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Evaluation of the Diagnostic Value of Maternal Testosterone Concentration During Gestation for Determination of Fetal Gender in Horses

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ABSTRACT

BACKGROUND: Ascertainment of fetal sex is of importance in equine industry owing to economic reasons. As a result, various methods have been developed for sex determination of fetus in horse; however, the current techniques have some limitations. Recently, evaluation of maternal testosterone concentration has been suggested as an easy and inexpensive method for diagnosis of fetal sex, but the findings are discrepant in different species.

OBJECTIVES: The aim of present study was to measure concentration of circulating testosterone in mares carrying male and female fetuses in order to assess the diagnostic value of maternal testosterone concentration for sex determination of equine fetus.

METHODS: Blood samples were collected from mares (n = 20) at months three, six and nine of pregnancy. The samples were centrifuged and stored at -20 ºC until hormonal analysis of testosterone concentration using an ELIZA kit. The gender of foals was determined at birth based on observation of external genitalia.

RESULTS: Neither testosterone concentration in the third, sixth and ninth months of pregnancy nor the cumulative concentration of testosterone differ between mares with male and female fetuses ($P > 0.05$). However, testosterone concentration changed during pregnancy in all mares regardless of the gender of their fetus and it was higher at month six than months three and nine ($P < 0.0001$).

CONCLUSIONS: In conclusion, the present study showed that maternal testosterone concentration could not be used for sex determination of fetus in horse. Yet the current study revealed dynamics of testosterone concentration over various stages of gestation in mares.

KEYWORDS: Androgen; Equine; Foal; Pregnancy; Sex determination

Introduction

The gender of offspring has been indicated in various species due to scientific and economic reasons, because of which numerous studies have been conducted to understand mechanisms contributing to offspring sex allocation (Abouhamzeh *et al*., 2020; Gharagozlou *et al*., 2016; Mozaffari Makiabadi *et al*., 2022). The sex of offspring could also be of importance in horse considering the horse breed and its application (Gharagozlou *et al*., 2014; Rezagholizadeh *et al*., 2015; Samper *et al*., 2012a; Samper *et al*., 2012b). For instance, breeders of polo horses prefer to have female foals whereas male foals are favorable for show jumping (Gharagozlou *et al*., 2014; Rezagholizadeh *et al*., 2015; Samper *et al*., 2012a; Samper *et al*., 2012b).

Given the significance of foal gender in equine industry, various methods have been developed so as to ascertain sex of fetus during pregnancy (Busato *et al*., 2021). In this context, ultrasonography could be used for diagnosis of fetal sex during gestation, but the period for application of ultrasonography is limited and it could not be used throughout pregnancy(Busato *et al*., 2021). Furthermore, analysis of cell-free fetal DNA in maternal circulation has been successfully used to determine fetal sex; however, this technique requires special equipment which restrict its usage in equine industry (de Leon *et al*., 2012).

Considering the limitations of prevailing techniques for determination of fetal sex, Kibushi *et al*. (2016) tested measurement of maternal testosterone concentration as an alternative method for diagnosis of fetal sex in bovine and observed higher concentration of testosterone in cows carrying male fetuses than cows carrying female fetuses. Moreover, the cut-off concentration testosterone for prediction of calf gender was of acceptable sensitivity and specificity (Kibushi *et al*., 2016). A former study by Meulenberg and Hofman, (1991) in human

has also showed greater concentration of testosterone in women with male fetuses than women with female fetuses. However, application of this method in equine resulted in discrepant outcomes as testosterone concentration, which was evaluated using radioimmunoassay technique, was higher in mares carrying female fetus than mares carrying male fetuses (Busato *et al*., 2021). The findings of the study by Busato *et al*. (2021) was unexpected since it is the testes of male fetuses producing testosterone, but not the ovaries of females fetuses, during gestation (Legacki *et al*., 2017; Scarlet *et al*., 2021).

Therefore, the present study was conducted to reexamine concentration of testosterone in mares pregnant with male and female fetueses to eluciadte whether maternal measurment of testototerone could serve as diagnostic method for fertal sex determination in equine.

