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The effect of sex and time of day on testosterone concentrations in equine saliva and serum

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RESEARCH ARTICLE

Abstract

In terms of exercise, testosterone is important for the growth and maintenance of skeletal muscle mass. Sampling saliva could be a non-invasive alternative to blood sampling for the quantification of testosterone levels in horses. The objective of this study was to compare testosterone concentrations in saliva and serum (sampled simultaneously) from horses of different sexes and at different times throughout the day. A total of 67 warmblood riding horses (21 geldings, 22 mares and 24 stallions) were included in the study. Saliva and blood samples were collected in the morning (06:00-08:00), at midday (11:00-13:00) and in the evening (17:00-19:00). The results demonstrated a weak correlation between saliva and serum testosterone concentrations (r_s=0.25, P=0.04). Stallions had higher serum testosterone concentrations than mares and geldings (P<0.001), but there was no significant effect of sex on salivary testosterone concentrations. The time of day did not affect the concentration of testosterone in either saliva or serum. In conclusion, our results indicate that saliva samples cannot be recommended for measuring testosterone levels in horses. However, further research is needed to identify the disturbing factors.

Keywords: horse, hormone, gelding, mare, stallion, testosterone

1. Introduction

Testosterone is a key anabolic hormone with numerous physiological functions in the body. It is especially important in the growth and maintenance of skeletal muscle, bone and red blood cells during exercise (Crewther *et al.*, 2006; Zitzmann and Nieschlag, 2001). Immunoassays designed to measure salivary testosterone in humans are well documented (Granger *et al.*, 1999), and saliva sampling can be used as a non-invasive alternative to blood sampling for the quantification of testosterone concentrations (Cook *et al.*, 2012; Nunes *et al.,* 2011). Furthermore, it allows for straightforward repeated sampling.

A correlation between testosterone concentrations in saliva and blood has also been documented in captive monkeys under anaesthesia (Arslan, 1984) and in monkeys trained for the sampling of blood and saliva (Kutsukake *et al.*, 2009), whereas no such correlation was found in guinea pigs (Fenske, 1996). Saliva from monkeys was sampled by passive drool (Arslan, 1984; Kutsukake *et al.*, 2009) and cotton swabs were used for the guinea pigs (Fenske, 1996).

Currently, testosterone concentrations in horses are typically measured in the blood (Cox *et al.*, 1973; Soma *et al.*, 2008), but Khalil *et al.* (2009) have also shown that faeces are suitable for measuring differences in equine testosterone concentrations. Saliva samples have been used in one study with a limited number of horses (n=3), but it was concluded that salivary mucopolysaccharides interfered, giving unreliable results (Boudene *et al.*, 1976). Whether saliva can reliably be used to assess testosterone concentrations in the blood requires further study. Blood testosterone concentrations from stallions follow a diurnal rhythm, peaking in the morning and with a nadir in the evening (Sharma, 1976), but no existing literature describes a diurnal rhythm in the saliva or blood testosterone concentrations of geldings or mares.

The objectives of the present study were to: (1) investigate a potential correlation between testosterone concentrations in serum and saliva; (2) assess the effect of sex; (3) determine whether diurnal variation could be measured in serum and salivary testosterone concentrations in geldings and mares. It was hypothesised that: (1) there is a positive correlation between serum and salivary testosterone concentrations; (2) saliva samples will demonstrate the expected difference in testosterone levels between the sexes; (3) there is a diurnal rhythm in the secretion of salivary testosterone as well as in serum testosterone concentrations in stallions, though not in mares or geldings.

2. Materials and methods

The experimental procedures followed Danish national legislation, in addition to guidelines on the protection of vertebrate animals, approved by the member states of the Council of Europe (Anonymous, 1986). Furthermore, this study conformed to the 'Guidelines for Ethical Treatment of Animals in Applied Animal Behaviour and Welfare Research' upheld by the ethics board of the International Society of Applied Ethology (www.applied-ethology.org).

