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Article

Measurement of circulating insulin-like peptide 3 and testosterone concentrations in pre-pubertal, tropical male goats

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Abstract: The present study attempted to: (1) simplify the existing enzyme immunoassay (EIA) for the measurement of insulin-like peptide 3 (INSL3) concentrations in goats (2) to measure circulating INSL3 and testosterone in pre-pubertal Jamnapari X local crossbred goats (3) to examine the relationships among INSL3 concentration, testosterone concentration, scrotal circumference and body weight during the pre-pubertal age. Serial blood samples were collected from normal pre-pubertal male Jamnapari X local crossbred goats (n = 6) at the ages of 19 to 28 weeks. Serum INSL3 was measured using a recently reported EIA with modifications to the original procedure. The detection ranges of the INSL3 and testosterone assays were 0.08 to 80 ng/mL and 0.01 to 40 ng/mL, respectively. The intra-assay coefficient of variations were 3.79% for INSL3 (n = 6) and 3.72% (n = 6) for testosterone. Serum INSL3 concentrations ranged from 13.62 ± 3.25 to 22.45 ± 6.09 ng/mL (mean ± SEM) in pre-pubertal goats. Those concentrations increased ($p < 0.05$) from 20 (13.62 ± 3.25 ng/mL) to 22 (22.45 ± 6.09 ng/mL) weeks of age. Testosterone concentrations ranged from 0.30 ± 0.07 to 1.22 ± 0.43 ng/mL in pre-pubertal goats. A significant drop was observed in testosterone concentrations at 23 weeks of age. INSL3 was correlated ($r = 0.58$; $p < 0.05$) with scrotal circumference while no significant correlation was observed among other tested parameters. A rapid, sensitive EIA system was simplified to quantify INSL3 in goats, by simplifying the existing procedure. Different serum INSL3 and testosterone dynamics were found from 19 to 28 weeks of age of the goats. Compared with testosterone, INSL3 dynamics seemed to be more consistent with the age of pre-pubertal goats and showed a relationship with the testicular growth.

Keywords: enzyme immunoassay; INSL3; male goats; pre-pubertal; serum; testosterone

1. Introduction

Insulin-like peptide 3 (INSL3), along with testosterone, is a major secretory product of Leydig cells in mature testes of all male mammals (Ivell *et al.*, 2013), including male goats (Siqin *et al.*, 2013). This hormone plays an important role in reproductive physiology of male animals and humans (Bay and Andersson, 2011), and these functions include the initiation of transabdominal phase of testicular descend (Nef and Parada, 1999; Zimmermann *et al.*, 1999) and suppression of germ cell apoptosis (Kawamura *et al.*, 2004), as shown in experimental animals. Readily detectable levels of INSL3 have been reported in many mammalian species including humans (Anand-Ivell *et al.*, 2006; Bay *et al.*, 2005; Bullesbach *et al.*, 1999; Ferlin *et al.*, 2006; Wikstrom *et al.*, 2006), dogs (Pathirana *et al.*, 2012), cattle (Hannan *et al.*, 2015; Kawate *et al.*, 2011) and goats

(Hannan *et al.*, 2016; Hannan *et al.*, 2017b), and peripheral concentrations of INSL3 changes with GnRH stimulation of LH release (Hannan *et al.*, 2015; Hannan *et al.*, 2016), age (Anand-Ivell *et al.*, 2006; Bay *et al.*, 2007; Pathirana *et al.*, 2012) and testicular abnormalities (Bay *et al.*, 2005; Pathirana *et al.*, 2012). Therefore, it has been suggested that blood INSL3 concentrations can be used as a biomarker to assess the Leydig cell function. Furthermore, INSL3 has been used as a testis-specific biomarker for the assessment of pubertal development (Anand-Ivell *et al.*, 2009; Ferlin *et al.*, 2006; Johansen *et al.*, 2014; Wikstrom *et al.*, 2006). Due to differential patterns of regulation, the measurement of both INSL3 and testosterone in the same animal may provide an added benefit in assessing Leydig cell function in vivo. However, the concentrations of INSL3 in peripheral blood of goats in tropics are yet to be reported. The only available reports are on a native Japanese miniature goat breed, Shiba (Hannan *et al.*, 2016; Hannan *et al.*, 2017a; Hannan *et al.*, 2017b). With this background, it is noteworthy to investigate the endocrine changes during the pre-pubertal age of male goats by measuring both circulating INSL3 and testosterone.

