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Effects of lactation period on some blood parameters and ANAE/AcP-ase enzyme activities in domestic Donkeys

Efectos del período de lactancia sobre algunos parámetros sanguíneos y actividades de la enzima ANAE/AcP-asa en asnos domésticos

***Mehmet Faruk Aydin*[1,*], *İhsan Kisadere*[2]**

[1]*Department of Histology and Embryology, Faculty of Veterinary Medicine, Balıkesir University, Balıkesir 10050, Türkiye*
[2]*Department of Physiology, Faculty of Veterinary Medicine, Balıkesir University, Balıkesir 10050, Türkiye*
**Corresponding Author: mfaydin@balikesir.edu.tr.*

ABSTRACT

It was aimed to research the influences of the lactation period on some hematological (red blood cells, white blood cells, and its subtypes, hematocrit, hemoglobin, mean erythrocyte hemoglobin, mean corpuscular hemoglobin concentration, mean erythrocyte volume, and blood clot count) and biochemical parameters (alanine amino transferase, aspartate amino transferase, gamma glutamyl transferase, blood urea nitrogen, and creatinine) as well as α - naphthyl acetate esterase and activity of the enzymes acid phosphatase positive lymphocytes percentages in domestic donkeys. For this purpose, 20 female donkeys were selected and divided into two equal groups as control (non-lactating, n = 10) and lactating (n = 10). They were in the middle of lactation (three to six months old), ranging in age from five to fourteen. Following the routine clinical examination, blood samples were taken from the animals in order to measure the activities of the α - naphthyl acetate esterase / activity of the enzymes acid phosphatase enzyme as well as several blood parameters. As a result, red blood cells, hematocrit, and hemoglobin values were higher in lactating donkeys than non-lactating donkeys ($P < 0.05$). Serum aspartate amino transferase and gamma glutamyl transferase values were found to be statistically higher in lactating animals ($P < 0.05$). Although enzyme activities were high in lactating donkeys, α - naphthyl acetate esterase and activity of the enzymes acid phosphatase positive lymphocyte percentages were found to be similar between the groups ($P > 0.05$). In conclusion, the middle-lactation phase in female donkeys was found to have an impact on several blood parameters, but not on the activity of enzymes.

Key words: biochemistry; Equus asinus; hematology; lactation

RESUMEN

El objetivo fue investigar la influencia del período de lactancia en algunos parámetros hematológicos (glóbulos rojos, leucocitos y sus subtipos, hematocrito, hemoglobina, hemoglobina media, hemoglobina media, volumen volumétrico medio y plaquetas) y bioquímicos (alanina aminotransferasa, nitrógeno ureico en sangre y creatinina sérica), así como en el porcentaje de linfocitos positivos para en asnos hembras domésticas. Para ello, se seleccionaron 20 burras y se dividieron en dos grupos iguales: control (no lactantes, n = 10) y lactantes (n = 10). Se encontraban en la mitad de su período de lactancia (de tres a seis meses de edad), con edades comprendidas entre los cinco y los catorce años. Tras el examen clínico de rutina, se tomaron muestras de sangre de las animales para medir la actividad de la enzima, así como diversos parámetros sanguíneos. Como resultado, los valores de glóbulo rojo, hematocrito y hemoglobina fueron mayores en las asnos hembras lactantes que en las no lactantes ($P < 0,05$). Se observó que los valores séricos de fueron estadísticamente superiores en animales lactantes ($P < 0,05$). Si bien la actividad enzimática fue alta en asnos hembras lactantes, los porcentajes de linfocitos positivos para fueron similares entre los grupos ($P > 0,05$). En conclusión, se observó que la fase media de la lactancia en asnos hembras influyó en varios parámetros sanguíneos, pero no en la actividad enzimática.

Palabras clave: Bioquímica; Equus asinus; hematología; lactancia

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INTRODUCTION

Historical processes indicate that humans domesticated donkeys (*Equus asinus*) about 5,000 years ago. The donkey is one of the domesticated animals of the equine family, as is widely known. They have been significant throughout human history and all phases of evolution [1].