Horses

A total of 67 privately owned horses (21 geldings, 22 mares and 24 stallions) from 10 different yards were included in the study. The age of the horses ranged from 3 to 24 years (mean age ± standard deviation SD: geldings: 8.3±3.1, mares: 8.4±2.7, stallions: 9.4±5.7 years). All horses were warmblood riding horses (Hanoverian, Oldenburger, Holstein, Danish or Dutch Warmblood), bred and trained for either dressage or showjumping. Samples were obtained during the non-breeding season (January), and the mares were non-pregnant and anoestrous, as confirmed by ultrasonography.

Sampling of blood and saliva

Blood and saliva samples were collected from each of the 67 horses while stabled in individual boxes in their home environment. Sampling was performed in the morning (06:00-08:00), at midday (11:00-13:00) and in the evening (17:00-19:00). Within each yard, samples from all horses were collected on the same day, but different yards were sampled on different days. Blood samples were obtained by venipuncture of the vena jugularis with the use of S-Monovette 7.5-ml Z tubes (Sarstedt, Nümbrecht, Germany), followed by centrifugation (2,000×*g* for 10 min). Serum was stored at -18 °C until analysis. A saliva sample was obtained using synthetic swabs (Salivette®, Nümbrecht-Rommelsdorf, Germany) immediately after blood sampling. The swabs were placed under and over the tongue with the help of an arterial clamp for approximately 1 min, until they were soaked with saliva. The swabs were then placed in a polypropylene tube and stored at -18 °C within 20 min of sampling. Saliva samples were further processed by thawing and centrifugation (2,500×*g*). Saliva were then transferred to new tubes and again frozen until analysis. The recovery of the synthetic swabs (Salivette) in the freezing/thawing cycle was separately determined (n=10) by adding tritium labelled testosterone. It was found to be 52.3±2.5%. Saliva and blood samples were obtained either prior to feeding or at least 1 h after feeding, and at least 2 h after exercise, when the horses were resting in their boxes.

Analysis of testosterone in saliva and serum

Serum samples (0.5 ml) were extracted with diethyl ether (5 ml) by shaking for 30 min, followed by centrifugation (2,500×*g* for 15 min), freezing (-20 °C) and decanting the ether phase into a new vial. After evaporating the ether to dryness (at 40 °C under a stream of nitrogen), extracts were redissolved in assay buffer and an aliquot measured in a testosterone enzyme immunoassay (EIA; see below). An aliquot of the saliva samples was directly measured using the same assay (assay readings were corrected for testosterone loss of the swabs). The antibody was raised in a rabbit against testosterone-3-CMO-bovine-serum-albumin, and a biotin-streptavidin system was utilised as label. Details of the assay procedure and the testosterone EIA, including cross-reaction of the antibody, can be found elsewhere (Möhle *et al.*, 2002; Palme and Möstl, 1994). The sensitivity of the EIA was 0.2 pg/well. The intra- and inter-assay coefficients of variation were 7.9 and 12.9%, respectively. All samples were run in duplicate.

Statistics

The statistical analysis was performed using the SigmaPlot statistics package, version 13.0 (Systat Software Inc., Chicago, IL, USA). The normality of the data was assessed using the Shapiro-Wilk test, and variance homogeneity was assessed via the Brown-Forsythe test. Data did not fit normality and/or had unequal variance therefore the non-parametric Friedman Repeated Measures Analysis of Variance on Ranks was used to analyse changes in testosterone concentrations throughout the day.

The average of the morning, midday and evening samples of testosterone in saliva and serum were calculated for each horse and a Kruskal-Wallis One Way Analysis of Variance on Ranks was used to analyse the effect of sex on testosterone concentration. Post-testing was performed with Dunn's Method (All Pairwise Multiple Comparison Procedures). Spearman's rank correlation coefficient was used to test for correlations between average testosterone concentrations in saliva and serum. *P*-values <0.05 were considered to be significant. Data was not normally distributed and therefore values are presented as medians (25^{th} and 75^{th} percentiles).

3. Results

All horses tolerated the procedures well, and sampling of saliva and blood was straightforward. There was a weak correlation between salivary and serum testosterone concentrations (r_s=0.25, *P*=0.04), and the average salivary and serum concentrations of testosterone from each horse (n=67) are shown in Figure 1.

