSL3 concentrations increased ($P<0.01$) 1 h after the treatment
223 and remained significantly high until 2 h ($P<0.05$) (Fig. 2B). From 3 h to 5 h INSL3
224 concentrations did not differ significantly when compared with the pre-treatment value.
225 However, a significant increase ($P<0.05$) of INSL3 concentrations was again observed
226 at 6 h.

227 Mean plasma testosterone concentrations were increased ($P<0.01$) at all time
228 points when compared with the pre-treatment value. Testosterone levels rose at 1 h post
229 treatment and remained significantly high until the end of sampling at 6 h (Fig. 2C). The
230 mean increasing rate (maximum/pre-treatment concentration) of testosterone
231 concentrations (7.6 ± 2.2-fold, $n=6$) after administration of GnRH analogue was higher
232 ($P<0.01$) than that of INSL3 (1.6 ± 0.2-fold, $n=6$).

3.3. Effect of hCG treatment on INSL3 and testosterone secretion

A single injection of hCG was administered to determine the effect of sustained levels of LH on INSL3 secretion. Mean plasma INSL3 and testosterone concentrations after administration of hCG are presented in Fig. 3. Plasma INSL3 concentrations increased ($P<0.01$) 2 h after treatment and remained significantly high ($P<0.05$) till the next sampling at 4 h. When compared to control no significant changes were observed at 8 h and 1 day after treatment. However, INSL3 concentrations again increased significantly on days 2 through 8 (day 2, $P<0.01$; day 4, $P<0.01$; day 8, $P<0.05$), approaching pre-treatment level on day 12 (Fig. 3A).

A dramatic increase ($P<0.01$) of mean plasma testosterone concentrations after treatment was observed from 2 h and continued till day 4. Thereafter, concentrations started to decrease but remained significantly elevated ($P<0.01$) until day 8, reaching basal level on day 12 post-treatment (Fig. 3B). After administration of hCG, the mean increasing rate (maximum/pre-treatment concentration) of testosterone concentrations (10.4 ± 2.2-fold) was higher ($P<0.001$) than that of INSL3 (1.8 ± 0.2-fold).

4. Discussion

For many species including bulls [14, 15] the pulsatile release of LH from the anterior pituitary stimulates immediate pulsatile secretion of testosterone from the testicular Leydig cells. Conversely, it has been reported that secretion of INSL3 is not acutely regulated by LH [22], but is stimulated by the long-term trophic effects of LH in men [1, 6–9]. However, the short-term secretory pattern of INSL3 and its relationship with LH with frequent blood sampling has not been reported. To the best of our knowledge, this is the first study to evaluate circulating INSL3 levels at 15-min intervals. We found that the nature of releasing INSL3 from the testicular Leydig cells into the general circulation of beef bulls is pulsatile, and a temporal relationship between LH and INSL3 secretion exists.

The secretion of INSL3 in the general circulation of beef bulls is pulsatile with an average pulse frequency of about 4 in an 8 h sampling session. The mean increasing rate of INSL3 pulses are much smaller than those of testosterone pulses, suggesting that INSL3 can act as a less-fluctuating marker than testosterone to evaluate the testicular Leydig cells status in bulls.

The frequency of testosterone pulses in the present study is in accordance with the previous reports [14, 15] in bulls. It has been reported that LH pulses precede testosterone pulses in bulls [14], which has been the case not only for testosterone

pulses but also for INSL3 pulses as shown in our present study. We noticed that 70% of INSL3 pulses peak within 1 h period from the peak of an LH pulse, indicating that in most cases INSL3 pulses are associated with LH pulses. The fewer number of testosterone pulses compared with LH pulses in an 8 h sampling session demonstrate that not all LH pulses are capable of generating a testosterone pulse, and therefore, there might be a minimum threshold value for an LH pulse to initiate a testosterone pulse whereas in case of INSL3 pulses, it seems that compared with testosterone pulses a comparatively lower minimum threshold value of LH pulses is required.

