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Analysis of reproductive hormones and morphometric attributes in Thamankaduwa White male cattle

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Abstract: Despite a few attempts in exploring genetic variability, management systems, and morphometric descriptions, Thamankaduwa White cattle in Sri Lanka have not been subjected to any evaluation of their endocrinological distinctiveness, especially related to reproduction. The main aims of the present study were to: (1) investigate the dynamics of circulating insulin-like peptide 3 (INSL3) and testosterone in Thamankaduwa bulls during development, and (2) assess the association among INSL3, testosterone and selected morphometric parameters, namely, body weight, height at withers, body length, chest girth of Thamankaduwa White cattle in Sri Lanka. The blood samples were collected from male animals (n = 41) under three age categories; 3-6 months (Group I; n = 12), 6-12 months (Group II; n = 14) and > 12 months (Group III; n = 15), along with their morphometric measurements. Serum INSL3 and extracted testosterone concentrations were measured by using a competitive ELISA. The detection ranges of INSL3 and testosterone assay were 0.078-80 ng/mL and 0.04-40 ng/mL, respectively. Intra- and inter-assay coefficient of variations of INSL3 and testosterone assays were 6.9% (n = 6) and 16.4% (n = 6), and 12.5% (n = 3) and 11.9% (n = 4), respectively. Serum INSL3 and testosterone concentrations ranged between 1.44 - 19.85 ng/mL, and 0.003 ng/mL - 2.81 ng/mL, respectively. The mean serum INSL3 concentrations did not differ between Gr. I and II ($p > 0.05$) but were significantly high ($p < 0.05$) in Gr. III. There was a significant association ($R^2 = 0.65$; $p < 0.05$) between serum INSL3 and testosterone concentrations in Thmankaduwa White males. No strong associations were observed among hormones and the morphometric parameters tested. In conclusion, the dynamics of INSL3 and testosterone concentrations were compatible and correlated with each other in Thamankaduwa White male cattle.

Keywords: ELISA, INSL3, morphometric attributes, native white cattle, testosterone

1. Introduction

Sri Lankan native white cattle is mostly described as one of the local zebu cattle types which originated from a cross between local Sri Lankan cattle with imported Indian cattle [1]. This is also known as the Thamankaduwa White cattle which is specifically characterized by its white coat, black color tail switch, and hooves. This cattle type is predominately available in the Eastern, South Eastern, and North Central regions of Sri Lanka [2,3]. Even though the genetic resources, management practices, morphometric analysis and milk attributes of Thamankaduwa White were little addressed [3- 7], the reproduction physiology of these animals has not been investigated to sufficient depth yet.

Insulin-like peptide 3 (INSL3), along with testosterone, is a predominate secretory product of Leydig cells of mature testes as well as in fetuses of all mammals [8]. INSL3 concentrations have been discovered in many mammals including humans [9-12], beef cattle [13-15], Norwegian Red bulls [16], dogs [17], goats [18-21], and sheep [22]. Furthermore, [23] reported that the set of body and reproductive tract morphometry was beneficial in assessing the growth of young bulls.

Lack of investigations on endocrinological changes in Thamankaduwa White cattle left with no comparison of the reproductive efficiency of this native cattle type with other cattle breeds. Thus, the existing background creates a research gap in identification of

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endocrine changes and assess the associated morphometric changes to support the breeding programs involved in this important native cattle type in Sri Lanka. The present study aimed to: (1) To measure the circulating INSL3 and testosterone concentrations during development in Thamankaduwa White male cattle (2) To examine the relationship between each hormone concentration and morphometric parameters in Thamankaduwa White male cattle in Sri Lanka.

