10.22059/IJVM.2022.336095.1005220

Journal of Iranian Veterinary Medicine
Volume 16- Issue 4

Original Article
Online ISSN: 2252-0554

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

OBJECTIVES: The aim of the present study was to measure the 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°C 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 at months three and nine ($P<0.0001$).

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

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

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Received: 2021-12-23

Accepted: 2022-02-14

How to Cite This Article

Akbarinejad, V ., Gharagozlou, F., Mobedi, E., Gholamali, E. (2022). Evaluation of the Diagnostic Value of Maternal Testosterone Concentration During Gestation for Determination of Fetal Gender in Horses. *Iranian Journal of Veterinary Medicine, 16*(4), 372-379.

Introduction

The gender of offspring has been indicated in various species due to scientific and economic reasons. 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 horses, 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 the equine industry, various methods have been developed to ascertain the sex of the fetus during pregnancy (Busato *et al*., 2021). In this context, ultrasonography could be used to diagnose fetal sex during gestation, but the period for application of ultrasonography is limited and 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 restricts its usage in the equine industry (de Leon *et al*., 2012).

Considering the limitations of prevailing techniques for determining fetal sex, Kibushi et al. (2016) tested the measurement of maternal testosterone concentration as an alternative method for diagnosing fetal sex in bovines. They observed a higher testosterone concentration in cows carrying male fetuses than in cows carrying female fetuses. Moreover, the cut-off concentration of testosterone for the prediction of calf gender was of acceptable sensitivity and specificity (Kibushi *et al*., 2016). A former study by Meulenberg and Hofman (1991) on humans also showed a greater concentration of testosterone in women with male fetuses than in women with female fetuses. However, application of this method in equines resulted in discrepant outcomes as testosterone concentration, which was evaluated using the radioimmunoassay technique, was higher in mares carrying female fetuses than mares carrying male fetuses (Busato *et al*., 2021). The findings of the study by Busato *et al*. (2021) were unexpected since it is the testes of male fetuses producing testosterone, but not the ovaries of female fetuses, during gestation (Legacki *et al*., 2017; Scarlet *et al*., 2021).

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

Materials and Methods

Study Design and Animals

The present study was approved by Animal Ethics Committee at the University of Tehran in terms of animal welfare and ethics. The study was cross-sectional, in which blood samples were collected from 20 mares of ages (8.80 ± 1.38 years) 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 in Qazvin province, Iran. Breedings were performed by a natural covering of mares using an individual stallion in the herd. Pregnancy diagnosis was implemented using rectal ultrasonographic examination 14 to 16 days after confirmation of ovulation by ultrasonographic examination. Given that breeding dates and pregnancy diagnosis were precisely recorded in the herd, the timepoints associated with the third, sixth, and ninth months of pregnancy could easily be determined for blood sampling in this study. The gender of the foal was ascertained at birth based on observation of external genitalia.

Blood Sampling and Testosterone Assay

Blood samples were collected from the jugular vein of mares at the third, sixth, and ninth months of gestation. The samples were centrifuged for 15 minutes at 2000 rpm, and the resultant serum was maintained at -20°C until hormonal assessment. Testosterone was evaluated using an ELIZA kit (Roche

Diagnostics, Mannheim, Germany) based on manufacturer instructions. The applied testosterone ELIZA kit's detection limit, intra-assay CV, and inter-assay CV were 12 pg/mL, 2.9 %, and 4.8 %, respectively.

Statistical Analysis

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

Results

The 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 was higher at the sixth month of pregnancy than in the third and ninth months 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 the 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, the parity of mares had no significant impact on the sex ratio of foals ($P>0.05$; Table 1), which implied that maternal age's effect on the sex ratio of offspring was not parity-related.

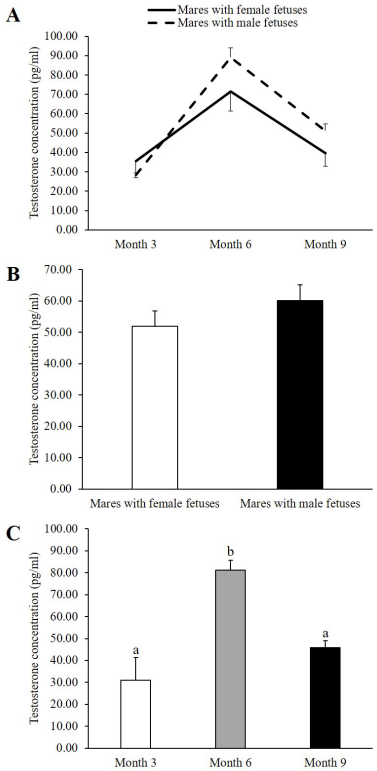

Figure 1. A) The interaction effect of fetal gender by time on testosterone concentration in pregnant mares. B) The main effect of fetal gender on testosterone concentration in pregnant mares. C) The main effect of gestation time on testosterone concentration in pregnant mares. [ab]Various letters indicate a significant difference ($P<0.0001$).

