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Original article

Pre-analytical factors affect the accurate measurement of testosterone concentrations in plasma and serum of goats

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Abstract

Several pre-analytical factors may influence the accurate measurements of testosterone (T) and therefore, these factors must be a significant concern. This study aimed to examine the effects of 1) time of sample collection, 2) delay to centrifugation, 3) sample matrix types, and 4) device and duration of sample storage on the T concentrations. Blood samples were collected from 34 bucks of Kacang goats. For testing the effect of collection time, 12 pairs of morning and afternoon samples were collected. For testing the effect of delayed centrifugation, 24 samples were subjected to treatments: (i) centrifuged < 1 hour after collection (control group), (ii) centrifuged 6, 12, and 24 hours after collection (test groups). For testing the different sample matrix types, 10 samples were processed as serum and plasma. For testing the effect of sample storage device and duration, 60 samples were subjected to treatments: i) frozen at -20^{O}C (control group), ii) stored in a cooler box, a styrofoam box, and a thermos-flask for two, four, and six days (test groups). T concentrations were measured using a validated testosterone ELISA kit. Concentrations of plasma testosterone (pT) from morning samples were significantly higher compared to afternoon samples ($p<0.05$). Delayed centrifugation for up to 24 h decreased significantly on pT concentrations ($p<0.05$). The concentrations of T from serum and plasma did not differ and showed a strong correlation ($r=0.981$). Storage device and duration affected the T concentrations compared to frozen samples ($p<0.05$) which T concentrations were stable for up to 4 days in a styrofoam box and a thermos-flask and up to 6 days in a cooler box. In conclusion, the measurement accuracy and stability of T concentrations in goats are affected by collection time, delay to centrifugation, and device and duration of storage.

Key words: centrifugation time, collection time, ELISA, goats, sample storage

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Introduction

The enzyme-linked immunosorbent assay (ELISA) technique is now widely used for measuring hormone concentrations. This technique is advantageous, because it is simple, rapid, convenient, and particularly free of radioisotope waste (Sakamoto et al. 2017). Several sample types can be used such as blood (Gholib et al. 2020a), urine (Du et al. 2017), feces (Gholib et al. 2020b), saliva (Bloomer 2015), and hair (Stradaioli et al. 2017). Using blood samples to measure hormone concentrations allows being fully clothed without adding anticoagulant to produce a serum or added anticoagulant and centrifuged as soon as possible to get a plasma (Evans et al. 2001, Hornakova et al. 2017). Both of the samples must be prepared and stored at low temperatures immediately before assaying them (Bielohuby et al. 2012). However, immediate blood preparation and storage are not always feasible in fieldwork. Moreover, samples are often transported from the field to the laboratory or from a laboratory to another laboratory for analysis. Hence, questions emerge about the suitability of sample preparation or preservation under these less ideal conditions.

Previous studies have shown that several pre-analytical factors related to biological factors (e.g., diurnal rhythm, age, sex, seasonal variation, etc.) and technical factors (e.g., handling of samples, different blood matrices, the stability of storage samples, etc.) could affect blood hormone measurements (Hegstad-Davies 2006, Raff and Sluss 2008, Ceglarek et al. 2010). Some hormone concentrations vary with day time in humans and animals such as testosterone (T) (Fukuda 1990, Sekoni et al. 1990, Raff and Sluss 2008, Brambilla et al. 2009, Urbanski 2011), cortisol (Urbanski 2011, Pawluski et al. 2017, Gholib et al. 2020b), and progesterone (Steinetz et al. 1990, Thuroczy et al. 2003). Accordingly, the time of sample collection was reported to be crucial. Moreover, during plasma or serum preparation, delayed time of centrifugation of blood samples leads to variation in the concentrations of progesterone (De Castro et al. 2004), adrenocorticotrophic hormone (Preissner et al. 2004, Christensen et al. 2016), and parathyroid hormones (Omar et al. 2001). Moreover, storage conditions can also affect the stability of hormone concentrations (Lewis et al. 2013, Prutton et al. 2015). Having seen the evidence above, it is thus important to evaluate if these pre-analytical factors introduce systematic bias on measuring T concentrations in goats.

