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Equine Alpha-fetoprotein Levels in Thoroughbred Mares with Normal and Pathological Pregnancies

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ABSTRACT

***Background*:** Alpha-fetoprotein (AFP) is a fetal protein that was first defined in 1956 and has been used since the 1970s to identify high-risk pregnancies for abnormalities in gynecology. First description of fetal protein was by Bergstrand and Czar in 1956 in humans and named as ''alpha-fetoprotein'' (AFP). AFP was found in the electrophoresis of fetal serum (pH 8.6) and migrated from serum albumin faster than slow alpha-1-globulin, and was located in the alpha globulins region. AFP has also been used as a tumor marker in many neoplastic formations and was later shown to be present in other mammalian species. The aim of our study was to determine the concentrations of maternal serum alpha-fetoprotein (MSAFP) during pregnancy of Thoroughbred mares and to investigate the possible differences of MSAFP in normal and pathological pregnancy.

***Materials, Methods & Results*:** The horses (*Equus caballus*) in this study were Thorougbred mares (n = 66) permanently housed at the Karacabey Stud of the Turkish Jockey Club. The mares were subjected to regular health checks. The mares that were healthy on clinical examination, had regular cyclic activity and were bred by different stallions during the 2019 official breeding season (15 February - 30 June) were randomly selected for inclusion in the study. The mares were divided into two groups: Mares with normal pregnancies (group I, n = 42) and mares with pathological pregnancies (group II, n = 24). Group II was divided into subgroups of embryonic loss (n = 11), abortion (n = 10), preterm birth (n = 2), and premature placental abruption (n = 1). The possible relationships between mean MSAFP and foal sex, birth weight, time of birth, and IgG concentration in colostrum were also investigated. The mean MSAFP concentration was 46.78 ± 0.87 pg/mL in normal pregnancies and 70.69 ± 3.86 pg/mL in pathological pregnancies ($P \leq 0.001$). No positive association was found between mean MSAFP concentration and colostrum quality or birth weight ($P > 0.05$).

***Discussion*:** In human medicine, alpha-fetoprotein is actively used in clinical practice as a tumour marker and as a screening test for early diagnosis of pregnancy pathologies in women. Pregnancy pathologies and embryonic losses cause great economic losses in horse breeding. Considering that the breeding season is limited, especially in thoroughbred racehorse breeding, it is important for breeders to identify mares that will experience pregnancy pathologies in the future during the breeding season. In our study, MSAFP concentrations were found to be higher than normal in mares with pregnancy pathologies such as embryonic death, abortion and premature placental separation, which is consistent with other studies. It was reported that a negative correlation was found between the age and gestational age of mares and MSAFP, but no correlation was found between the sex, birth weight, chest and leg circumference of foals and MSAFP concentration. Studies have shown that MSAFP may be a marker for pregnancy pathologies. In a study investigating the effects of seasons on MSAFP concentrations of mares, it was reported that MSAFP concentrations were higher in summer months compared to spring months ($P = 0.0378$) and MSAFP concentrations were higher in the last month of pregnancy compared to the previous months ($P = 0.0117$).These findings are supported by the results of our study. By measuring MSAFP concentrations, pathological pregnancy can be predetermined, interventions can be made and it can be possible to obtain one foal per year.

Keywords: alpha-fetoprotein, thoroughbred mares, embryo loss, abortion, anomaly, foal.