Material and Methods

Study design and animals

The present study was approved by Animal Ethics Committee at University of Tehran in terms of animal welfare and ethics. The study was a cross-sectional study, in which blood samples were collected from 20 mares with various ages (8.80 ± 1.38 year) and parities (including 7 nulliparous and 13 parous mares). To ease the statistical analysis of the effect of age on dependent variables, mares were divided into two age categories including mares with ≤ 10 years old age (n = 12) and mares with > 10 years old age (n = 8). The mares were housed in a warm-blood horse farm located in Qazvin province, Iran. Breedings were performed by natural covering of mares using an individual stallion in the herd and pregnancy diagnosis was implemented using rectal ultrasonographic examination 14 to 16 days after confirmation of ovulation by ultrasonographic examination. Given that dates of breeding and pregnancy

diagnosis were precisely recorded in the herd, the timepoints associated with third, sixth and ninth month of pregnancy could easily be determined for blood sampling in this study. The gender of foal was ascertained at birth based on observation of external genitalia.

Blood sampling and testosterone assay

Blood samples were collected from jugular vein of mares at third, sixth and ninth month of gestation. The samples were centrifuged for 15 minutes at 2000 rpm and the resultant serum were maintained at -20°C until hormonal assessment. Testosterone was evaluated using an ELIZA kit (Roche Diagnostics, Mannheim, Germany) based on manufacturer instructions. The detection limit, intra-assay CV and inter-assay CV of applied testosterone ELIZA kit were, 12 pg/ml, 2.9 % and 4.8 %, respectively.

Statistical Analysis

Data associated with testosterone concentration were analyzed using GLM procedure by a repeated measures model. Multiple comparisons were conducted using LSMEANS statement. Data associated with sex ratio of foals were analyzed using GENMOD procedure including function link logit in the model. All analysis were conducted in SAS version 9.4 (SAS Institute Inc., Carry, NC, USA). Differenced were considered significant at $P < 0.05$.

Results

Concentration of testosterone in mares was not affected by the interaction effect of fetal gender by time ($P > 0.05$; Figure 1A) and the main effect of fetal gender ($P > 0.05$; Figure 1B). But maternal testosterone concentration was affected by the main effect of time and it was higher at the sixth month of pregnancy as compared with the third and ninth month of pregnancy ($P < 0.0001$; Figure 1C). Moreover, concertation of testosterone during pregnancy was not different

between ≤ 10 years old and > 10 years old mares ($P > 0.05$; Figure 2A), and between nulliparous and parous mares ($P > 0.05$; Figure 2B).

Irrespective of testosterone concentration, analysis of data associated with sex ratio of offspring revealed that ≤ 10 years old mares were more likely to produce male foals as compared with > 10 years old mares (odds ratio = 9.00, 95% confidence interval = 1.14-71.04; $P < 0.05$; Table 1). However, parity of mares had no significant impact on sex ratio of foals ($P > 0.05$; Table 1), which implicated that the effect of maternal age on sex ratio of offspring was not parity-related.

Discussion

Given the importance of fetal sex diagnosis during pregnancy in equine industry (Gharagozlou *et al*., 2014; Rezagholizadeh *et al*., 2015; Samper *et al*., 2012a; Samper *et al*., 2012b) the present study was conducted to assess the association of maternal circulating testosterone concentration with fetal gender in mares. In this context, the present study showed no significant difference in serum testosterone concentration between mares carrying male and female fetuses at third, sixth and ninth months of gestation, implicating that maternal circulating testosterone may not serve as an useful indicator of fetal sex in equine. In a recent study, Busato *et al*. (2021) found comparable concentrations of testosterone in male-fetus- and female-fetus-baring mares at sixth and seventh months of pregnancy, as similar to the present study; however, they observed higher concentrations of testosterone in mares carrying female fetuses than those carrying male fetuses at fifth and eighth months of gestation (Busato *et al*., 2021). The contradictory findings of these two studies might be related to different measurement methods applied by each of these studies to analyze testosterone concentration, which require further

studies to be elucidated. Unlike equine, greater concentrations of testosterone was found in females carrying male fetuses than those carrying female fetuses in bovine (Kibushi *et al*., 2016) and human (Meulenberg & Hofman, 1991). These phenomena imply disparity among species either at fetal level and development of gonads or at maternal level, particularly placental function and physiology, which needs further research to become understood.