Upon treatment with GnRH, we noticed that similar to LH and testosterone, INSL3 concentrations also increased significantly within 1 h. The increasing rate (maximum/pre-treatment concentration) by GnRH stimulation is much lower for INSL3 than for testosterone. A similar lower increasing rate of INSL3 pulses than testosterone pulses was observed under physiological condition in experiment 1 with 15-min intervals sampling. These results show that LH pulses precede INSL3 pulses in most cases. The significant increase of INSL3 concentrations within 1 h period of GnRH treatment in experiment 2 suggests that the INSL3 secretion is acutely regulated by LH. Administration of hCG, which has LH-activity, provides additional evidence regarding this issue. After hCG treatment, a significant increase of both testosterone and INSL3

287 levels was observed that sustained over a longer period of time. For both hormones, the
288 concentrations increased shortly after treatment, remained high till day 8, but again the
289 increasing rate is much smaller for INSL3 than for testosterone. Maintaining a
290 significant higher concentration of INSL3 and testosterone for a longer period of time
291 by hCG than GnRH treatment is probably due to the sustained longer activity of hCG
292 [25]. Previously, a significant increase of testosterone in the general circulation of bulls
293 has been shown after GnRH and hCG treatments [16–19]. In the present study, the
294 simultaneous increase of INSL3 and testosterone concentrations within 1 to 2 h after
295 those treatments, provide another new information that LH acutely regulates the
296 secretion of INSL3 in bull plasma. This acute regulation of INSL3 by LH in bulls is the
297 novel finding of our present study and is in difference to previous studies in men. One
298 study showed that when men were treated with hCG and peripheral blood was taken
299 daily for 8 days, testosterone concentrations increased after hCG treatment, but INSL3
300 did not change [22] whereas other studies showed that hCG can increase INSL3
301 concentrations in blood after 4 or 10 days of treatment when endogenous LH secretion
302 was inhibited by androgen analogues or GnRH antagonist [26, 27].

303 In conclusion, the secretion of INSL3 in the general circulation of beef bulls
304 occurs in a pulsatile manner. Endogenous and exogenous LH can stimulate INSL3

secretion soon after the treatment, suggesting the acute regulation of INSL3 by LH in bulls. Moreover, the increasing rate of INSL3 pulses is much smaller than those of testosterone pulses, and therefore we suggest that INSL3 can be used as a less-fluctuating marker than testosterone when testing the functions of testicular Leydig cells in bulls.

Acknowledgements

The authors thank NIDDK, NIH, USA and Dr. A. F. Parlow, National Hormone & Pituitary Program, Harbor, UCLA Medical Center, for providing the bovine LH (AFP11743B).

References

[1] Ivell R, Balvers M, Domagalski R, Ungefroren H, Hunt N, Schulze W. Relaxin-like factor: a highly specific and constitutive new marker for Leydig cells in the human testis. Mol Hum Reprod 1997;3:459–66.

[2] Adham IM, Burkhardt E, Benahmed M, Engel W. Cloning of a cDNA for a novel insulin-like peptide of the testicular Leydig cells. J Biol Chem 1993;268:26668 –72.

[3] Nef S, Parada LF. Cryptorchidism in mice mutant for Insl3. Nat Genet 1999;22:295

9.

[4] Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, et al. Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. Mol Endocrinol 1999;13:681–91.

[5] Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, et al. Paracrine regulation of mammalian oocyte maturation and male germ cell survival. Proc Natl Acad Sci U S A 2004;101:7323–8.

[6] Foresta C, Bettella A, Vinanzi C, Dabrilli P, Meriggiola MC, Garolla A, et al. Insulin-like factor 3: a novel circulating hormone of testis origin in humans. J Clin Endocrinol Metab 2004;89:5952–8.

[7] Bay K, Hartung S, Ivell R, Schumacher M, Jürgensen D, Jorgensen N, et al. Insulin-like factor 3 serum levels in 135 normal men and 85 men with testicular disorders: relationship to the luteinizing hormone-testosterone axis. J Clin Endocrinol Metab 2005;90:3410–8.

[8] Ferlin A, Garolla A, Rigon F, Rasi CL, Lenzi A, Foresta C. Changes in serum insulin like factor 3 during normal male puberty. J Clin Endocrinol Metab 2006;91:3426–31.