At present, various methods are used to quantify hormones in blood of many species. Among these, enzyme immunoassay (EIA) is a reliable, widely used biochemical technique. Plasma INSL3 concentrations of several species, i.e., dogs (Pathirana *et al.*, 2012), cattle (Hannan *et al.*, 2015; Kawate *et al.*, 2011) and goats (Hannan *et al.*, 2016; Hannan *et al.*, 2017a) have been previously measured through immunoassays which include an INSL3 extraction step using a vacuum centrifuge. A simplified EIA protocol which requires less expensive equipment is important for the research studies conducted in developing countries with a resource-limited setup. The objectives of the present study were to (1) simplify the existing EIA protocol for the measurement of INSL3 concentrations in goats, (2) measure the circulating INSL3 and testosterone in pre-pubertal Jamnapari × local crossbred goats and (3) examine relationships among serum hormone (INSL3 and testosterone) concentrations, scrotal circumference and body weight in male goats at pre-pubertal age.

2. Materials and Methods

2.1. Animals, body measurements and blood sampling

Jamnapari × local crossbred male kids (n = 6) born on the same day in the Faculty Teaching Farm, Faculty of Agriculture, University of Ruhuna were used for this study. All kids were normal at birth and had no apparent abnormalities and remained healthy throughout the experiment. All goats were at 19 weeks of age at the beginning of the study. After restraining of the animals, scrotal circumference was measured at the age of 19, 22 and 28 weeks by using a flexible tape, and the body weight was also recorded. Serial blood samples (three to five milliliters) were collected at weeks 19, 20, 22, 23 and 28 from a jugular vein puncture. Serum was separated by following a 2000 × *g* centrifugation for 20 min and was stored in microcentrifuge tubes at -20 ^{o}C prior to hormone assays. The samples were thawed just prior to the INSL3 and testosterone assays. The protocol of the experiment was approved by the Research Ethics Committee of the Faculty of Agriculture, University of Ruhuna, Sri Lanka.

2.2. INSL3 assay

A competitive EIA was developed to quantify INSL3 by modifying the previously described procedures for goats (Hannan *et al.*, 2016) and cattle (Kawate *et al.*, 2011). Previously developed INSL3 EIA was modified by omitting the INSL3 extraction procedure which involved a vacuum centrifugation step of more than 3 h. In brief, Strip wells were coated with 100 µL of anti-mouse IgG (5 µg/mL in 0.05M sodium bicarbonate; pH 9.7), and non-specific binding sites were blocked overnight with 2% BSA, 0.02% ProClin 950 in 0.01M PBS, pH 7.4 (assay buffer). Fifty-microliter of each standard or serum sample and 50µL of anti-bovine INSL3 (1:1,000,000 dilution in assay buffer; 2-8F (Bullesbach and Schwabe, 2002), a gift from Dr. E.E. Büllesbach, Medical University of South Carolina, USA) were then dispensed into the wells and incubated for 2 h at room temperature. Subsequently, 50 µL of biotinylated human INSL3 peptide (1 ng/mL in assay buffer) was added and incubated for a further 1 h. The wells were washed three times with saline containing 0.05% Tween-20 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 were incubated for another 30 min with 100 µL 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution. The reaction was stopped by adding 100 µL of 2M sulfuric acid, and optical density was measured at 450 nm with a 630 nm reference using a microplate reader (UT-2100C, MRC, Israel).