Nevertheless, temporal dynamics of serum testosterone was observed over the course of gestation in mares as circulating testosterone elevated from month three to six of pregnancy and declined afterwards up to month nine of gestation. In this sense, it has been reported that level of blood testosterone increased in mares during pregnancy up to months seven and eight of gestation, at which circulating testosterone peaked (Silberzahn *et al*., 1984). Furthermore, Satué *et al*. (2019) revealed that maternal blood testosterone in pregnant mares initiated to increase in months two and three of pregnancy, plateaued between months four and six of pregnancy, decreased from month seven to nine of pregnancy, and experienced a peak in month 10 of pregnancy (Satué *et al*., 2019). On the other hand, Legacki *et al*. (2016) mapped alterations of steroid hormones during equine pregnancy using mass spectrometry and reported that testosterone concentration ranged between 0.10 and 0.34 ng/ml from week 6 to 14 of gestation, but it was not debatable afterwards (Legacki *et al*., 2016). Taken together, although all studies substantiated alterations of circulating testosterone in pregnant mares during gestation, the pattern of these changes seems to be discrepant among various studies, which might be due to the timepoints selected for blood sampling and/or the methodology and kits used for hormonal analysis.

Another interesting result of the present study was the effect of maternal age on fetal sex. It was found that younger mares were more likely to produce male offspring as compared with

older mares. It should be noted that this effect of maternal age did not depend on the history of experiencing pregnancy. Moreover, the effect of stallion on the fetal sex ratio has been reported previously (Gharagozlou *et al*., 2014) but in the present study, all investigated mares were bred by a single stallion, and so the effect of stallion on sex ratio was not a confounding factor in this study. In line with findings of present research, a study by Santos *et al*. (2015) showed that the percentage of males dwindled with increase in age of mares (Santos *et al*., 2015). Many changes in mares occur during aging, including changes in the development of ovarian follicles and oocytes (Ginther *et al*., 2008; Rambags *et al*., 2014; Rizzo *et al*., 2019) embryonic and fetal growth (Cuervo-Arango *et al*., 2018; Derisoud *et al*., 2021; Squires *et al*., 1999) and even the structure and function of the uterus (Ousey *et al*., 2012), which can be considered as potential influencing factors (Busato *et al*., 2021). With regard to potential contributing mechanisms, factors affecting sex ratio of offspring could be divided into two categories of preconceptional and postconceptional determinants. With the former, androgens, estrogens and immune system of female reproductive tract have been reported to skew sex ratio of offspring through impacting gametes and/or their interaction with oviduct (Almiñana *et al*., 2014; Emadi *et al*., 2014; Gharagozlou *et al*., 2016). With the latter, maternal body condition and glucose concentration have been observed to dimorphically affect the survival of male and female embryos, thereby impacting sex ratio (Cameron *et al*., 2008). Nevertheless, the exact mechanisms underlying the effect of maternal age on sex ratio of offspring warrants further studies to be deciphered.

Conclusion

The present study showed no significant difference in concentrations of maternal testosterone between mares carrying male fetuses and mares carrying female fetuses at the third,

sixth and ninth months of gestation. Therefore, it appeared that evaluation of circulating testosterone in pregnant mares could not serve as a diagnostic method for determination fetal sex in horses.