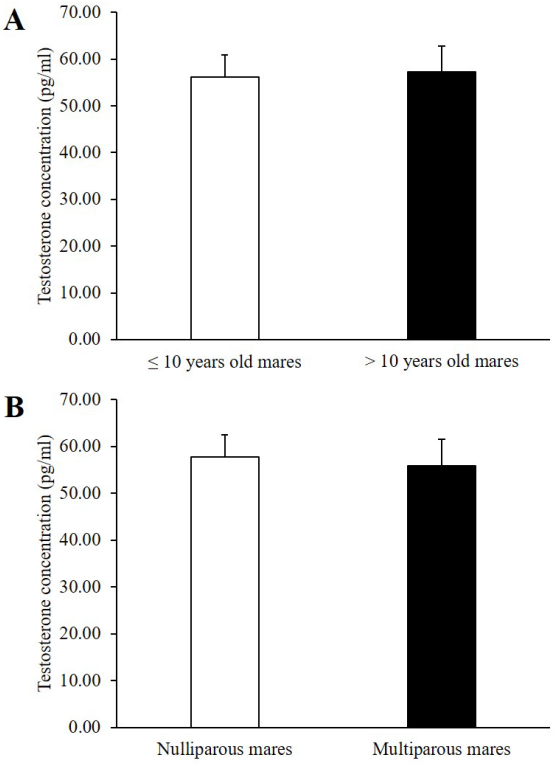

Figure 2. A) Concentration of circulating testosterone in ≤ 10 and > 10 years old mares during pregnancy. B) Concentration of circulating testosterone in nulliparous and parous mares. [ab]Various letters indicate a significant difference ($P<0.05$)

Table 1. Effect of age and parity of mares on the 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

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 the third, sixth, and ninth months of gestation, implicating that maternal circulating testosterone may not serve as a 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 the sixth and seventh months of pregnancy, 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 requires further studies to be elucidated. Unlike equines, greater testosterone concentrations were found in females carrying male fetuses than in those carrying female fetuses in bovines (Kibushi *et al*., 2016) and humans (Meulenberg & Hofman, 1991). These phenomena imply disparity among species either at a fetal level and development of gonads or at the maternal level, particularly placental function and physiology, which needs further research to become understood.

Nevertheless, temporal dynamics of serum testosterone were 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. In this sense, it has been reported that the blood testosterone level 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. They 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 afterward (Legacki *et al*., 2016). 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 a 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 a stallion on sex ratio was not a confounding factor in this study. In line with present research findings, a study by Santos *et al.* (2015) showed that the percentage of

males dwindled with the increase in the 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). Concerning potential contributing mechanisms, factors affecting the sex ratio of offspring could be divided into two categories of preconceptional and postconceptional determinants. With the former, androgens, estrogens, and the immune system of the female reproductive tract have been reported to the skewed sex ratio of offspring by 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 the sex ratio (Cameron *et al*., 2008). Nevertheless, the exact mechanisms underlying the effect of maternal age on the sex ratio of offspring warrants further studies to be deciphered.

Conclusion

The present study showed no significant difference in maternal testosterone concentrations 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 determining fetal sex in horses.

Acknowledgments

The current research was supported by the University of Tehran (Grant number: 7508010/6/21). The authors would like to express their gratitude to the staff at the warm-blood horse farm (Qazvin, Iran) for their kind assistance.

Conflict of Interest

The authors declared no conflict of interest.

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10.22059/IJVM.2022.336095.1005220

Iranian Journal of Veterinary Medicine

Abstracts in Persian Language

Online ISSN 2252-0554

مجله طب دامی ایران، ۱۴۰۱، دوره ۱۶، شماره ۴، ۳۷۹-۳۷۲

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

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(دریافت مقاله: ۰۲ دی ماه ۱۴۰۰، پذیرش نهایی: ۲۵ بهمن ۱۴۰۰)

زمینه مطالعه: تعیین جنسیت جنین در صنعت اسب به دلایل اقتصادی از اهمیت برخوردار است. بنابراین، روش‌های مختلفی برای تعیین جنسیت جنین در اسب توسعه پیدا کرده است، ولی تکنیک‌های حاضر دارای محدودیت‌هایی هستند. اخیراً، ارزیابی غلظت تستوسترون مادری به‌عنوان روشی ساده و ارزان برای تشخیص جنسیت جنین پیشنهاد شده است، اما یافته‌ها در گونه‌های مختلف متناقض هستند.

هدف: هدف مطالعه حاضر اندازه‌گیری غلظت تستوسترون گردش خون مادیان‌های آبستن با جنین‌های نر و ماده به‌منظور سنجش ارزش تشخیصی غلظت تستوسترون مادری برای تعیین جنسیت جنین اسب بود.

روش کار: نمونه‌های خون در ماه‌های سه، شش و نه آبستنی از مادیان ها (تعداد = ۲۰) اخذ شد. نمونه‌ها سانتریفیوژ شده و تا زمان آنالیز هورمونی غلظت تستوسترون با استفاده از کیت الایزا در منفی ۲۰ درجه سانتی‌گراد ذخیره شدند. جنسیت کره‌ها در زمان تولد و بر اساس مشاهده اندام تناسلی خارجی تعیین شد.

نتایج: غلظت تستوسترون در ماه‌های سوم، ششم و نهم آبستنی و نه غلظت تجمعی تستوسترون تفاوتی میان مادیان‌های دارای کره نر و ماده نداشت ($P > ۰/۰۵$). اما غلظت تستوسترون در طول آبستنی در تمامی مادیان‌ها فارق از جنسیت جنین آنها تغییر کرد و در ماه شش بالاتر از ماه‌های سه و نه بود ($P<۰/۰۰۰۱$).

نتیجه‌گیری نهایی: در نتیجه، مطالعه حاضر نشان داد که غلظت تستوسترون مادری نمی‌تواند 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

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