Age Related Changes in Serum Concentrations of Insulin-like Peptide 3 and Testosterone in Male German shepherd Dogs

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Abstract
The insulin-like peptide 3 (INSL3; previously known as relaxin-like factor) and testosterone are major circulating hormones secreted by Leydig cells in mammalian testes. Along with testosterone, serum INSL3 concentrations have emerged as a novel clinical marker of Leydig cell function in humans. However, its role in dogs in relation to the age is scanty. The objectives were to: (1) to measure INSL3 and testosterone concentrations in male German shepherd dogs and to compare the changes with age (2) to identify the relationships among INSL3, testosterone, age and body weight. Blood samples were taken from normal male German Shepherd dogs from prepubertal age to advanced age (1.5 months to 11 years; n=46), and from pre-pubertal (n=5), pubertal (n=5), post-pubertal (n=15) middle age (n=17) and advanced age (n=4). German shepherd serum INSL3 and testosterone were measured using enzyme immunoassays. The detection ranges of the INSL3 and testosterone assays were 0.08 to 80ng/ml and 0.01 to 40ng/ml, respectively. Serum INSL3 concentrations increased ($P<0.05$) from pre-pubertal age to post-pubertal age, remained similar during post-pubertal and middle ages, and significantly reduced ($P<0.05$) from middle to advanced age. Testosterone concentrations increased ($P<0.05$) drastically from pre-pubertal age to pubertal age reaching a plateau, and no significant alteration was observed from pubertal to advanced age. The coefficients of determination of best regression curves between serum INSL3 and testosterone levels, INSL3 and age, INSL3 and body weight, testosterone and age, and testosterone and body weight were 0.065 ($n=46$, $P>0.15$), 0.11 ($n=46$, $P=0.075$), 0.055($n=46$, $P>0.15$), 0.53 ($n=46$, $P<0.0001$) and 0.59 ($n=46$, $P<0.0001$). In conclusion, INSL3 concentrations showed a clear decline with increasing old age of German shepherd dogs whereas testosterone did not show such trend. Different serum INSL3 and testosterone dynamics were found in relation with age and body weight.

Keywords: Enzyme immunoassay, German shepherd dog, INSL3, Serum, Testosterone
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Introduction
Insulin-like peptide 3 (INSL3; formerly, relaxin-like factor) and testosterone are major secretory products of Leydig cells in testis (Ivell *et al.,* 1997). The novel hormone INSL3 is expressed in Leydig cells of many mammalian species (Ivell *et al.,* 1997). Secretion of INSL3 is dependent on the maturation of Leydig cells. Along with testosterone, serum INSL3 concentrations have emerged as a novel clinical marker of Leydig cell function in humans (Bay and Andersson, 2010). 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.* Circulating concentrations of the above two hormones are worth studying in domestic animals, with a view to evaluate Leydig cell function. For the first time, we measured the plasma concentrations INSL3 and testosterone in small breed dogs (Pathirana *et al.,* 2012). Furthermore, circulating concentrations of INSL3 and testosterone are worth studying in large breed dogs such as German shepherd, as age-related INSL3 dynamics are yet to be investigated in large breeds. The objectives of the present study were to determine the changes of serum INSL3 with age in male German shepherd dogs, and to relate them to testosterone concentrations. Furthermore, serum INSL3 and testosterone concentrations, age and body weight were compared to identify relationships among INSL3, testosterone, age and weight in male German Shepherd dogs.

Materials and Methods
Animals and sample collection
Forty-six German shepherd male dogs [pre-pubertal (n=5), pubertal (n=5), post-pubertal (n=15) middle age (n=17) and advanced age (n=4)] employed in this study were presented to a private animal hospital close to the university. All dogs were privately owned, and the owners' consent was obtained before the collection of samples.The ages of animals ranged from 1.5 months to 11 years. The body weights were

ranged from 2 to 35 kg. Collected blood samples were immediately dispatched to the laboratory on ice. The blood was centrifuged at 3500 rpm for 5 min in the laboratory, and separated serum was stored at -18^{0}C until hormone Analyses.

INSL3 assay

Serum INSL3 concentrations were measured using an enzyme immunoassay (EIA). Previously developed INSL3 EIA was employed with minor modifications using bovine INSL3 standards instead of human INSL3 standards (Pathirana *et al.,* 2011).The minimum detection limit of the assay was 0.08ng/ml, and detection was reliable in the range 0.08 to 80 ng/ml. The intra- and inter-assay coefficients of variation were 11.0 (n = 2-4) and 10.0 (n = 4), respectively.

Testosterone Assay

Extraction of testosterone from canine serum was performed according to the procedure described previously (Pathirana *et al.,* 2011). For extracted standards or extracted samples, testosterone EIA using the HRP-labeled testosterone and anti-testosterone antibody was performed essentially according to the method described previously (Pathirana *et al.,* 2011). The minimum detection limit of the assay was 0.01ng/ml, and detection was reliable in the range 0.01 to 40 ng/ml. The intra- and inter-assay coefficients of variation were 8.1 (n = 3) and 17.7 (n = 3), respectively.