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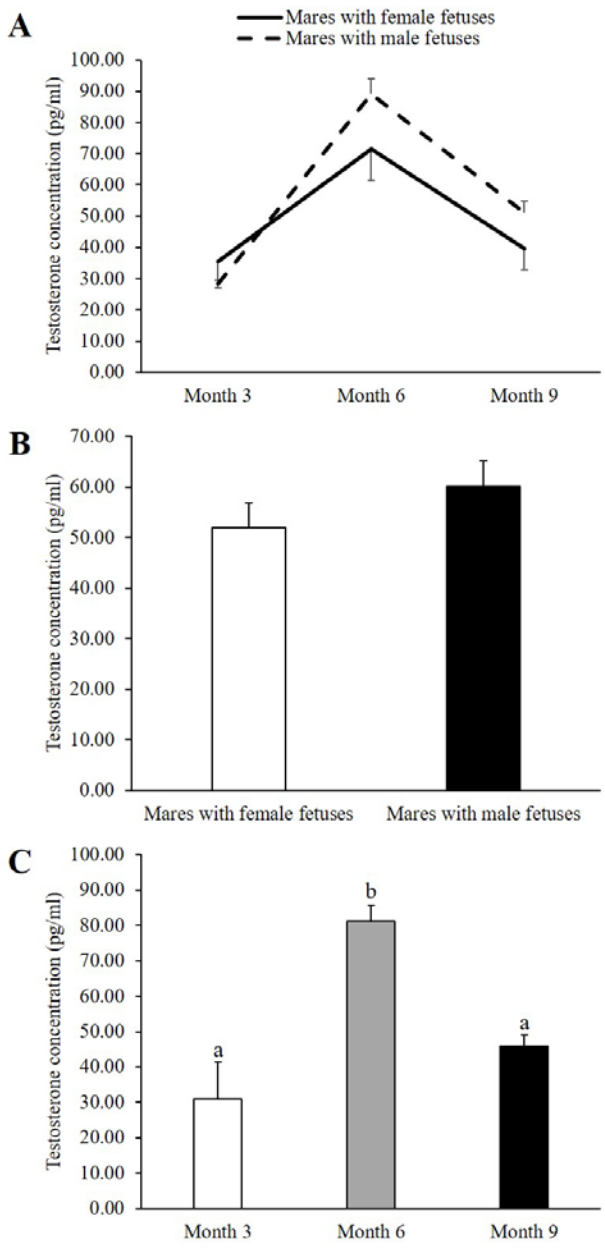

Figure 1. A) The interaction effect of fetal gender by time on concentration of testosterone in pregnant mares. B) The main effect of fetal gender on concentration of testosterone in pregnant

mares. C) The main effect of time of gestation on concentration of testosterone in pregnant mares. [ab]Various letters indicate significant difference ($P < 0.0001$).