2.3. Testosterone assay

Extraction of testosterone from goat serum was performed according to the procedure described previously (Kawate *et al.*, 2011; Pathirana *et al.*, 2012). For extracted standards or extracted samples, testosterone EIA

using the HRP-labeled testosterone and anti-testosterone antibody was performed using a method described previously (Kawate *et al.*, 2011; Pathirana *et al.*, 2012). The minimum detection limit of the assay was 0.01 ng/mL, and detection was reliable in the range 0.01 to 40 ng/mL. The intra-assay coefficient of variations was 3.72% (n = 6) for testosterone.

2.4. Statistical analysis

Differences in mean INSL3 and testosterone concentrations among groups (age in weeks) were compared using pairwise comparisons of the generalized linear models (GZLM; SPSS version 20.0, IBM Corporation, Somers, NY, USA) procedure by the least significant difference (LSD) post hoc test. Correlations among hormone concentrations (INSL3 and testosterone), testicular circumference and body weight in pre-pubertal goats were estimated by using the Pearson correlation coefficient (IBM SPSS Statistics 20.0). Data were expressed as mean ± SEM, with differences considered significant at $P < 0.05$.

3. Results

The existing EIA system (Hannan *et al.*, 2016; Kawate *et al.*, 2011) was simplified to measure circulating INSL3 in male goats, by omitting the extraction procedure which requires a vacuum centrifugation step. The removal of vacuum centrifugation step shortened the assay procedure for more than 3 h. The minimum detection limit of the assay was 0.08 ng/mL and the percent binding (B/B0, 96.8 ± 1.6%) at this limit was significantly less ($P < 0.05$) than B0, as observed in three consecutive standard curves. The sensitivity range of the assay was 0.08 to 80 ng/mL (Figure 1A). The simplified EIA system demonstrated a parallel drop in percent binding between INSL3 standards compared with a serially diluted (two to eight fold) serum sample from a male goat (Figure 1B). The intra-assay coefficient of variation was 3.79 % (n = 6) for INSL3 and the intra-assay variation observed was quite acceptable.

As measured by the present EIA system, serum INSL3 concentrations ranged from 13.62 ± 3.25 to 22.45 ± 6.09 ng/mL in pre-pubertal goats (Figure 2A). There were no significant fluctuations in INSL3 levels during the tested period, except the increase ($P < 0.05$) observed from week 20 (13.62 ± 3.25) ng/ml to 22 (22.45 ± 6.09 ng/mL) of age.

The detection range of the testosterone assay was 0.01 to 40 ng/mL. The intra-assay coefficient of variations was 3.72% (n = 6) for testosterone. Testosterone concentrations ranged from 0.30 ± 0.07 to 1.22 ± 0.43 ng/ml in pre-pubertal goats and did not differ from 19 to 22 weeks of age (Figure 2B). Interestingly, the testosterone concentration dropped ($P < 0.05$) on week 23 and increased ($P < 0.05$) by more than three-fold by week 28.

There was a significant correlation ($r = 0.58$; $P < 0.05$) between INSL3 concentration and scrotal circumference (ranged from 7.5 to 18.0 cm) in male pre-pubertal goats from 19 to 28 weeks of age. Correlations among testosterone concentration and scrotal circumference, INSL3 concentration and testosterone concentration, each hormone concentration and body weight were statistically non-significant. Body weight was increased with age from week 19 to 28 (Figure 3). Although a 23.4% increase in scrotal circumference was noted during the study period, it was not significant ($P > 0.05$; Figure 3). Obviously, the scrotal circumference and the body weight (ranged from 8.5 to 19.9 kg) was correlated ($r = 0.65$; $P < 0.05$) during the measured period.