[9] Wikstrom AM, Bay K, Hero M, Andersson A-M, Dunkel L. Serum insulin-like

341 factor 3 levels during puberty in healthy boys and boys with Klinefelter syndrome. J
342 Clin Endocrinol Metab 2006;91:4705–8.
343 [10] Anand-Ivell R, Wohlgemuth J, Haren MT, Hope PJ, Hatzinikolas G, Wittert G, et al.
344 Peripheral INSL3 concentrations decline with age in a large population of
345 Australian men. Int J Androl 2006;29:618–26.
346 [11] Büllesbach EE, Rhodes R, Rembiesa B, Schwabe C. The relaxin-like factor is a
347 hormone. Endocrine 1999;10:167–9.
348 [12] Pathirana IN, Yamasaki H, Kawate N, Tsuji M, Büllesbach EE, Takahashi M, et al.
349 Plasma insulin-like peptide 3 and testosterone concentrations in male dogs:
350 changes with age and effects of cryptorchidism. Theriogenology 2012;77:550–7.
351 [13] Kawate N, Ohnari A, Pathirana IN, Sakase M, Büllesbach EE, Takahashi M, et al.
352 Changes of plasma concentrations of insulin-like peptide 3 and testosterone from
353 birth to pubertal age in beef bulls. Theriogenology 2011;76:1632–8.
354 [14] Stumpf TT, Wolfe MW, Roberson MS, Kittok RJ, Kinder JE. Season of the year
355 influences concentration and pattern of gonadotropins and testosterone in
356 circulation of the bovine male. Biol Reprod 1993;49:1089–95.
357 [15] Finnerty M, Enright WJ, Roche JF. Testosterone, LH and FSH episodic secretory
358 patterns in GnRH-immunized bulls. J Reprod Fertil 1998;114:85–94.

[16] D'Occhio MJ, Aspden WJ. Characteristics of luteinizing hormone LH and testosterone secretion, pituitary responses to LH-releasing hormone LHRH and reproductive function in young bulls receiving the LHRH agonist deslorelin: effect of castration on LH responses to LHRH. Biol Reprod 1996;54:45–52.

[17] Mongkonpunya K, Hafs HD, Convey EM, Tucker HA, Oxender WD. Serum luteinizing hormone, testosterone and androstenedione in pubertal and prepubertal bulls after gonadotropin releasing hormone. J Anim Sci 1975;40:682–6.

[18] Sundby A, Tollman R, Velle W. Long-term effect of hCG on plasma testosterone in bulls. J Reprod Fert 1975;45:249–54.

[19] Murase T, Okuda K, Sato K. Assessment of bull fertility using a mucus penetration test and a human chorionic gonadotrophin stimulation test. Theriogenology 1990;34:801–12.

[20] Saito H, Sawada T, Yaegashi T, Goto Y, Jin J, Sawai K, et al. Kisspeptin-10 stimulates the release of luteinizing hormone and testosterone in pre- and post-pubertal male goats. Anim Sci J 2012;83:487–92.

[21] Falvo RE, Buhl AE, Reimers TJ, Foxcroft GR, Dunn MH, Dziuk PJ. Diurnal fluctuations of testosterone and LH in the ram: effect of hCG and gonadotrophin-releasing hormone. J Reprod Fert 1975;42:503–10.

[22] Bay K, Andersson AM. Human testicular insulin-like factor 3: in relation to development, reproductive hormones and andrological disorders. Int J Androl 2011;34:97–109.

[23] Büllesbach EE, Schwabe C. The primary structure and the disulfide links of the bovine relaxin-like factor (RLF). Biochemistry 2002;41:274–81.

[24] Johnson ML, Pipes L, Veldhuis PP, Farhy LS, Boyd DG, Evans WS. AutoDecon, a deconvolution algorithm for identification and characterization of luteinizing hormone secretory bursts: description and validation using synthetic data. Anal Biochem 2008;381:8–17.

[25] Boime I, Ben-Menahem D. Glycoprotein hormone structure-function and analog design. Recent Prog Horm Res 1999;54:271–88.

[26] Bay K, Matthiesson KL, McLachlan RI, Andersson A-M. The effects of gonadotropin suppression and selective replacement on insulin-like factor 3 secretion in normal adult men. J Clin Endocrinol Metab 2006;91:1108–11.

[27] Roth MY, Lin K, Bay K, Amory JK, Anawalt BD, Matsumoto AM, et al. Serum INSL3 is highly correlated with intratesticular testosterone in normal men with acute, experimental gonadotropin deficiency stimulated with low-dose hCG: a randomized-controlled trial. Fertil Steril 2013;99:132–9.

395

396 **Figure legends**

397

398 **Fig. 1.** Changes of plasma LH, INSL3 and testosterone concentrations in blood samples

399 taken from two individual representative beef bulls (A: #1, B: #2). Blood samples were

400 taken at 15-min intervals for 8 h. The peaks for INSL3 (⋯►), LH (→), and testosterone

401 (○) pulses were determined by the Pulse XP software.