To our knowledge, a standardized protocol of sample handling for hormone analysis in goats has not been published. Therefore, in an arrangement to monitor the reproduction of goats, we conducted a study to test the stability of T concentrations in response to several pre-analytical factors. First, we examined the influence of the daytime of sample collection (morning versus afternoon) on T concentrations. Second, we performed an experiment to test the effect of delayed centrifugation to simulate the condition when immediate sample preparation is not possible. Third, we compared T concentrations derived from different sample matrices (serum versus plasma). Finally, we compared different storage devices (e.g., freezer at -20°C, cooler box, styrofoam box, and thermos-flask) and duration (2, 4, and 6 days) to simulate sample transport, e.g., from field to laboratory or from a laboratory to another laboratory for hormone measurements.

Materials and Methods

Ethical approval

The use of all experimental animals in the present study was approved by the Institutional Committee of Animal Ethics of the Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh, Indonesia (Ref: 80/KEPH/XII/2020).

Study animals

Plasma and serum were collected from 34 adult males of Kacang goat (buck), aged two to three years, weighing between 15 and 20 kg. Bucks were collected from a smallholder farmer at Aceh Besar District, Aceh Province, Indonesia. They were reared using a semi-intensive feeding system. In the morning until afternoon (08:00 to 17.00), bucks were placed in the pasture for daily grazing. After that, bucks were placed in stables which were equipped with feeding and water trough.

Collection and processing of blood samples

Blood samples were collected by a veterinarian from the jugular vein. After that, the blood was processed as plasma and/or serum depending on the experimental procedure (see below). For plasma collection, an anticoagulant agent, *ethylenediaminetetraacetic acid* (EDTA) was added to the blood samples (1 mg EDTA per 1 ml blood). Afterward, blood was centrifuged at 1200 x g for 10 min. at 4°C. Plasma was then separated from red blood cells by pipetting, transferred to polypropylene tubes (Eppendorf Safe-Lock tubes), and stored at -20°C for hormone analysis. For serum collection, the blood was collected without adding an anticoagulant agent. After collection, the blood was then allowed to clot at room temperature at 25°C for 30 minutes until two hours. The serum was then separated from the red blood cells by centrifugation

Table 1. Design of the different experiments conducted in this study and the number of the sample used for each.

Experiments	N
Experiment 1. Effect of sample collection times	
Morning	12
Afternoon	12
Experiment 2. Effect of delay to centrifugation	
Control (0 hours, immediately centrifuged after sample collection)	6
6 hours	6
12 hours	6
24 hours	6
Experiment 3. Effect of the different type of sample matrix, serum versus plasma	
Serum	10
Plasma	10
Experiment 4. Effect of different storage devices and duration	
Control (stored in a freezer at -20^{O}C, K0)	6
Cooler box for 2 days (C2)	6
Cooler box for 4 days (C4)	6
Cooler box for 6 days (C6)	6
Styrofoam box for 2 days (S2)	6
Styrofoam box for 4 days (S4)	6
Styrofoam box for 6 days (S6)	6
Thermos flask for 2 days (T2)	6
Thermos flask for 4 days (T4)	6
Thermos flask for 6 days (T6)	6

at 1200xg for 10 min. at 4°C. After that, the serum was immediately transferred to polypropylene tubes (Eppendorf Safe-Lock tubes) and stored at -20°C for hormone measurements. The number of samples used for each experiment is presented in Table 1.

Experiment 1 testing the effect of collection time of blood samples on testosterone concentrations

To test whether T concentrations vary with daytime, 12 paired of the morning (06:00 to 08:00) and afternoon (12:00 to14:00) blood samples were collected from 12 goats (Table 1). In total 2 ml blood samples were collected with anticoagulant, EDTA, and filled into 2 ml micro-tubes (Eppendorf Safe-Lock tubes). Blood samples were then prepared as plasma using the method described above and stored at -20^{O}C until hormone analysis.