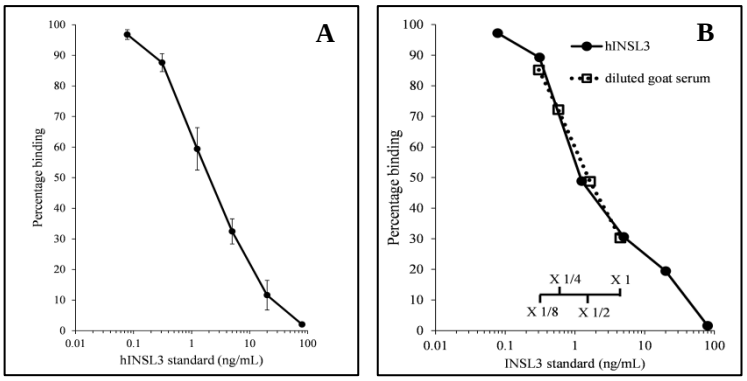

Figure 1. (A) Calibration curve of the enzyme immunoassay for INSL3 showing the reliable detection range, 0.08–80 ng/ml. Mean percentage B/B0 ± SEM of 3 curves. (B) Parallelism for human INSL3 standards with two- to eight-fold serial dilutions of the serum sample from a male goat. Standards for human INSL3 ranged from 0.08 to 80 ng/ml. hINSL3, human INSL3.

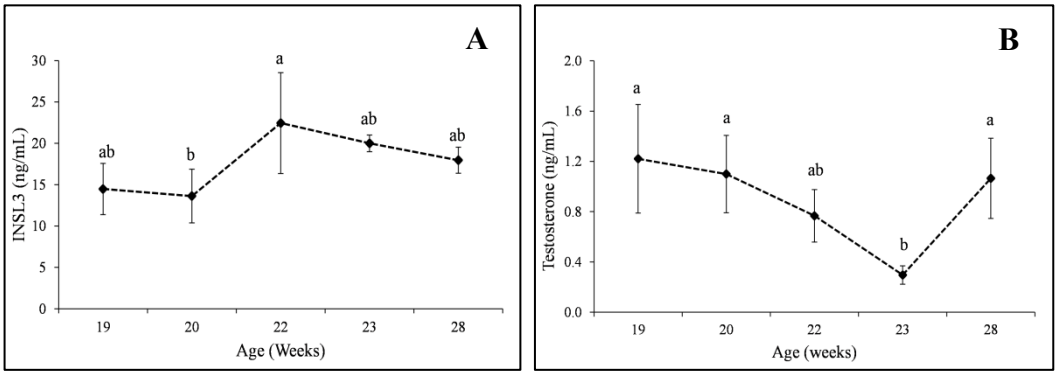

Figure 2. Mean ± SEM serum concentrations of INSL3 (A) and testosterone (B) in pre-pubertal goats (n = 6 at each week). [a–b] within a hormone, means without a common superscript differs (P < 0.05).

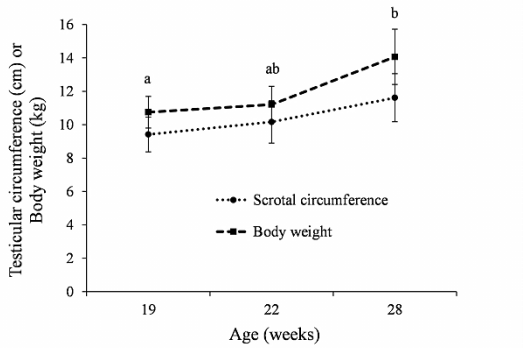

Figure 3. Scrotal circumference and body weight change in goats from 19 to 28 weeks of age. Data are expressed as mean ± SEM (n = 6 at each week). [a– b] Different superscripts indicate the significant difference for mean body weight at each week (P < 0.05).