402

403 **Fig. 2.** Plasma LH (A), INSL3 (B), and testosterone (C) concentrations in response to

404 GnRH treatment (0.5 μg/kg) in beef bulls. Data are expressed as mean ± SEM (n=6). *P

405 <0.05, **P <0.01 compared with the pre-treatment value of the corresponding hormone

406 at 0 h.

407

408 **Fig. 3.** Plasma INSL3 (A), and testosterone (B) concentrations in response to hCG

409 treatment (5 IU/kg) in beef bulls. Data are expressed as mean ± SEM (n=6). *P <0.05,

410 **P <0.01 compared with the pre-treatment value of the corresponding hormone at 0 h.

411

Figure

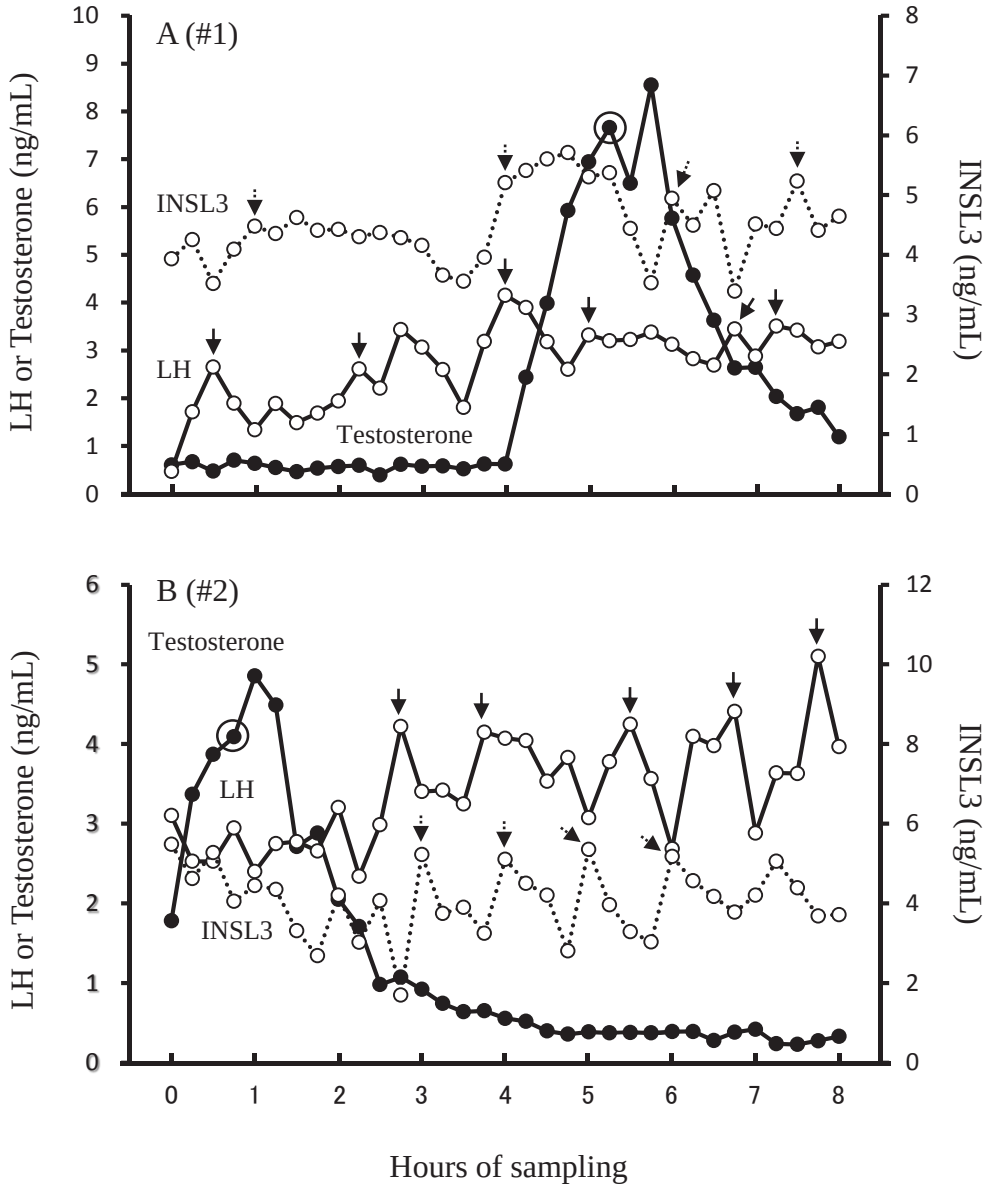
A (#1)
INSL3
LH
Testosterone
LH or Testosterone (ng/mL)
INSL3 (ng/mL)
B (#2)
Testosterone
LH
INSL3
Hours of sampling