2. Materials and Methods

2.1. Animals, blood sampling and body measurements

Thamankaduwa White male animals (n = 41) raised in six smallholder semi-intensively managed farms in Chenkalady veterinary range (Latitude: 7° 46' 59.99" N and Longitude: 81° 35' 59.99" E), Batticaloa District, Eastern Province, Sri Lanka were used to this study. Blood samples were drawn from healthy animals of three age groups (Group I: 3–6 months, n = 12; Group II: 6-12 months, n = 14; Group III: more than 12 months, n = 15), and all were apparently normal. Samples were collected from jugular vein puncture in to plane vacutainer tubes and were centrifuged at 2000 × g for 20 minutes just after brought them into the laboratory. The Serum was separated and stored in microcentrifuge tubes at -20 °C until the hormone assays. Morphometric parameters were simultaneously measured during the collection of blood samples. The chest girth (CG), height at withers (HW), and body length (BL) were measured by using a flexible measuring tape, while the body weight (BW) was calculated using the weigh band (Dalton Supplies Ltd.) according to the obtained chest girth. The Ethical clearance for the study was obtained from the Research Ethics Committee, Faculty of Agriculture, University of Peradeniya, Sri Lanka (ECC/2023/R/062).

2.2. Hormone assays

2.2.1. INSL3 assay

Serum INSL3 was measured using a homologous bovine competitive enzyme immunoassay (EIA) as described by [13] for cattle and [21] for goats, with modifications. Eight-well strips (SPL Life Sciences, South Korea) were coated with 100 µL/well by using anti-mouse IgG (5 µg/mL in 0.05 M sodium bicarbonate; pH 9.7; KPL Lab Inc.) and kept for 2 hours at room temperature. The wells were then drained and washed three times by using 200 µL/well of washing saline (Sodium Chloride). Non-specific binding sites were blocked by using assay buffer 200 µL/well (AB I; 0.01 M Phosphate buffer which contained 0.15 M sodium chloride, 0.25 % skim milk and ProClin 950 (Sigma-Aldrich); pH 7.4) and kept overnight at 4 °C for blocking the wells which were drained immediately before the assay. Then, 50 µL of each standard or serum sample and 50 µL of anti-bovine INSL3 (1: 4000; a generous gift from Prof. E. E. Bullesbach, Medical University of South Carolina, USA) were dispensed into each well and kept two hours for incubation at room temperature followed by 50 µL of biotinylated human INSL3 peptide (1 ng/mL in AB I, 1: 5000; a generous gift from Prof. N. Kawate, Osaka Metropolitan University, Japan) dispensing and allowing again one hour for incubation. Subsequently, the wells were drained and washed 3 times with 200 µL/well of saline (0.15 M sodium chloride containing 0.05 % Tween 20). Then, 100 µL of HRP-labeled streptavidin (100 ng/mL in AB I, 1: 5000; KPL Lab Inc.) was added to each well and kept for 30 min at room temperature for incubation. After 30 min the wells were drained and washed three times using saline and kept another 30 min with 100 µL of substrate solution containing the 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma-Aldrich). Finally, the reaction was stopped by adding 50 µL of 2 M sulfuric acid, and the optical density (OD) was measured at 450 nm with a 630 nm reference using a microplate reader (UT-2100C, MRC, Israel).

The minimum detection limit of the assay was 0.078 ng/mL and the sensitivity range was 0.078 - 80 ng/mL. Intra and Inter-assay coefficient of variations were 6.9 % (n = 6), and 16.4 % (n = 6), respectively.

2.2.2. Testosterone extraction

Testosterone extraction prior to the testosterone assay was performed according to the previously described protocol by [17], with modifications. Briefly, various concentrations of testosterone standards (0.01-

40 ng/mL) were diluted with the assay buffer (AB II; 0.01 M Phosphate buffer containing 0.15 M sodium chloride, 0.1 % BSA and 0.02 % Proclin 950; pH 7.4). Native white cattle serum samples (250 µL/ sample) were dispensed into glass tubes and mixed with 2.5 mL of diethyl ether by vortexing for 5 min followed by centrifugation at 3500 rpm for 5 min to separate the upper ether phase from the lower water phase and kept at -18 °C allowing the lower water phase to freeze. Then, the upper phase was separated into another glass tube, and allowed to evaporate using a heat block at 40 °C. After that, the dried extracts were dissolved in 250 µL of AB II by vigorous vortexing. Simultaneously, the standards were also extracted.