Experiment 2 testing the effect of delayed centrifugation of blood samples on the stability of testosterone concentrations

To test whether the delay to centrifugation from the time of collection affects the stability of T concentrations, six blood samples were collected from six different goats (2 ml per individual, collected with EDTA). Upon collection, each blood sample was divided into 4 aliquots and filled into 1.5 ml micro-tubes (Eppendorf Safe-Lock tubes; total 24 aliquots; 0.5 ml blood per tube). For the control group, aliquots were centrifuged less than an hour following collection. Aliquots in the test group were stored at room temperature before centrifugation. Each test group was subsequently centrifuged 6, 12, and 24 hours after blood collection (Table 1). After aliquots were centrifuged, plasma was stored at -20°C and then assayed at the same time for measuring T concentrations.

Experiment 3 testing the effect of different types of sample matrix (serum versus plasma) on testosterone concentrations

To test whether the type of sample matrix (serum versus plasma) influences measured T concentrations, 10 blood samples were collected from 10 goats (2 ml per individual). Each blood sample was divided into two aliquots (1 ml per aliquot, Table 1). To one aliquot, 1 mg EDTA was added and then centrifuged immediately after collection to get a plasma. The other aliquot was allowed to clot at ambient temperature and then centrifuged to get the serum. Plasma and serum were then stored at -20°C and then assayed at the same time for measuring T concentrations.

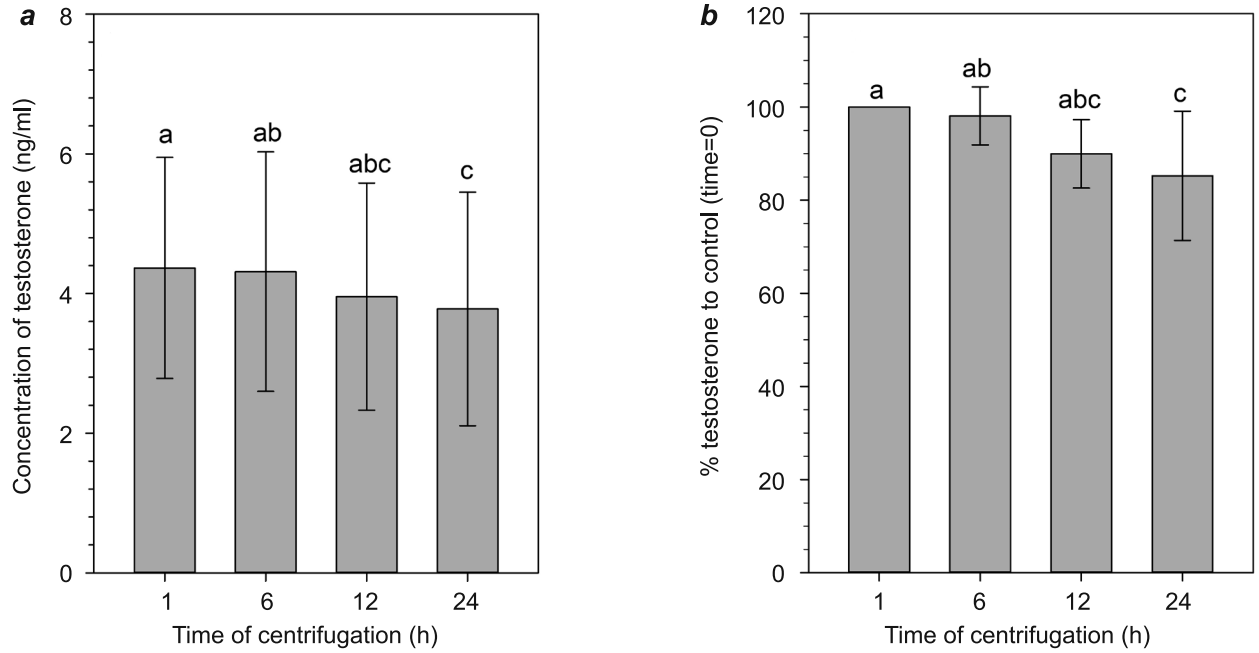

Fig 1. pT concentrations of blood samples centrifuged at different times. Values represent absolute (mean±SD) of pT concentrations (a), and percentages (mean±SD) of pT relative to control 100% (b). Different superscripts above the histogram indicate a significant difference between groups ($p<0.05$).