Fig. 1

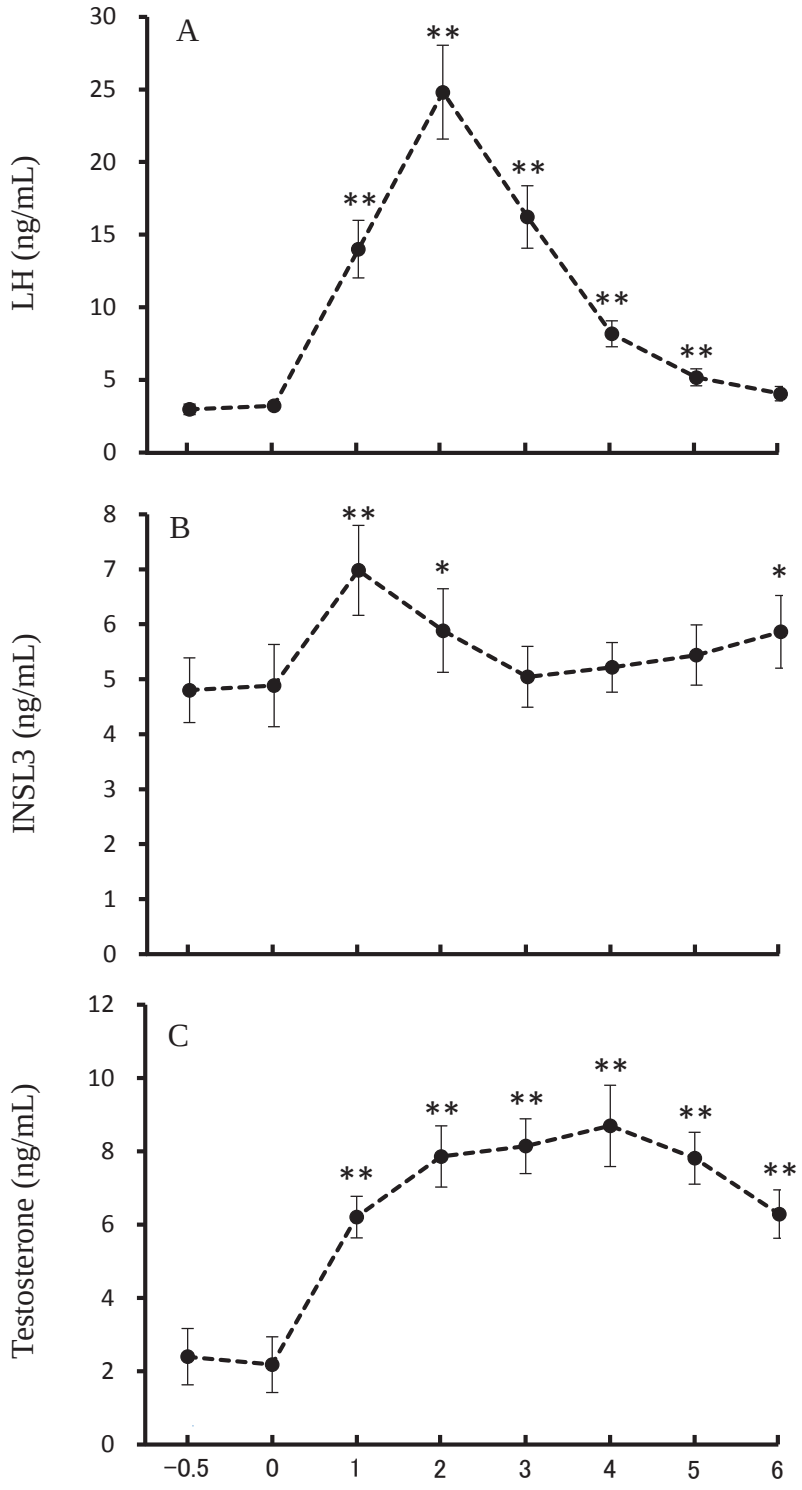
A
B
C
LH (ng/mL)
INSL3 (ng/mL)
Testosterone (ng/mL)
Hours after GnRH treatment
−0.5
0
1
2
3
4
5
6