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INTRODUCTION

The fetal protein was first described in humans in 1956 by Bergstrand and Czar [2] and named "alpha-fetoprotein" (AFP). The average molecular weight of AFP has been determined to be 69,000 (65,000-72,000) daltons [10,15,18,26] and has been reported to be 68,350 daltons [16] in horses. AFP has been used in human medicine to detect foetal abnormalities since the 1970s and is now routinely used in pregnancy as a "triple test" (second trimester screening test) [1]. AFP is synthesised mainly from the yolk sac and fetal liver, but small amounts are also synthesised from the placenta, fetal gastrointestinal tract and kidneys [17]. Later in pregnancy, the foetal liver is the main organ where AFP is synthesised [11]. Studies show that AFP is also present in mammalian amniotic fluid and allantoic fluid during pregnancy and is rarely increased by maternal neoplasia [8,14,19,20]. Fetal elevations in AFP concentrations occur in twin/multiple pregnancies, fetal tumours, anomalies and deaths, while maternal elevations are due to tumour diseases, endogenous biological changes, carcinomas originating from liver tissue and hepatitis [13,25]. Recent studies have reported that AFP is an important parameter in the detection of pathological pregnancy in mares [4,5,22-24]. The aim of this study was to determine the mean MSAFP concentrations in pregnant mares throughout pregnancy and to determine whether MSAFP can be used as a screening test for pregnancy-related complications.

MATERIALS AND METHODS

Animals

All animal experiments were performed according to the following guidelines in compliance with the Ethics Committee of the Faculty of Veterinary Medicine, Istanbul University-Cerrahpaşa: approved protocol ♯2019/34. The horses (*Equus caballus*) in this study were Thorougbred mares (n = 66) permanently housed at the stud of the Türkiye Jockey Club Karacabey. The mares at the stud were regularly treated with internal and external parasite treatments, influenza, tetanus and EHV-1/4 follow-up programmes. Mares included in the study were randomly selected from those that were healthy at clinical examination, had regular cyclic activity, and were covered by different stallions during the 2019 official breeding season (February 15 to June 30). Body condition scores of the mares ranged from 5-6 (Body Condition Score Card; Kentucky Equine Research, USA), and mean age was 10.71 ± 0.47 (4–17 years). All mares were kept in individual stalls (3.5 m × 3.5 m) and were allowed to exercise in the paddocks during the day (8–9 h on average). They were fed 3.5–4 kg of mixed feed (50 kg of oats soaked five hours before feeding, then 15 kg of barley paste and 12 kg of soybeans added) and roughage (alfalfa and meadow grass) three times a day (at 06:00, 15:00, and 23:00) and received *ad libitum* fresh water.

Blood was drawn from the pregnant mares initially on days 14-16 after ovulation and then every month of gestation until delivery or loss of pregnancy (embryonic loss, abortion, premature placental abruption, or preterm delivery). The mares were divided into two groups according to their pregnancy status: the parturient group (group I; n = 42) and the pregnancy loss/abortion group (group II; n = 24). Two of the mares in group I and four of the mares in group II were maiden mares. The lactation status and birth numbers of the mares in previous years are shown in Table 1. The placenta of aborted mares were examined for its shape and inverted to look inside the allantoic surface of the chorioallantois to examine for any lesions.

Table 1: Number of previous births and lactation information of mares.

No.	NPB	L	No.	NPB	L	No.	NPB	L
#1	1	+	#23	2	-	#45	7	+
#2	2	+	#24	4	+	#46	1	+
#3	2	+	#25	11	+	#47	6	+
#4	3	-	#26	2	-	#48	6	+
#5	5	+	#27	0	-	#49	2	+
#6	1	+	#28	4	+	#50	1	+
#7	2	+	#29	9	+	#51	3	+
#8	6	+	#30	7	+	#52	3	-
#9	3	+	#31	7	-	#53	10	+
#10	1	-	#32	1	+	#54	2	+
#11	6	+	#33	6	+	#55	9	-
#12	4	+	#34	5	+	#56	0	-
#13	7	+	#35	3	+	#57	7	+
#14	9	+	#36	7	+	#58	0	-
#15	3	+	#37	0	-	#59	7	+
#16	5	+	#38	1	+	#60	3	+
#17	12	+	#39	8	+	#61	1	+
#18	4	+	#40	3	+	#62	1	+
#19	0	-	#41	3	-	#63	2	+
#20	7	-	#42	8	+	#64	3	-
#21	2	+	#43	2	+	#65	7	+
#22	2	+	#44	0	-	#66	7	+

L: Lactation status, NPB: Number of Previous Births.