Experiment 4 testing the effect of different storage devices and duration of plasma on testosterone concentrations

To test whether different devices and duration of plasma storage affect the measured T concentrations, six blood samples were collected from six goats (3 ml/individuals). Blood samples were then prepared as plasma. Each plasma sample was divided into 10 aliquots and filled into 1.5 ml microtubes (Eppendorf Safe-Lock tubes; total 60 aliquots; 0.1 ml per tube; Table 1), closed tightly and sealed with parafilm. These aliquots were used for the following treatments: (i) aliquots stored in a freezer at -20°C up to the time of hormone measurements (as control group /K0), (ii) aliquots stored in a cooler box (Marina Cooler 6s, Lion Star) for two, four, and six days (as C2, C4, C6), (iii) aliquots stored in a styrofoam box for two, four, and six days (as S2, S4, S6), and (iv) aliquots stored in thermos-flask (food container, Houssina) for two, four, and six days (as T2, T4, T6). To each cooler box, styrofoam box, and thermos-flask were added with the ice gel packs. The temperatures in the test group on the second, fourth, and sixth day of storage were 8.2°C, 15.6°C, and 21.7°C, in the cooler box; 12.1°C, 22.4°C, and 28.5°C, in styrofoam box; and 7.8°C, 17.4°C, and 26.9°C, in a thermos-flask, respectively. After the different treatments, all aliquots were assayed at the same time for measuring T concentrations.

Measurement of testosterone concentrations

Testosterone concentration was measured using a commercial testosterone ELISA kit produced by DRG Instruments GmbH, Germany (EIA-1559). This assay has been validated previously for Kacang goats (Gholib et al. 2016), and other animals (Akmal et al. 2019, Gholib et al. 2020a). The assay uses a mouse monoclonal anti-testosterone antibody and horseradish peroxidase (HRP) as an enzyme conjugate. Testosterone measurements were performed in accordance with the manufacturer's instructions and as described by Gholib et al. (2016).

The coefficients of variation (CVs) of intra-assay of high and low-value QC were 6.51% and 5.63%, respectively (n=10). The coefficients of variations (CVs) of inter-assay of high and low-value QC were 9.32% and 8.32%, respectively (n= 4).

Data analysis

Before performed the data analysis, data distribution was tested for normality using the Shapiro-Wilk test. Because data from experiments 1 to 4 did not significantly deviate from a normal distribution ($p>0.05$), parametric tests were used to analyze them. For experiment 1, a paired-samples t-test was conducted to compare T concentrations obtained from morning versus afternoon samples. For experiment 2, the relative change in T concentrations relative to time 0 h (control) of centrifugation was calculated as $(a_n\text{-}x_n / x_n)*100$,