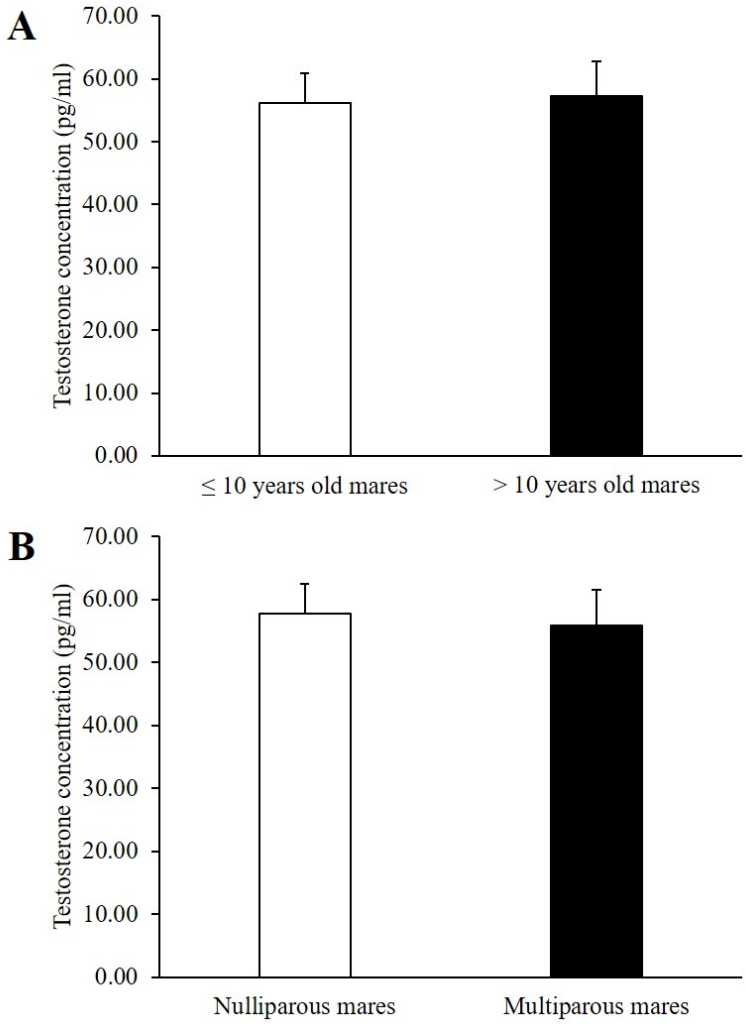

Figure 2. A) Concentration of circulating testosterone in ≤ 10 and > 10 years old mares during pregnancy. B) Concentration of circulating testosterone in nulliparous and parous mares. [ab]Various letters indicate significant difference ($P < 0.05$).

Table 1. Effect of age and parity of mares on sex ratio of offspring. Values in parenthesis are actual numbers.

Effect	Class	Sex ratio (%)	OR	95% CI	*P* value
Age	≤ 10 years old	75.00 (8/12)	9.00	1.14-71.04	0.04
	> 10 years old	25.00 (2/8)	—	—	—
Parity	Nulliparous	57.14 (4/7)	1.14	0.18-7.28	0.89
	Parous	53.85 (7/13)	—	—	—

OR: odds ratio; CI: confidence interval.

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ارزیابی ارزش تشخیصی غلظت تستوسترون مادری حین آبستنی برای تعیین جنسیت جنین در اسب

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چکیده

زمینه مطالعه: تعیین جنسیت جنین در صنعت اسب به دلایل اقتصادی از اهمیت برخوردار است. بنابراین، روش های مختلفی برای تعیین جنسیت جنین در اسب توسعه پیدا کرده است، ولی تکنیک های حاضر دارای محدودیت هایی هستند. اخیراً، ارزیابی غلظت تستوسترون مادری به عنوان روشی ساده و ارزان جهت تشخیص جنسیت جنین پیشنهاد شده است، اما یافته ها در گونه های مختلف متناقض هستند.

هدف: هدف مطالعه حاضر اندازه گیری غلظت تستوسترون گردش خون مادیان های آبستن با جنین های نر و ماده به منظور سنجش ارزش تشخیصی غلظت تستوسترون مادری برای تعیین جنسیت جنین اسب بود.

روش کار: نمونه های خون در ماه های سه، شش و نه آبستنی از مادیان ها (تعداد = 20) اخذ شد. نمونه ها سانتریفیوژ شده و تا زمان آنالیز هورمونی غلظت تستوسترون با استفاده از کیت الایزا در منفی 20 درجه سانتی گراد ذخیره شدند. جنسیت کره ها در زمان تولد و بر اساس مشاهده اندام تناسلی خارجی تعیین شد.

نتایج: غلظت تستوسترون در ماه های سوم، ششم و نهم آبستنی و نه غلظت تجمعی تستوسترون تفاوتی میان مادیان های دارای کره نر و ماده نداشت ($P > 0/05$). اما غلظت تستوسترون در طول آبستنی در تمامی مادیان ها فارق از جنسیت جنین آنها تغییر کرد و در ماه شش بالاتر از ماه های سه و نه بود ($P < 0/0001$).

نتیجه‌گیری: در نتیجه، مطالعه حاضر نشان داد که غلظت تستوسترون مادری نمی تواند برای تعیین جنسیت جنین در اسب استفاده شود. اما مطالعه حاضر مبین تغییرات غلظت تستوسترون در مقاطع مختلف آبستنی در مادیان بود.

واژه‌های کلیدی: آندروژن؛ تکسمیان؛ کره؛ آبستنی؛ تعیین جنسیت