Pregnancy detection

Pregnancy diagnosis was performed on days 14-16 after ovulation by transrectal ultrasonography[1] with a linear probe at a frequency of 7.5 Mhz in B-mode. Mares were examined once a month for pregnancy by transrectal ultrasonography. Mares with early embryonic loss in the group II were pregnant at the first control examination (K1), but empty 24-26 days after ovulation at the second control examination (K2).

Blood sampling and storage

After the jugular area of the mares was disinfected, blood was collected using a Vacutainer blood collection tube[2]. Samples were transported from the stud to the laboratory at 2-4°C in an automatic refrigerator with a digital display[3]. After centrifugation[4] at 1814 *g* at 4°C for 10 min, serum was aliquoted and stored in a -86°C freezer[5] until analysis.

MSAFP Assays

MSAFP assays were quantified using the enzyme-linked immunosorbent assay (ELISA). First, serum samples were thawed at room temperature (17–20 °C). Samples were processed in the Thermo Scientific Multiskan FC Microplate Photometer ELISA[6] instrument in the laboratory of the Department of Medical Biochemistry, Faculty of Medicine, Bezmialem Foundation University. A phosphate-buffered saline tablet[7] was diluted with 200 mL of deionized water at room temperature (pH: 7.2), and a 380 µL PBS + 20 µL mixture of thawed serum samples was added to each Eppendorf before the process. Serum alpha-fetoprotein concentrations were determined using equine antibodies against alpha-fetoprotein[8]. The sensitivity of the assay was 6.25–400 pg/mL for alpha-fetoprotein, intra-assay coefficients of variation were < 10%, and inter-assay coefficients of variation were < 12%.

Determination of colostrum quality

Colostrum quality of each mare was evaluated using the Brix refractometer[9] to measure IgG content in colostrum immediately after foaling, before the foal was suckled. Briefly, colostrum samples (≈1 mL) were collected from mares after foaling, after the udders had been cleaned with warm water. After pouring out the first milking volumes, a drop of the colostrum sample was placed on the glass surface of the Brix refractometer so that it could spread without air bubbles, and the lid of the chamber was closed. Then the value in the refractometer was read and recorded by holding it to the light source [6].

Statistical analysis

In cases where independent data (for $n > 30$) had a normal distribution, a t test (independent samples t test) was used to compare independent groups. The Mann-Whitney U test was used to compare independent groups when it was determined that the data were independent (for $n > 30$) and did not have a normal distribution. The Pearson correlation test was used to examine the relationships between the parameters assessed. To investigate the relationship between the mean MSAFP values in the samples of group I and group II mares during pregnancy, a normality test was performed for $n = 66$ mares. The significance level was accepted as $P < 0.05$ (IBM Statistics for Windows[10], Version 25.0).

RESULTS

Clinical Data

All group I mares finished their pregnancies and gave birth to healthy foals within the 2020 birth season. The mares gave birth in March (n = 8), April (n = 19), May (n = 14), and June (n = 1). IgG concentrations in the mares' colostrum ranged from 13-32% as measured by Brix refractometer. Pregnancy pathologies in the group II are listed in Table 2. Mare #64 had an abortion in the 6th month of gestation with an anencephalic fetus (Figure 1). Mare # 65 gave birth prematurely with a red sac (Figure 2). Macroscopic examination of the placenta of aborted mares showed no abnormalities (all were in normal shape and had no lesions on the chorioallantoic surface) and were not sent to the laboratory for further examination (i.e. histopathology and microbiological evaluations).

Table 2. Diagnostic information of group II mares.

No	Diagnosis	No	Diagnosis
#37	Premature Birth	#55	Embryonic Loss
#44	Embryonic Loss	#56	Abortus at 2nd month
#45	Embryonic Loss	#57	Abortus at 3rd month
#46	Embryonic Loss	#58	Abortus at 2nd month
#47	Embryonic Loss	#59	Abortus at 5th month
#48	Embryonic Loss	#60	Abortus at 5th month
#49	Embryonic Loss	#61	Abortus at 5th month
#50	Abortus at 2nd month	#62	Abortus at 6th month
#51	Embryonic Loss	#63	Abortus at 6th month
#52	Embryonic Loss	#64	Abortus at 6th month
#53	Embryonic Loss	#65	Premature Birth*
#54	Embryonic Loss	#66	Premature Placental Seperation*

*Red bag delivery.