2.2.3. Testosterone assay

Testosterone concentrations were measured in the same set of samples using the method described by [13, 17] previously. In brief, previously coated wells with 100 µL/well of anti-rabbit IgG polyclonal antibody (2µg/mL in 0.05 M Sodium bicarbonate; pH 9.7; KPL Lab Inc.) and blocked by AB II, were drained just before the assay, and 50 µL of extracted testosterone standards or extracted samples, 50 µL of HRP-labeled testosterone (1: 1600; Cosmo Bio Co., Ltd., Japan) and 50 µL of anti-testosterone rabbit polyclonal antibody (1: 1500; Cosmo Bio Co., Ltd., Japan) were added and kept two hours for incubation. Then, the wells were drained and washed three times by using washing saline 200 µL/well following the step of adding 100 µL/well substrate containing TMB and kept for another 30 min. The reaction was stopped by adding 50 µL of 2 M sulfuric per each well and the optical density was measured as similarly mentioned in the INSL3 assay.

The minimum detection of the assay was 0.04 ng/mL and the detection was reliable between 0.04 - 40 ng/mL. Intra and Inter-assay coefficient of variation and percentage recovery were 12.5 % (n = 3), 11.9 % (n = 4), and 91.8 % (n = 2), respectively.

2.3. Statistical Analysis

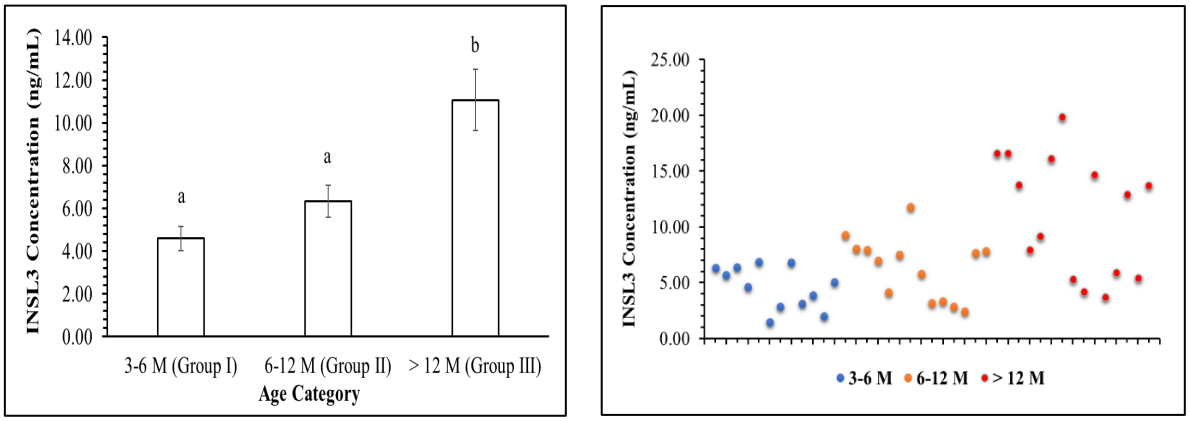
The changes in individual and mean INSL3 and testosterone hormone concentrations with age were assessed. Differences in mean INSL3 and testosterone concentrations among age groups (age in months) were compared using pairwise comparisons of the generalized linear models (GZLM; SPSS version 25.0, IBM Corporation, Somers, NY, USA) procedure by the least significant difference (LSD) post hoc test. Best regression curves among hormone concentrations (INSL3 and testosterone), and morphometric parameters were estimated by using the curve estimation procedure (SPSS version 25.0, IBM Corporation, Somers, NY, USA).

3. Results

3.1. Serum concentrations of INSL3 and testosterone in Thamankaduwa White male cattle

The values obtained for intra- and inter-assay CV and percentage recovery were within the acceptable range for both EIAs used during the present study. INSL3 standards had uniform inhibition at the same concentrations in the sensitivity range and the B/B0 values for serially diluted samples were parallel to the standards [13].