Donkeys were mostly used as load carriers and as riding animals in the past. The growing popularity of donkeys is attributed to the possible health benefits of donkey milk, even though they are still utilized for onotherapy, sports, labor, and rural transportation [2 , 3]. Their population has increased globally as a result of this phenomenon, particularly in Asia and Europe. Donkey meat is eaten by humans like other meats in several regions of the world, particularly in Asian societies [4 , 5].

Nearly all female mammals, including horses and donkeys, nurse their offspring after parturition by secreting milk from their mammary glands. This period is called as lactation. In general, the lactation period is divided into three periods, especially for donkeys and horses, as early, middle, and late lactation [6]. Previous studies on donkeys and horses have showen that the lactation period significantly affects many physiological, hematological, and biochemical parameters [7 , 8 , 9]. The primary causes of these changes can be identified as the mother's (a mule or female donkey) increased energy requirements during nursing, hormonal and immunological changes, stress, and environmental factors [10].

The health condition of the donkeys and also horses (alongside other animals) is frequently evaluated by using hematological markers. These markers help Veterinaries, specialists, and also researchers to diagnosis and prognosis of many equine diseases. The count of white blood cells (WBC) and its subtypes, hemoglobin (HGB) concentration, red blood cells count (RBC), hematocrit (HCT) value, mean erythrocyte volume (MCV), mean erythrocyte hemoglobin (MCH) and its concentration (MCHC), and blood clot count (PLT) are most frequently used hematological parameters in this area. Furthermore, a wide range of variables including species status, age, breed, diet, sex, disease, and seasonal fluctuations have been documented to have an impact on the pattern of these parameters [11 , 12].

In both clinical treatment and fundamental research, the evaluation of an animal's status can be facilitated by measuring its blood biochemical characteristics. Variations in blood biochemical markers are a useful tool for evaluating the health, welfare, and stress level of an animal since they are frequently suggestive of alterations in the animal's physiological state (e.g., gestation, parturition, and lactation). In this sense, it is critical to determine some enzyme levels to reveal the general condition of the liver and kidneys, especially in a different metabolic phenomenon such as lactation [13]. Gamma glutamyl transferase (GGT), blood urea nitrogen (BUN), alanine amino transferase (ALT), aspartate amino transferase (AST), and creatinine (CREAT) can be given as samples of these important enzyme types [14].

Based on the data gathered from earlier research, using enzyme histochemical techniques seems to be an easy and affordable process. The main purpose of enzyme histochemical techniques is to have crucial data regarding the identity, location, and quantity of an enzyme. Determining the activity of the enzymes acid phosphatase (AcP - ase) and α - Naphthyl acetate esterase (ANAE) is one of these important histochemical techniques. The primary purpose of these techniques is to categorize lymphocyte subsets. According to reports, ANAE appears later in the thymus during the T - lymphocyte maturation phase, while AcP - ase occurs early on [15 , 16].

Naphthyl acetate esterase belongs to the nonspecific esterase family, which is extensively present in different kinds of cells. According to research, both adult and immature T lymphocytes exhibit ANAE, a lymphocyte lysosomal enzyme that is specifically localized in lysosome membranes. Numerous research on the topic has highlighted how crucial ANAE is for differentiating peripheral blood samples, particularly those containing T and B cells and monocytes [16 , 17].

Moreover, AcP - ase, like ANAE, is a lysosomal enzyme. For many cell types, especially mammalian T-lymphocytes, this enzyme is unique. It has also been noted in earlier research that fetal thymocytes exhibit AcP - ase enzyme activity [18]. Important illnesses, including T cell lymphoproliferation, have also been diagnosed with this technic [19]. According to earlier research, a variety of physical and metabolic activities can influence the activities of the ANAE and AcP - ase enzymes in living organisms including donkeys and horses. The rates of these indicators have also been found to be influenced by species, age, sex, and stress levels [11 , 12 , 20 , 21].

The purpose of the study was to examine how the lactation period affected the percentages of ANAE and AcP-ase positive lymphocytes as well as certain hematological (RBC, WBC, HCT, HGB, MCH, MCHC, MCV, and PLT) and biochemical (ALT, AST, GGT, BUN, and CREAT) parameters in domestic donkeys.