Data analysis

Evaluation of INSL3 and testosterone data were performed by generalized linear models (GENLIN) of SPSS version 20.0 (IBM Corporation, Somers, NY, USA) to assess the effects of age of the dogs. Differences in hormone concentrations age among various groups were compared using pair wise comparisons of the GENLIN procedure by the least significant difference (LSD) post hoc test. To evaluate changes in plasma hormone concentrations with the age, samples were categorized into five age groups. Best regression curves were estimated among two hormone concentrations, age and weight using the Curve Estimation Procedure of Regression Analysis (IBM SPSS Statistics 20.0). Data were expressed as mean ±SEM, with differences considered significant at $P < 0.05$.

Results and Discussion

There was an effect of age group on serum INSL3 ($P < 0.0001$) and testosterone ($P < 0.0001$) concentrations in male German Shepherd dogs. Serum INSL3 concentrations increased ($P < 0.05$) from pre-pubertal age to post-pubertal age and remained similar during post-pubertal and middle ages. It was significantly reduced ($P < 0.05$) from middle to advanced age (Fig.1A). Anand-Ivell *et al.* (2006) reported an age-related decrease in serum INSL3 concentrations in humans, and suggested this decline reflects the properties of Leydig cell population. In small breed dogs, we previously reported that INSL3 concentrations peak at pubertal age (6 months to 1 year) and those concentrations do not differ among advanced age (more than 10 years) compared with other age groups (Pathirana *et al.,* 2012). The difference of INSL3 peaks between small and large breed dogs indicate that INSL3 secretion seems to depend on Leydig cell maturation, as testicular maturation is delayed in large breed dogs (Johnston *et al.,* 2001). Testosterone concentrations increased ($P < 0.05$) drastically from pre-pubertal age to pubertal age, and thereafter, reached a plateau (Figure 1B). We previously reported a same pattern in testosterone dynamics in small breed dogs (Pathirana *et al.,* 2012). Early peak of testosterone compared with INSL3 may be attributed to tight regulation and maintenance of testosterone secretion by hypothalamic-pituitary-gonadal axis.

The coefficients of determination (R^2) of best regression curves between serum INSL3 and testosterone levels, INSL3 and age, INSL3 and body weight, testosterone and age, and testosterone and body weight were 0.065 (n=46, $P > 0.15$), 0.11 (n=46, P=0.075), 0.055(n=46, $P > 0.15$), 0.53 (n=46, $P < 0.0001$) and 0.59 (n=46, $P < 0.0001$).The low R^2 values between INSL3 and testosterone levels were possibly due to different patterns of release of two hormones during pre-pubertal to pubertal and middle to advanced ages. The INSL3 levels and age showed a nearly significant relationship with a low R^2 value whereas testosterone levels had high R^2 values with both age and body weight. INSL3 secretion may depend on testicular maturity and

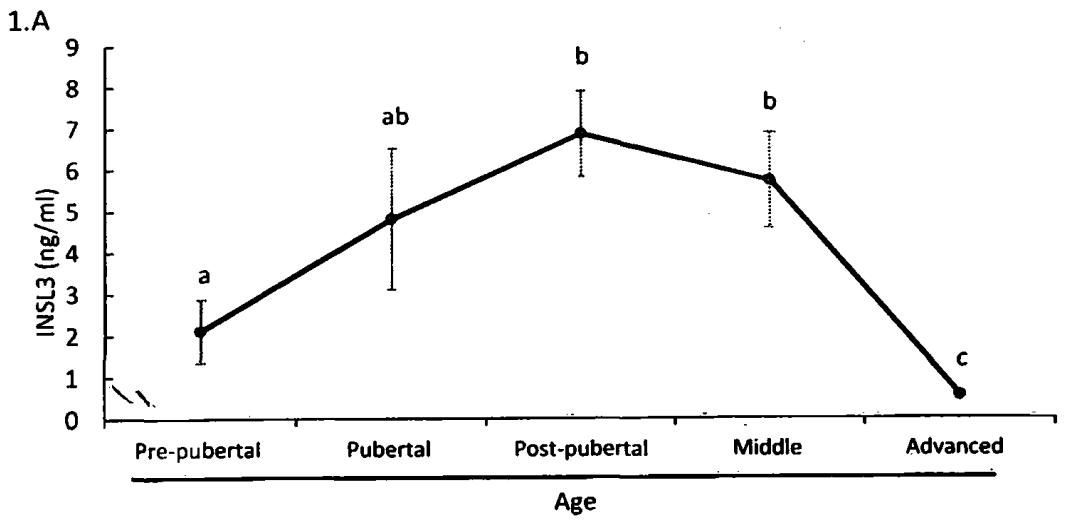

Figure 1: Mean ± SEM serum concentrations of INSL3 (A) and testosterone (B) in various age groups of normal dogs

Results are shown for pre-pubertal age (1-6 months, n = 5), pubertal age (6–12 months, n = 5), post-pubertal age (1–5 years, n = 15), middle age (5–9 years, n = 17) and advanced age (>9 years, n = 4).$^{a-c}$ within a hormone, means without a common superscript differs ($P < 0.05$)

age rather than testosterone which tend to rely on the age and body weight.

In conclusion, INSL3 concentrations showed a clear decline with increasing old age of German shepherd dogs whereas testosterone did not. Different serum INSL3 and testosterone dynamics were found in relation with age and body weight.

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