Fig. 2

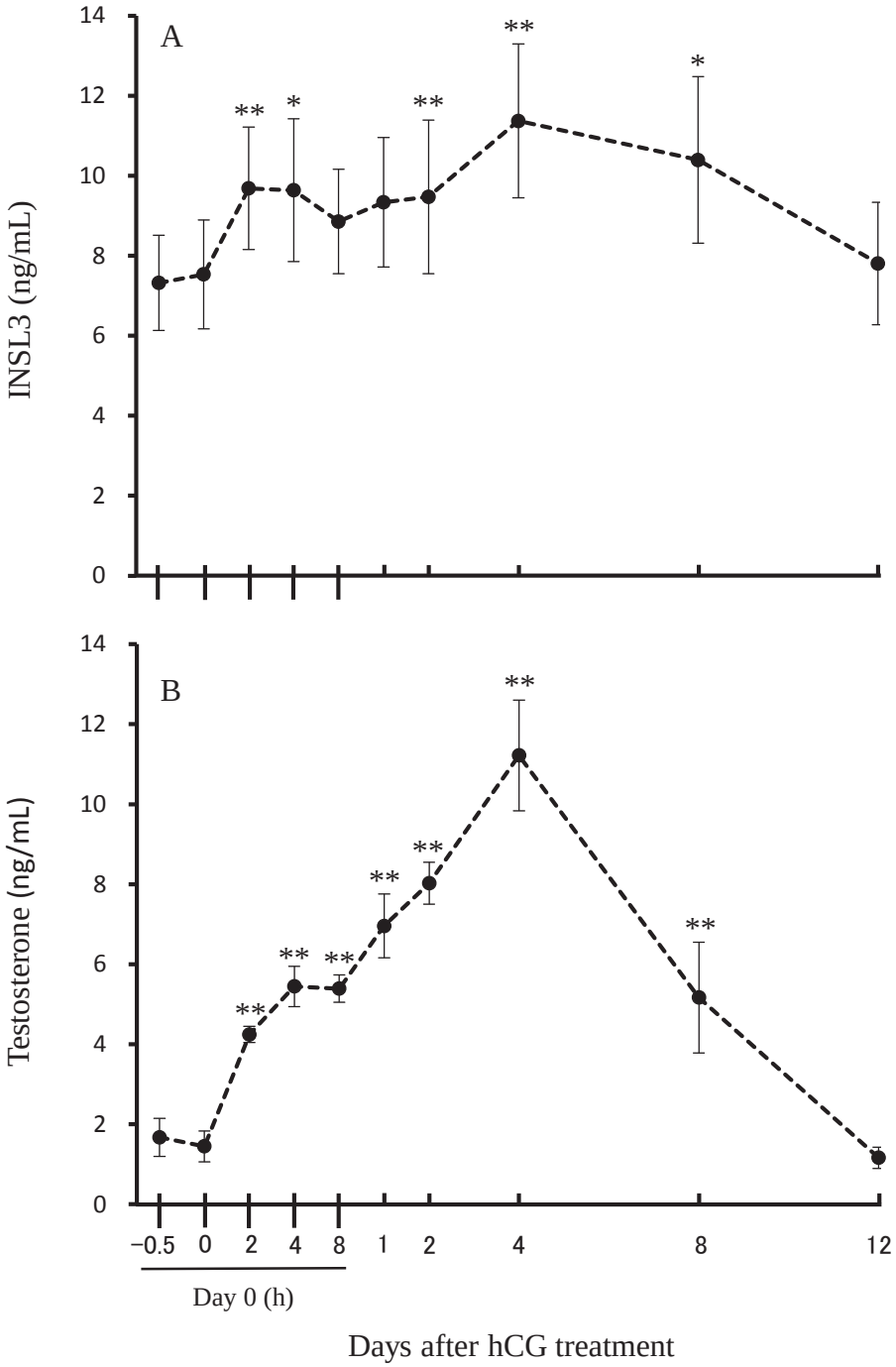
A
B
INSL3 (ng/mL)
Testosterone (ng/mL)
0
2
4
6
8
10
12
14
**
*
−0.5
0
2
4
8
1
2
4
8
12
Day 0 (h)
Days after hCG treatment

Fig. 3