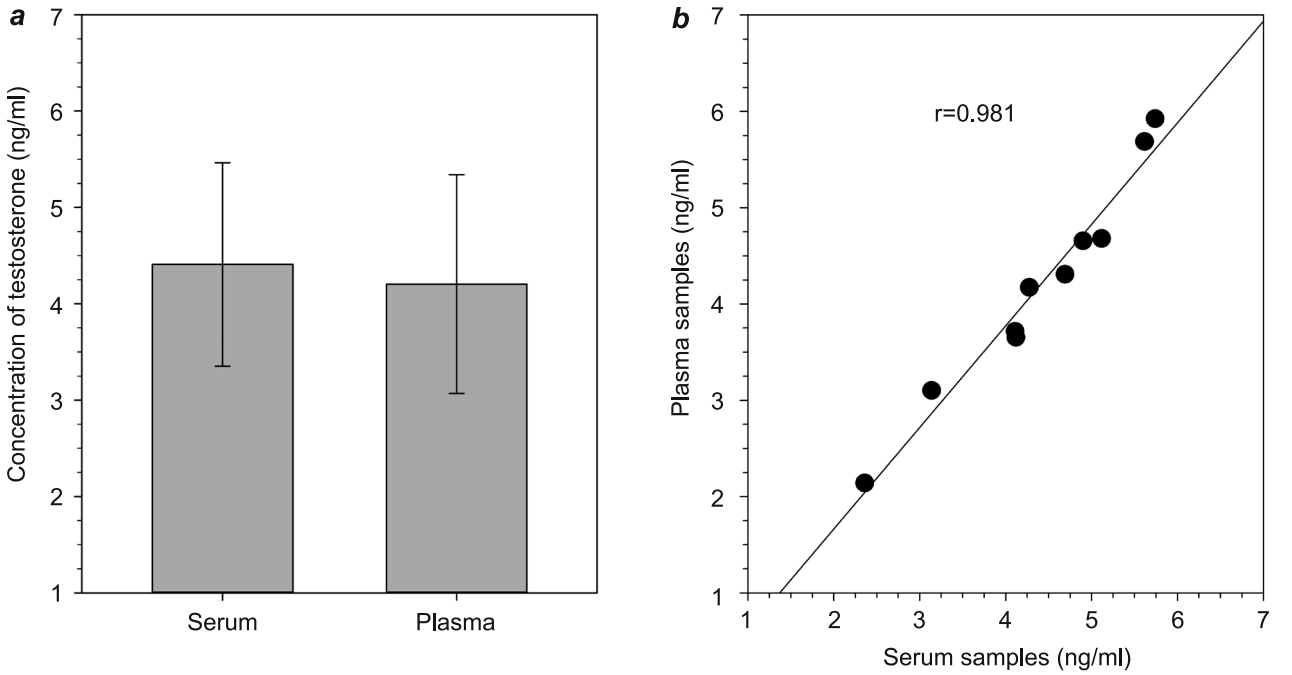

Fig 2. a) Concentrations of testosterone (mean±SD) in serum and plasma of Kacang goats, b) correlation of testosterone concentrations between serum and plasma samples.

where a_n is the nth sample value in each duration (6, 12, and 24 h) and x_n is the value at time 0 (control) of the nth sample. A one-way repeated-measures ANOVA was conducted with Bonferroni as a post-hoc test to determine if T concentration changes of test groups were different from the control group. For experiment 3, T concentrations in serum and plasma were compared in an independent-samples t-test. In addition, a Pearson correlation test was performed to examine if T concentrations in serum and plasma correlated. For experiment 4, the relative change in T concentrations relative to control (always frozen at -20°C) was calculated as $(a_n\text{-}x_n / x_n)*100$, where a_n is the n^{th} sample value in each storage place (cooler box, styrofoam box, thermos-flask) and x_n is the control value of the nth sample. To evaluate the percentual change in T concentration depending on storage device and duration, a one-way repeated-measures ANOVA was conducted following by a post-hoc analysis using the Bonferroni test. A significance level of all statistical tests was set to $\alpha = 0.05$. Results of statistical analyzes were presented as mean and standard deviation (SD). Data analyses were conducted using IBM SPSS 25. All graphs were created using Sigma Plot 12.5.

Results

Experiment 1 testing the effect of sample collection time on testosterone concentrations

The mean (±SD) of pT concentrations in the morning and afternoon was 5.08±1.33 ng/ml and 3.66±1.74 ng/ml, respectively. Concentrations of plasma testosterone (pT) from blood samples collected in the morning were significantly higher compared to the pT concentrations collected in the afternoon ($p<0.01$).