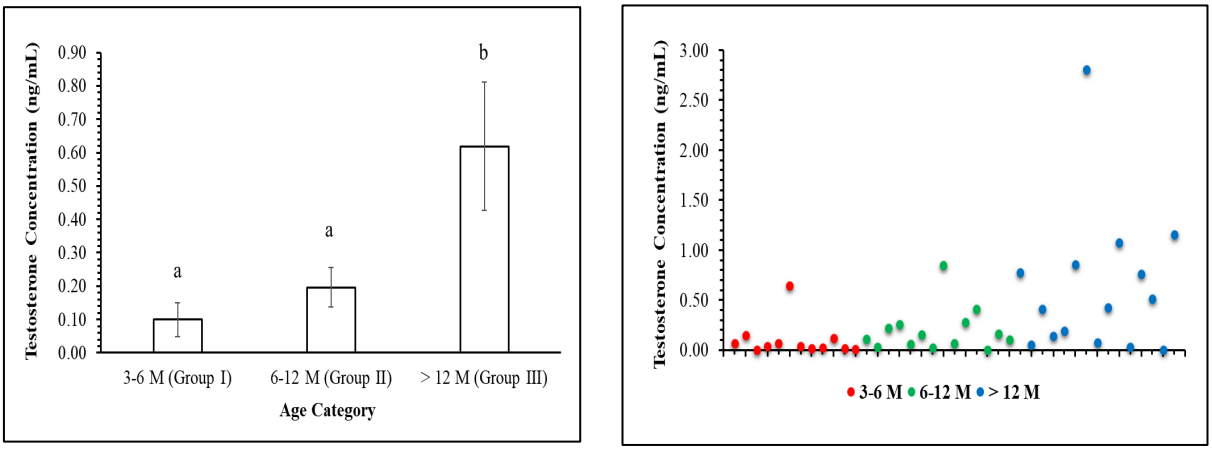
The serum INSL3 concentrations during the development of Thamankaduwa White cattle ranged between 1.44 to 19.85 ng/mL. The mean INSL3 concentration increased from the age group I (4.58 ± 0.55 ng/mL; n = 12) to age group II (6.34 ± 0.75 ng/mL; n = 14) ($p > 0.05$) with an increment factor of 1.4, and from age group II to age group III (11.06 ± 1.43 ng/mL; n = 15) ($p < 0.05$) with an increment factor of 1.7 (Figure 1a). The highest individual concentration of INSL3 (19.85 ng/mL) was observed in group III whereas the lowest (1.44 ng/mL) was observed in group I (Figure 1b).



(**a**) (**b**)

Figure 1. (a) Mean serum INSL3 concentration (mean ± SEM); (b) Individual INSL3 dynamics among age group I (3-6 M; n = 12), II (6-12 M; n = 14) and III (> 12M; n = 15) of native white cattle. [a-b] Mean with different superscripts significant at $p < 0.05$

Serum testosterone concentrations also followed the same pattern of dynamics of INSL3 concentrations (Figure 2A). The mean testosterone concentration was increased from age group I (0.10 ± 0.05 ng/mL) to age group II (0.20 ± 0.06 ng/mL) but it was not statistically significant. However, it was increased ($p < 0.05$) from age group II to III (0.62 ± 0.19 ng/mL). The highest individual concentration of testosterone (2.81 ng/mL) was observed in group III whereas the lowest (0.04 ng/mL) was observed in group I (Figure 2B).



(**a**) (**b**)

Figure 2. (a) Mean serum testosterone concentration (mean ± SEM); (b) Individual testosterone dynamics among age group I (3-6 M; n = 12), II (6-12 M; n = 14) and III (> 12M; n = 15) of native white cattle. [a-b] Mean with different superscripts significant at $p < 0.05$

3.2. Regression analyses among INSL3, testosterone and morphometric measurements

In the Thanakaduwa White male animals, the R^2 value of the of the best regression curve was 0.65 (n = 41, $p < 0.05$; Figure 3).