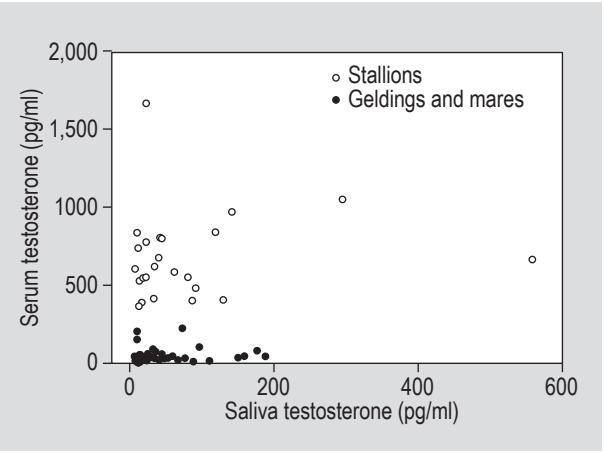

Figure 1. Average of morning, midday and evening samples of saliva (x-axis) and serum (y-axis) testosterone concentrations in stallions (n=24) and geldings and mares (n=43) (r_s=0.25, *P*=0.04).

There was an effect of sex on the concentrations of testosterone in serum, with stallions having higher concentrations than mares and geldings (*P*<0.001; Table 1). However, this effect of sex was not observed in the salivary testosterone concentrations. No diurnal rhythm was detected in testosterone concentrations, either in serum or in saliva (Table 2).

4. Discussion

Salivary testosterone reflects the free fraction of testosterone in blood that diffuses through the salivary glands into saliva. Saliva samples are frequently used as a non-invasive substitute for blood samples to measure testosterone levels in humans (Maso *et al.*, 2004; Nunes *et al.*, 2011), and it has been reported that salivary testosterone levels correlate well with free testosterone levels in serum when sampled simultaneously (Vittek *et al.*, 1985). To our knowledge, only one published study has measured salivary testosterone in horses (Boudene *et al.*, 1976), and the authors concluded that testosterone could not be measured accurately in saliva due to interference with mucopolysaccharides. However,

Table 1. The effect of sex (geldings, mares and stallions) on testosterone concentrations in serum and saliva. Values are presented as medians [25th; 75th percentiles].

	Sex	n	Median [0.25; 0.75][1]	*P*-value
Serum (pg/ml)	geldings	21	33.7 [18.7; 51.4] [b]	<0.001
	mares	22	41.5 [32.4; 56.4] [b]	
	stallions	24	613.0 [494; 804] [a]	
Saliva (pg/ml)	geldings	21	15.0 [10.4; 62.9]	0.23
	mares	22	26.0 [14.9; 58.0]	
	stallions	24	37.1 [16.9; 90.0]	

[1] Values in the same column with different superscript letters differ significantly (*P*<0.05).

Table 2. The effect of time (morning, midday and evening) on testosterone concentrations in serum and saliva. Values are presented as medians [25th; 75th percentiles].

	Sex	n	Time	Median [0.25; 0.75]	*P*-value
Serum (pg/ml)	stallions	24	morning	607 [388; 958]	0.76
		24	midday	668 [389; 991]	
		24	evening	519 [372; 710]	
	mares and geldings	43	morning	36.2 [16.0; 51.0]	0.80
		43	midday	31.0 [16.0; 54.4]	
		43	evening	37.4 [19.4; 57.0]	
Saliva (pg/ml)	all	67	morning	25.1 [10.0; 76.2]	0.23
		67	midday	14.8 [10.0; 44.1]	
		67	evening	17.3 [10.0; 44.5]	

studies in humans have found that a freeze-thaw cycle followed by centrifugation removes mucopolysaccharides from the saliva sample (Arregger *et al.*, 2007), but that centrifugation might decrease salivary testosterone levels in comparison to unprocessed saliva (Durdiaková *et al.*, 2013). Therefore we applied such a freezing/thawing cycle to the saliva samples prior to the analysis with the testosterone EIA and added a recovery experiment to correct for testosterone loss of the whole saliva collection method. Unfortunately, also in our study the correlation between testosterone concentrations in serum and saliva was weak.