4. Discussion

The EIA is a simple, sensitive and specific analytical tool once hormone measurements are considered. However, expensive equipment limits its applications especially in developing world. The present paper reports a simplified version of the existing EIA procedure (Hannan *et al.*, 2016) to measure INSL3 in goats. The established EIA procedure excluded the previously reported INSL3 extraction procedure using a vacuum centrifugation and reduced the assay duration by more than 3 h. The present EIA system showed an improved minimum detection limit of 0.08 ng/mL and maintained a broad INSL3 detection range of 0.08 to 80 ng/mL. The minimum detection limit and the detection range of first reported INSL3 EIA were 0.31 ng/mL and 0.31 to 20 ng/mL, respectively (Hannan *et al.*, 2016). The minimum detection limit of the present EIA was almost similar to recently reported time-resolved fluorescence immunoassay (TRFIA) which showed an improved sensitivity (Hannan *et al.*, 2017a).

To the best of our knowledge, there are no previous reports on INSL3 measurements in goats reared in the tropics. The circulating INSL3 levels have only been published in a native Japanese miniature goat breed, Shiba (Hannan *et al.*, 2016; Hannan *et al.*, 2017a; Hannan *et al.*, 2017b). The serum INSL3 concentrations reported in the present study were quite comparable to that of Shiba goats of the same age. The INSL3 concentrations of Jamnapari × local crossbred goats did not differ much during the tested period, except the significant increase observed from 20 to 22 weeks. In contrast, INSL3 concentrations have been increased approximately in two times from 18 to 28 weeks in Shiba goats (Hannan *et al.*, 2017a). The variation in INSL3 concentrations between two studies is possibly due to the difference in breeds and in management practices. The goats of the former study had been managed for experimental purposes inside an experimental room, while the goats of the present study were managed under routine farm management procedures. Possibly, the process of sexual maturity varied under the two management conditions, especially with feeding management.

The pattern of testosterone secretion was different from that of INSL3 during the study period. A marked drop (three- to four-fold) in testosterone concentrations was observed at 23 weeks compared with weeks 19, 20 and 28. Several previous studies have reported similar observations in Shiba goats after 20 weeks (Tani *et al.*, 1992)

and 26 weeks (Hannan *et al.*, 2017a) of age, and also in Black Bengal goats during 12 to 20 weeks (Georgie *et al.*, 1985) of age. It is understood that testosterone is acutely regulated by the hypothalamic-pituitary-gonadal (HPG) axis and subjected to acute episodic fluctuations of LH. Especially, the HPG axis is activated during puberty. We have recently showed that INSL3 is also acutely regulated by LH in goats (Hannan *et al.*, 2016); however, the amplitude of INSL3 rise is much lower than that of testosterone.

In the present study, a significant positive correlation was observed between serum INSL3 concentrations and the testicular circumference of Jamnapari × local crossbred goats from 19 to 28 weeks of age. Previously, Hannan *et al*. (Hannan *et al.*, 2017a) has also shown a strong positive correlation between the plasma concentrations of INSL3 and the scrotal circumference in Shiba goats. In humans, increasing INSL3 concentrations were associated with increasing testicular volume during pubertal development (Ferlin *et al.*, 2006). In contrast to INSL3 concentrations, testosterone concentrations were not significantly correlated with testicular circumference in tested goats in the present study. In line with this observation, a weak correlation has been observed between plasma testosterone and testicular circumference in Shiba goats (Hannan *et al.*, 2017a). It has been argued that circulating INSL3 concentrations better reflect the status of Leydig cells than testosterone concentrations, when single time-point blood samples are considered (Hannan *et al.*, 2017a). The acute dependence of testosterone secretions on pulsatile secretions of LH regulated by HPG axis, compared with INSL3 secretions, may also be critical in this scenario.

5. Conclusions

A rapid and highly sensitive enzyme immunoassay was developed to quantify INSL3 in goats by modifying an existing EIA procedure. Different serum INSL3 and testosterone dynamics were found during 19 to 28 weeks of age. The scrotal circumference was significantly correlated with INSL3 concentrations. Compared with testosterone concentrations, INSL3 concentrations seemed to be more consistent with the growth of pre-pubertal goats. Further studies in a broad age range with more frequent sampling would be needed to confirm these results in tropical goat breeds.

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Conflict of interest

None to declare.

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