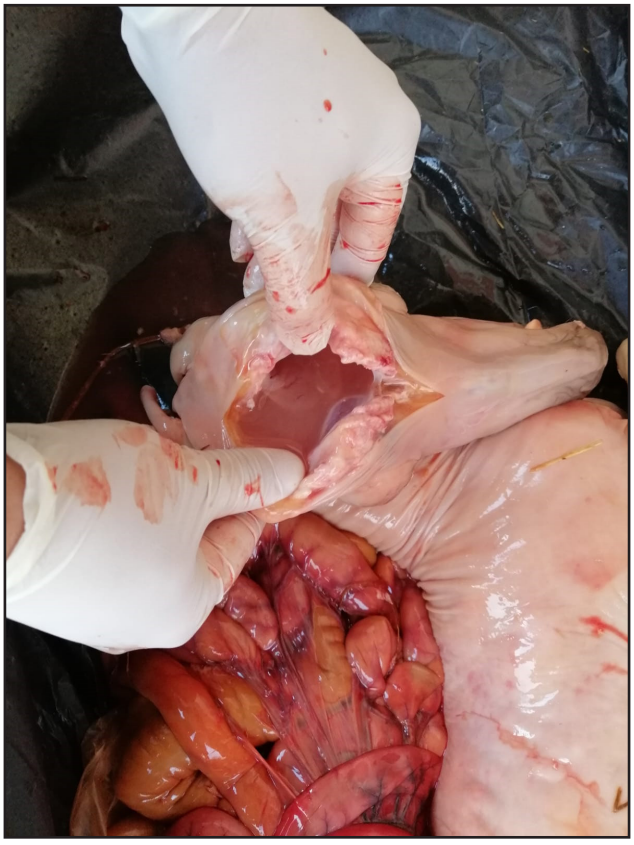

Figure 1. Mare #64 had an abortion in the 6th month of gestation with an anencephalic fetus.

Figure 2. Mare # 65 gave birth prematurely with a red bag.

MSAFP Concentrations

No correlation was found between mare age and MSAFP concentration ($P > 0.05$). In group I, the mean MSAFP concentration in the first trimester of gestation (from the first sample to 4 months) was 41.61 ± 1.89 pg/mL, in the second trimester of gestation (5–8 months) 54.04 ± 2.15 pg/mL, and in the third trimester of gestation (9–11 months) 44.39 ± 2.25 pg/mL. MSAFP concentrations were higher at 1st month in group II mares (n = 5) with early embryonic loss than in group I mares (n = 42) ($P = 0.045$). In addition, mean MSAFP values at 2-month pregnancies in group II were higher in mares (n = 3) with early fetal death than in group I (n = 42) ($P = 0.002$). While the mean MSAFP concentration in one mare (♯57) that suffered an abortion in the 3rd month of gestation was 94.06 pg/mL in the group II, it was quite high compared with the MSAFP concentration in group I (42.33 pg/mL). The mean MSAFP concentration of the preterm mares in group II (n = 2) was significantly higher than the mean MSAFP concentration at 9 months of pregnancy of the mares in group I ($P = 0.002$). The mean MSAFP concentration of a mare in the group II with premature placental abruption was 70.87 pg/mL. Both the MSAFP concentration at the 10th month (55.95 pg/mL) and the mean MSAFP concentration (70.87 pg/mL) of a mare in group II with premature placental abruption were higher than the mean MSAFP concentration of group I (47.51 pg/mL) and the MSAFP concentration at the 10th month (43.88 pg/mL) in group I. The MSAFP concentration of a mare (#64) who had an anencephalic fetus abortion at 6 months of gestation is given in Figure 3.

MSAFP Comparisons

The difference in MSAFP values between group I and group II is $P = 0.001$ (46.78 ± 0.87 pg/mL, 70.69 ± 3.86 pg/mL, respectively).