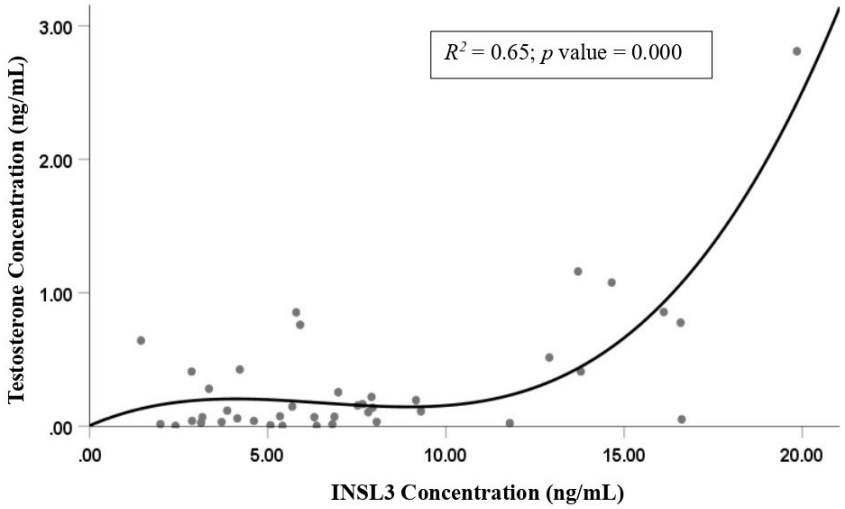


Figure 3. Best regression curves between serum concentrations of insulin-like peptide 3 (INSL3) and testosterone in native white cattle bulls during development (0 to > 12 months of age; n = 41)

Despite the weak associations, significant relationships were observed between the serum INSL3 concentration and BW, CG, and BL ($p < 0.05$) except with HW ($p > 0.05$) (Table 1). Similarly, there were weak associations noticed between the serum testosterone level and BW, CG, HW, and BL ($p < 0.05$; Table 2). However, the body length exhibited the highest significant association with INSL3 among all the tested characteristics, while the chest girth showed the highest significant association with testosterone.

Table 1. Estimated R^2 values and p values of best regression curves between serum INSL3 concentrations and morphometric characteristics of native white cattle bulls in Sri Lanka (*$p < 0.05$; n = 41)

	Body weight	Height at withers	Chest girth	Body length
R^2 value	0.193*	0.183	0.213*	0.321*
p value	0.045	0.055	0.030	0.002

Table 2. Estimated R^2 values and p values of best regression curves between serum testosterone concentrations and morphometric characteristics of native white cattle bulls in Sri Lanka (*$p < 0.05$; n = 41)

	Body weight	Height at withers	Chest girth	Body length
R^2 value	0.250*	0.245*	0.259*	0.236*
p value	0.013	0.015	0.011	0.018

4. Discussion

In the present study, we report to the best of our knowledge, the first measurement of circulating INSL3 and testosterone in Thamankaduwa White cattle and the association between those hormones and morphometric measurements. [21] suggested that the measurement of both INSL3 and testosterone in the same animal may provide an added benefit in assessing Leydig cell function due to its differential patterns of regulations. [13] found that there was an effect of age on INSL3 concentrations in prepubertal Japanese black beef bulls. Those findings further revealed that the plasma INSL3 concentration did not differ significantly from prepubertal (3 to 6 months) to early pubertal age (6 to 12 months) followed by a significant elevation from early to late (12 to 18 months) and late to post-pubertal age (18 to 22 months). Additionally, [16] reported that, even though the mean serum INSL3 concentrations didn't significantly differ over age

categories 2-3, 4-5, 6-7, 8-10, and 11-13 months, respectively, there was an increasing trend over time where the highest concentration was observed at 8-10 months age category in Norwegian Red bulls. Hence, the present findings of INSL3 concentrations of Thamankaduwa White cattle type in Sri Lanka were closely aligned with the previous findings of Japanese black beef bulls whereas it was more or less similar to the findings of Norwegian Red bulls. Furthermore, the INSL3 dynamics followed similar trends as found in the present study in male sheep [22], male Saanen goats [24], Jamnapari X Local crossbred goats [21], and Kottukachchiya crossbred goats [25] during development. However, the serum INSL3 concentrations were very high just after the birth of humans and sharply declined within a few months thereafter and one year of age [26], and continued until 10 years followed by a sharp increment during puberty [9,12]. Even in rats, the serum INSL3 concentrations were in minor quantities during 2 days before and after birth which continued until 10 days after birth followed an increment until puberty [27,28]. However, the decrement trends of serum INSL3 concentrations between infancy to puberty seemed to be absent in Thamankaduwa White cattle males. This is in strong agreement with the previous findings of Japanese black beef bulls as well [13].