Experiment 2 testing the effect of delayed centrifugation of blood samples on the stability of T concentrations

Delayed centrifugation of blood samples after collection for up to 24h significantly affected pT concentrations ($p<0.05$, Fig. 1a). Concentrations of pT from blood samples centrifuged 6 h, 12 h, and 24 h after collection decreased gradually by 1.92%, 10.06%, and 14.77%, respectively compared to the control group (Fig. 1b). The Bonferroni test revealed that pT concentrations from blood samples centrifuged 24 h after collection were significantly lower compared to the control group ($p<0.05$). However, concentrations of pT from blood samples centrifuged at 6 h and 12 h after collection did not differ significantly from the control group ($p>0.05$).

Experiment 3 testing the effect of different sample matrices (serum versus plasma) on testosterone concentrations

The mean (± SD) of T concentrations in serum and plasma was 4.41 ± 1.06 ng/ml and 4.20 ± 1.14 ng/ml, respectively (Fig. 2a). Absolute T concentrations did not differ significantly between serum and plasma samples ($p>0.05$). Moreover, T concentrations measured

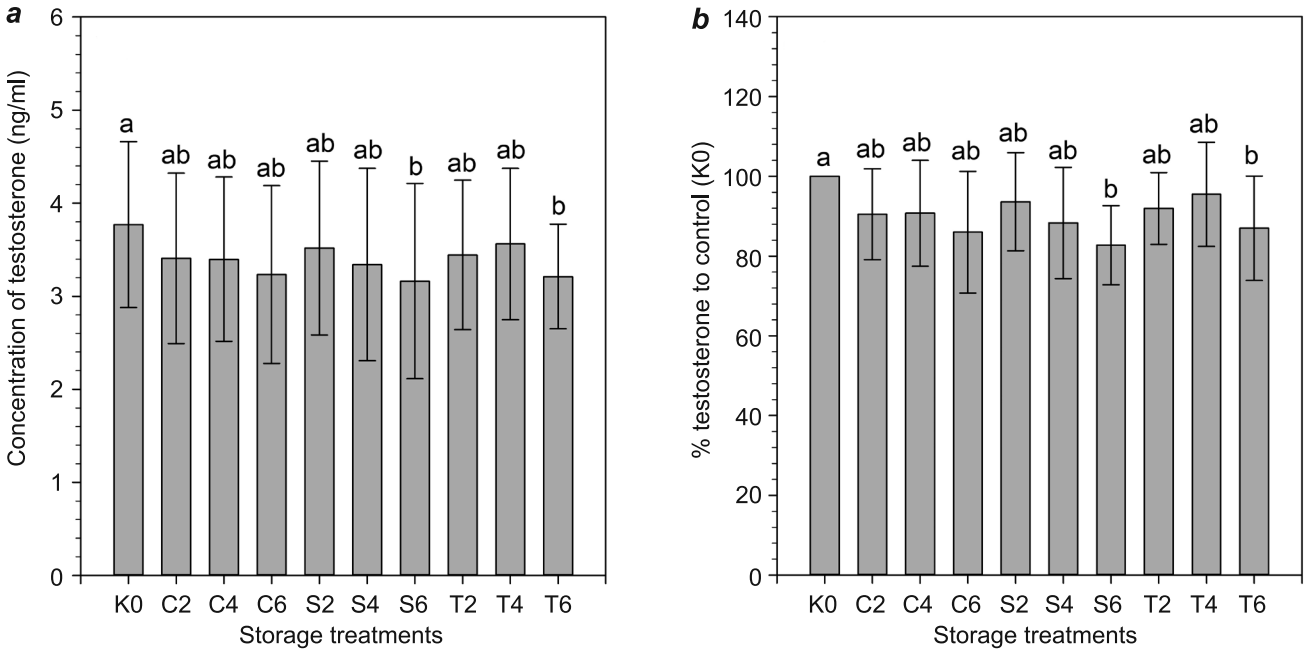

Fig 3. pT concentrations of plasma after short-term storage in various devices and different duration. Values represent absolute (mean±SD) of pT concentrations (a), and percentages (mean±SD) of pT relative to control 100% (b). K0 = control group, stored in a freezer at -20°C; C2,C4, and C6 = stored in a cooler box for 2,4, and 6 days; S2,S4, and S6 = stored in a styrofoam box for 2,4, and 6 days; T2,T4, and T6 = stored in a thermos flask for 2,4, and 6 days. Different superscripts above the histogram indicate a significant difference between groups ($p<0.05$).

from serum correlated strongly with T concentrations measured from plasma ($r=0.981$, Fig. 2b).