The weak correlation observed in the current study could be related to the saliva sampling method. In humans, the correlation between salivary testosterone and total testosterone in serum is dependent on the saliva-collection method (Fiers *et al.*, 2014; Granger *et al.*, 2004). Passive drool has been found to increase the correlation between serum and salivary testosterone significantly in both men (r=0.68) and women (r=0.52) compared to saliva sampled with cotton swabs (both sexes: r=0.43) (Fiers *et al.*, 2014). In contrast to humans and monkeys, passive drool is not a practical option in horses, but it might be possible if saliva production could be stimulated pre-sampling, thus avoiding sampling with cotton swabs. However, there are also conflicting results regarding the use of saliva stimulation with regard to testosterone measurements. In humans, stimulating the saliva flow by touching the tongue with cotton swabs treated with 2% citric acid increased saliva production, but did not affect the concentration of salivary testosterone in comparison to cotton swabs (Durdiaková *et al.*, 2013). Other studies state that citric acid should be avoided due to interference with the method used for testosterone analysis (Granger *et al.*, 2004; Shirtcliff *et al.*, 2001), and that stimulation of saliva flow increases salivary testosterone concentrations (Dabbs, 1991; Granger *et al.*, 2004). However, a high correlation has been observed between salivary testosterone and free testosterone concentrations in human serum, regardless of immunoassay method (Granger *et al.*, 2004; Riad-Fahmy *et al.*, 1982; Wang *et al.*, 1981).

Additional factors potentially affecting testosterone analysis include the handling and storage of samples. One study found that storing samples at room temperature or between -20 and -80 °C, for up to 1 month did not affect the testosterone concentrations (Durdiaková *et al.*, 2013), whereas another study found that testosterone concentrations increased by 20% after 4 weeks of storage at 4 °C, and decreased after 24 months of storage at -20 °C (Granger *et al.*, 2004). In the current study, saliva samples were stored at -18 °C within 20 min of sampling, and were analysed within 3 months, so storage is not expected to have influenced the results.

It is possible that there is a time lag before responses measured in blood can be measured in saliva. Human studies demonstrating a positive correlation between testosterone in saliva and blood were sampled simultaneously (Fiers *et al.*, 2014; Johnson *et al.*, 1987), and following exogenous testosterone administration, testosterone concentrations in saliva and serum rose abruptly and in parallel (Wang *et al.*, 1981). Salivary pH and flow differs between horses and humans, with equine salivary pH ranging from 7.4 to 7.9 (Alexander, 1966), compared to 5.5-6.0 in humans (Horner, 1976). Saliva is continuously produced in humans, but is stimulated when horses masticate (Alexander, 1966; Burgen and Emmelin, 1961), with the rate of saliva flow influencing electrolyte concentrations (Alexander, 1966). However, information on the influence of saliva flow (and pH) on testosterone concentrations in saliva of horses is lacking. However, this could be a potential reason for the lack of correlation between saliva and plasma testosterone concentrations. Thus future research should determine whether there is a time lag between saliva and blood in terms of testosterone concentrations in horses, in order to ensure that saliva is sampled at the optimal time.

As expected, serum testosterone concentrations were higher in stallions than mares and geldings, and it was anticipated that this difference also would be observed in saliva. However, this difference between the sexes could not be detected in the saliva samples. Average salivary testosterone concentrations from stallions were two times higher than the values measured in mares and geldings, but due to large variations, the differences were not significant. Further research is needed to locate the problem.

In humans, a diurnal rhythm in testosterone concentrations has been confirmed (Granger *et al.*, 2004), and this has also been measured in blood from stallions, with peak testosterone concentrations in the morning and a nadir in the evening or at night (Kirkpatrick *et al.*, 1976; Sharma, 1976). However, the results are conflicting, as others did not find any diurnal rhythm in blood testosterone concentrations (Bono *et al.*, 1982). This is similar to the results from the present study, where diurnal rhythms in serum and salivary testosterone levels could not be detected.

5. Conclusions

In conclusion, salivary testosterone concentrations did not correlate well with serum testosterone concentrations. Stallions had higher serum testosterone concentrations than mares and geldings, but there was no significant difference between the sexes in salivary testosterone concentrations. It was not possible to detect a diurnal rhythm in serum or salivary testosterone concentrations, regardless of sex. The results from this study indicate that blood samples are more reliable for measuring testosterone concentrations in

horses. Presently, saliva samples cannot be recommended for measuring testosterone levels in horses. However, further research is needed to identify the disturbing factors.

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