Serum testosterone concentrations also followed the same pattern of dynamics as INSL3 concentrations during the present study. There was a marked increment of testosterone concentration of Japanese black beef bulls from prepubertal (3-6 months) to early pubertal age (6-12 months) though it wasn't observed significant increment from early to late phase (12-18 months) [13]. Even though there was no evidence on the age at puberty of Thamankaduwa White bulls, [29,30] reported that the average age at puberty of Bos indicus beef bulls ranged from 16-18 months. In Brahman bulls, the serum testosterone level increment was observed between 12-14 months [23]. The increment of serum concentrations of both INSL3 and testosterone during puberty was suggested due to the HPG axis triggering during this particular age in mammals [8,12]. Hence, the present marked increment of INSL3 and testosterone concentrations from age group II to III in Thamankaduwa White cattle could be suggested due to puberty during this age. Furthermore, the testosterone of Saanen bucks did not differ in all those three age groups as considered in the present study, nevertheless, a significant increment was observed in Kottukachchiya crossbred bucks from below 06 months age category to 6-12 months age category [24,25]. [21] observed a significant drop in serum testosterone concentration on the 23rd week after birth and a three-fold increment on the 28th week after the birth of Jamnapari X Local crossbred goats. There was no difference from the 3-6 months phase to the 6-12 months phase. However, a remarkable elevation from the 6-12 months phase to the 12-24 months phase in male sheep [22]. Thus, the present findings for serum testosterone dynamics in Thamankaduwa White cattle in Sri Lanka were not in agreement with those findings recorded for Japanese black beef bulls and more or less similar to the findings of Saanen and Kottukachchiya crossbred buck. Nevertheless, the findings were comparable with those observed in male sheep during development. However, the minor concentrations of testosterone detected, compared to the serum INSL3 concentrations in Thamankaduwa White cattle were comparable to previous studies reported for the livestock species during development [18,22,24,25], except Japanese black beef bulls [13].

The R^2 value of the association between INSL3 and testosterone hormone concentrations in Thamankaduwa White cattle bulls in the present study was higher ($p < 0.05$) than that of the Japanese black beef during development. [13] concluded that the high R^2 value inferred a similarity in releasing patterns of those two hormones both of which are produced by Leydig cells. It further observed a higher R^2 value for the association between these two hormones during birth to 3 months of age than that of the period around pubertal age (3 to 22 months).

The morphometric measurements of Withers height and chest girth of Thamankaduwa White cattle during the present study were comparable with the previous findings [3,31]. All the farms visited to collect the samples are being managed under a semi-intensive system. The cattle are allowed to graze in nearby lands during the morning as a herd just after the milking and confined to a paddock during the night time. Since the morphometric measurements were taken without proper restraining facilities at the field conditions, the weak associations between the tested reproductive hormones and body measurements could be attributed to the precision of the measurement taken. Therefore, the weigh band was used to take the body weight in all groups even though it could be effectively used for mature animals. However, [32] found that the prediction of live weight using the heart girth measurements is acceptable from the prepubertal to postpubertal ages of cattle. [23], reported that there were positive correlations between testosterone level and girth ($r = 0.38$; $P < 0.01$), body weight ($r = 0.38$; $P < 0.01$), right testicle ($r = 0.23$; $P < 0.05$), left testicle ($r = 0.21$; $P < 0.01$) and testicular volume ($r = 0.22$; $P < 0.008$) in Brahman male cattle.

Owing to the unexpected practical difficulties, the association between scrotal circumference (SC) with serum INSL3 and testosterone levels in Thamankaduwa White cattle males could not be assessed in the present study. Therefore, further studies are recommended to assess the association among INSL3, testosterone and SC, because it has been already proven that there is a significant association between INSL3 and SC in several mammals including humans, Norwegian Red bulls, and several breeds of goats [12,16,21, 24,25].

5. Conclusions

Serum INSL3 and testosterone concentration dynamics were highly compatible and strongly correlated with each other in Thamankaduwa White male cattle. Future studies could aim at comparing reproductive hormones and reproduction-related attributes such as scrotal circumference and sperm quality parameters which would be beneficial to understand the Leydig cell functionality during the sexual development of Thamankaduwa White male cattle.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of the Faculty of Agriculture, University of Peradeniya, Sri Lanka and approved by the Ethics Committee of the Faculty of Agriculture, University of Peradeniya, Sri Lanka (ECC/2023/R/062).

Data Availability Statement: All the relevant data is available in the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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