Mean MSAFP concentrations (26.54 ± 0.88 pg/mL) 14-16 days after ovulation were lower in mares conceived in the spring than in mares conceived in the summer (40.44 ± 3.88 pg/mL) ($P = 0.0005$).

Mean MSAFP concentrations (32.41 pg/mL) of maiden mares throughout pregnancy were lower than the mean MSAFP concentrations (47.22 ± 1.78 pg/mL) of the multiparous mares in group I.

Some of the mares in group I had had foals in the previous season and were lactating (n = 33), while

others were non-lactating mares (n = 9). There was no difference between MSAFP concentrations in the first trimester of pregnancy, at the time of separation from foaling (6 months after foaling), and throughout the gestation period in lactating and non-lactating mares ($P > 0.05$). No correlation was found between MSAFP concentrations and colostrum quality.

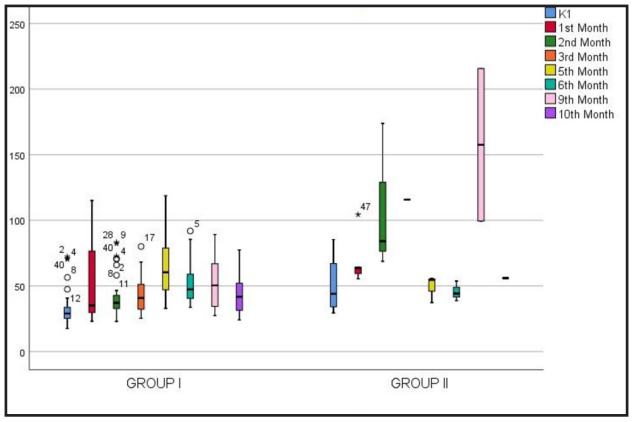

Figure 5. Monthly comparison of mean Maternal Serum Alpha-Fetoprotein concentrations (MSAFP) of the mares in group I (n = 42) and group II (n = 24) .

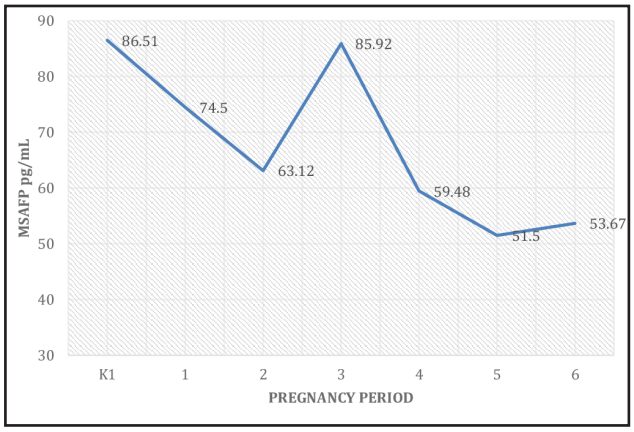

Figure 3. Maternal Serum Alpha-Fetoprotein (MSAFP) concentration of a mare (#64) with an anencephalic fetus anomaly.

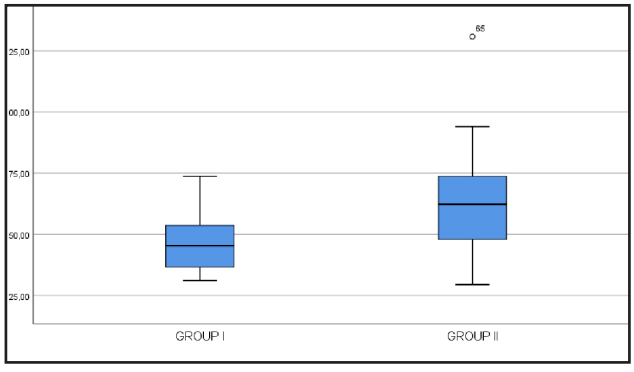

Figure 4. Statistical difference of mean Maternal Serum Alpha-Fetoprotein concentrations (MSAFP) of the mares in group I (n = 42) and group II (n = 24).