Experiment 4. testing the effect of different devices and duration for short-term storage of plasma on the stability of testosterone concentrations

Short-term storage of plasma in various devices and duration for up to six days showed a significant effect on pT concentrations compared to the control ($p<0.05$, Fig. 3a). The Bonferroni test indicated that pT concentrations from plasma stored in a styrofoam box and thermos-flask for six days (S6 and T6, respectively) were significantly lower compared to the control group/ K0 ($p<0.05$), but pT concentrations were not affected when stored for up to four days in either styrofoam box or thermos-flask ($p>0.05$). Interestingly, pT concentrations remained stable when stored in a cooler box for up to six days ($p>0.05$). On average, the percentual change of pT concentrations stored in a cooler box, styrofoam box, and thermos-flask was 9.5 to 14.0%, 6.4 to 17.3 %, and 8.1 to 13.0 %, respectively lower compared to the control groups (K0) after being stored for two, four, or six days (Fig. 3b).

Discussion

This is the first study to highlight the importance and directly evaluate the effects of pre-analytical factors prior to hormone measurements in serum and plasma of goats. Our results show that the time of blood collection affected the T concentrations with the morning samples having higher T concentrations than the afternoon samples. Moreover, delayed centrifugation of blood samples also influenced measured T concentrations, with T concentrations only being stable in blood samples up to 12 hours following collection. Our data also reveal that hormone analysis using plasma or serum generates similar absolute T concentrations. Finally, our study also indicates that T concentrations remained stable as long as samples were stored in a cooler box for up to 6 days and up to 4 days in a styrofoam box and a thermos flask.

With this study, we show that pT concentrations were 64.47% higher in morning samples (06:00 to 08:00) than in afternoon samples (12:00 to 14:00). The circadian activity of T secretions seems to be the reason for the observed pattern. Accordingly, our findings are in agreement with previous studies in humans (Raff and Sluss 2008, Brambilla et al. 2009) and animals (Fukuda 1990, Sekoni et al. 2008) where T concentrations were reported to be highest in the early morning and lowest in the afternoon and evening. Testosterone secretion displays a circadian rhythm with the peak concentrations of T occurring around the commencement of diurnal activity in the morning, and reduced T concentrations in the evening and overnight (Neumann et al. 2019). Testosterone is a gonadal

steroid stimulated by pituitary gonadotropins that are secreted in a pulsatile fashion under the influence of hypothalamic GnRH (Ouyang et al. 2018, Neumann et al. 2019). In males, T secretion appears to have a period of 2–3 h until it is decomposed in the blood after secretion (Heuett and Qian 2006). With the provided evidence for the circadian rhythm, future studies must standardize sampling collection time of day when developing reference ranges for T measurements in goats.

In addition to the daytime of sample collection, the delay to centrifugation of blood samples after collection must be of concern. Our study shows that T concentrations decrease with increasing delayed centrifugation after blood collection. Concentrations of T in the blood that was only centrifuged 24 hours after collection revealed was significantly reduced compared to the controls procedure (blood centrifuged less than one hour after collection). Interestingly, blood centrifuged at 6 and 12 hours after collection showed no difference in T concentration compared to the controls. This indicates that centrifugation should be performed as soon as possible after collection. However, if this is not possible, it is recommended to centrifuge the blood within 12 h of sample collection.

Our results are in line with several previous studies on steroid hormones such as testosterone (Fahmi et al. 1985, Khonmee et al. 2019), cortisol (Khonmee et al. 2019), and progesterone (Fahmi et al. 1985, Reimers et al. 1991), and also polypeptide tropic hormones such as ACTH (Prutton et al. 2015, Christensen et al. 2016). The expected factor that caused decreasing T concentrations is the degradation of hormone concentrations when stored in the cooler box before centrifugation. The intracellular components in the blood which has not been separated are very susceptible to degradation processes due to proteolytic enzyme activities (Bielohuby et al. 2012, Christensen et al. 2016). Therefore, it is important to perform blood centrifugation immediately after collection to obtain plasma and stored this plasma at a low temperature of -20°C or below.

In addition to hormone degradation, hemolysis may be an additional factor to cause a decrease in the T concentration as the result of delayed centrifugation. Our study provides evidence for hemolysis as the plasma obtained after delayed centrifugation is redder compared to plasma from blood that is centrifuged immediately after collection. Hemolysis can affect the results of blood chemistry measurements including hormones (Reimers et al. 1991, Moreno et al. 1998, Lippi et al. 2008, Yin et al. 2015). There are several possible mechanisms by which hemolysis affects hormone concentration. Wenk (1998) suggested that an unknown chemical interaction may have occurred between the red blood cell components and the test reactants and may consequently affect antibody-antigen binding. In addition, the release of hemoglobin, or other intracellular components, as a result of rupture of the erythrocyte membrane, can also cause degradation and a decrease in analyte concentration in the sample (Farrell and Carter 2016). Moreover, the presence of hemoglobin in the sample may interfere with the absorbance spectra of colorimetric assays (Reddy et al. 1997). Therefore, hemolysis should be avoided in hormone measurements.

Interestingly, absolute concentrations of T in plasma and serum did not significantly differ and correlated strongly in our study. These results indicated that the absolute concentration of testosterone in plasma and serum is similar. In addition, this strong correlation of T concentrations obtained from two types of samples indicates that it is possible in the future to compare values generated from two different samples matrix, plasma versus serum. Similar results were found in Asian elephants where absolute concentrations of steroid hormones (i.e., testosterone, progesterone, and cortisol) in plasma and serum were reported to be similar (Khonmee et al. 2019). Therefore, steroid hormones can be measured in both, plasma and serum samples.

The stability of pT concentrations is also affected by the storage devices and duration (Bielohuby et al. 2012). In the present study, short-term storage of plasma in various devices and duration led to different T concentration measurements. The pT concentrations were not significantly different between the control treatment and when plasma was placed in a cooler box for up to 6 days. In contrast, the pT concentration decreased gradually and was significantly lower after being stored for 6 days in a styrofoam box or thermos flask than the control treatment, but pT concentrations did not significantly differ from the control treatment after 4 days. These results indicate that storage and transport of plasma in a cooler box containing ice gel packs can maintain the stability of pT concentrations for up 6 days, whereas in a styrofoam box and thermos flask only for up to 4 days. Our results are similar to the reports by Evans et al. (2001) indicating that some hormones are stable for up to 5 days when stored at 4°C and 30°C. Decreasing testosterone concentrations gradually when stored at styrofoam box or thermos flask is expected because these could not maintain the temperature for up to 6 days, resulting in plasma degradation. Accordingly, the pT concentration was lower than the control (plasma stored in a freezer at -20°C). Degradation caused by blood enzymes is a factor for the instability of hormones (Lewis et al. 2013).

In sum, our study provides several recommenda-

tions regarding the standardization of pre-analytical factors prior to hormone measurement in goats. First, the time of sample collection must be standardized since T concentrations differ between morning and afternoon. Second, it is recommended to centrifuge blood samples immediately after collection or at least not later than 24 hours after collection. Third, either serum or plasma can be used for testosterone measurement depending on which sample matrix is easier to process in the field (e.g., availability of equipment). Finally, when samples have to be sent from the field to the laboratory or from a laboratory to another laboratory for hormone measurements, it is recommended to use a cooler box filled with ice gel packs and transport duration should not exceed 6 days.

Conclusion

Our results confirm that the measurement accuracy and stability of T concentrations in goats are affected by blood collection time, delay to centrifugation, and storage devices, and duration. Therefore, when using blood samples for T measurements, researchers need to take these pre-analytical parameters into account to obtain accurate T measurements.

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