Foals were born in the 2020 birth season; 15 (36%) foals were female and 27 (64%) were male. The mean MSAFP pregnancy value for mares that gave birth to male foals was 45.25 ± 2.19 pg/mL, and for mares that gave birth to female foals was 48.79 ± 2.82 pg/mL ($P > 0.05$). Foals weighed between 43-62 kg and the mean birth weight was 51.88 ± 0.73 kg. There was no correlation between foal weight and mean MSAFP concentration throughout gestation, the last trimester of gestation, and the last month of gestation ($P > 0.05$).

DISCUSSION

In human medicine, alpha-fetoprotein is used as a screening test for early detection of pregnancy pathologies in women and as a tumor marker in cancer patients [12,21,25]. Altough, MSAFP concentrations were found to be higher in pregnancy-related pathologies (i.e. embryonic loss, abortion, preterm delivery, and premature placental abruption) and this result is consistent with other studies [4,22] the mean MSAFP concentration in pregnancy-related pathologies in our study was lower (70.69 ± 3.86 pg/mL) than that in another study (152 ± 36.48 pg/mL) [22]. One study [7] emphasized that the breed factor should be considered when interpreting MSAFP values. There were differences in MSAFP values between black and white women; black women's MSAFP values were 10% higher than white women per week of gestation [7]. In previous studies [22-24] related horse breeds were European and Lipizzan warm-blooded, whereas in our study Thoroughbred belonged to hot-blooded breeds. As the authors, we consider that this difference in MSAFP concentrations between our study results and those of other studies may be due to the different horse breeds in the studies. In the future, more studies should be conducted with different MSAFP test kits and different horse breeds to minimize any MSAFP variations.

Two studies found a negative correlation between MSAFP and mare's age and gestational age [22,24], but no correlation was found between foal sex, birth weight, chest girth, and leg girth and MSAFP score ($P > 0.05$). In our study, however, no positive or negative correlation was found between the mare's age, gestational age, foal gender and birth weight and MSAFP values. In a study [24], seasons were found to have an effect on MSAFP value in

mares, and MSAFP value was higher in summer than in spring (P = 0.0378), and MSAFP value in the last month of gestation was higher than in the previous ones (P = 0.0117). Because the last months of gestation of the mares in our study coincided with the spring season, the relationship between MSAFP and the season of parturition could not be evaluated in our study. There was no correlation between MSAFP values and colostrum quality in group I (P > 0.05). No study comparing colostrum quality with MSAFP concentrations was found in the literature search conducted until the results of this study were determined. In our opinion, MSAFP has no effect on immune components in milk. AFP is synthesized by the fetal liver, released into the fetal blood, crosses the placenta, and enters the maternal circulation. This circulating AFP is elevated in some pregnancy-related pathologies in women [17,18], which is probably due to leakage from the placenta under these conditions. AFP is also elevated in twin pregnancies, embryonic loss, plasentitis and abortions in horses [4,5,9,22-24]. In our study, MSAFP concentrations were found to be higher in mares in the group II with embryonic losses, abortions (one anencephalic anomaly), and preterm births compared to group I mares with healthy pregnancies. An increase in MSAFP concentrations in pregnancy-related pathologies in our study is consistent with other studies, although placental abruption and preterm delivery were not observed in these studies [4,9,22,24]. In addition, the high MSAFP value (86.51 pg/mL) in mare #64 at the beginning of our study (K1 sampling) shows us that anencephalic anomaly can be found at the very early stages of pregnancy and this will enable termination of these pregnancies. Characteristic of high AFP in early pregnancy was used in human pregnancies before for the antenatal diagnosis of anencephaly [3].

In conclusion, with this study, MSAFP assessments were made at each month during pregnancy in pregnant thoroughbred mares, and these data will provide a clearer understanding of the scientific and clinical value of MSAFP in gestation in mares. By measuring MSAFP, information can be obtained about the state of pregnancy before embryonic loss, early and late abortions, preterm delivery, or early placental abruption occurs. In the authors' assessment, high-risk pregnancies can be easily diagnosed and terminated if mares' weekly MSAFP measurements are monitored during pregnancy, thereby improving mares' reproductive performance.

MANUFACTURERS

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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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