MATERIAL AND METHODS

Animals and sample collection

Twenty female donkeys were chosen as animal material and split into two equal groups: 10 lactating (n = 10) and 10 non-lactating (control, n = 10). Animals were from 5 to 14 years old, and into the middle lactation period (~ 3 - 6 months). Donkeys were housed at the Aegean Donkey Farm in Edremit District of Balikesir Province.

They were partially grazed and fed *ad libitum* with hay produced on the farm. No mineral or vitamin supplements were given during the study. Before the applications, general clinical examination techniques were utilized to identify the healthy state, and the animals did not exhibit any abnormal symptoms. A 1.2 mm × 38 mm needle was used to draw blood samples from the jugular vein into both heparinized and non-heparinized tubes. Then, the samples were quickly moved by the cold chain to the laboratory for analysis. For every animal, four smears were done by using blood samples [ANAE (2) and AcP - ase (2) stainings].

Hematological parameters

Abacus Junior Vet - 5 (Vienna, Austria) hematology analyzer was used to identify parameters (WBC and its subtypes, HGB, RBC, PLT, MCV, HCT, MCH, and MCHC) in female donkeys.

Biochemical parameters

Using an automated biochemical analyzer (BS - 400 PLUS, Mindray, China), the levels of serum ALT, AST, GGT, BUN, and CREAT were determined.

α - Naphthyl acetate esterase activity

Naphthyl acetate esterase was demonstrated with some modifications utilizing Aydin's approach no mineral or vitamin supplements were given during the study [12]. The produced smears were first fixed for three min at - 10 °C in a glutaraldehyde acetone solution. They were then air-dried at room temperature (24 °C) after being cleaned three times in distilled water. In order to prepare the ANAE solution, 0.8 mL of acetone (Merck, Germany) was used to dissolve 20 mg of α – naphthyl - acetate (N - 8505; Sigma, Germany). The afore mentioned solution was then supplemented with 4.8 mL of hexazotized pararosaniline.

Subsequently, 80 mL of buffered phosphate saline (pH = 5) was filled with dissolved α-naphthyl-acetate and hexazotized pararosaniline mix solution. After adding 1 N NaOH to the produced ANAE solution, the pH was finally adjusted to 5.8 and filtered. Following a 2 - hour (h) incubation period at 37 °C, the produced smears underwent three rinses with distilled water. Then, leukocyte nuclei were stained for 20 min using 1 % methyl green (Sigma, Germany) that had been produced in an acetate buffer with a pH of 4.2.

enzymes acid phosphatase activity

With minor modifications, AcP - ase activity was demonstrated utilizing Donmez and Sur's [16] methodology. The ready samples were fixed for ten min at 4 °C in formal - Ca. They were then cleaned with distilled water. The AcP - ase solution was made as follows: 1 mL of N,N - dimethyl formamide (Sigma, Germany), 13 mL of distilled water, 1.6 mL of hexazotized pararosaniline, and 5 mL of veronal acetate buffer (Michaelin's, pH = 5) were used to dissolve 10 mg of naphthol AS-BI phosphate (N-2125, Sigma, Germany).

The produced AcP - ase solution was filtered, and its pH was adjusted to five using one N sodium hydroxide. Following a 1 - h incubation period at 37 °C, the blood smears underwent three rounds of rinsing with distilled water. Leukocyte nuclei were then stained for 20 min with 1 % methyl green (Sigma, Germany) in acetate buffer (pH = 4.2).

Using a light microscope (Leica DM 2500, Wetzlar, Germany), all smear samples were examined. Two hundred lymphocytes were counted in each of the prepared smears showing ANAE and AcP - ase positives, and the percentage of cells exhibiting an enzyme-positive lymphocyte ratio was announced.

Statistical analysis

The SPSS 22.0 software was utilized to analyze the differences between the groups by an independent samples T-test. The suitability of the data for normal distribution was examined with the Shapiro - Wilk test. Since the data regarding ANAE and AcP enzyme activities did not comply with normal distribution, the Mann - Whitney U test was used to compare the two groups. (SPSS, Inc, Chicago, IL). P values less than 0.05 were regarded as significant.

RESULTS AND DISCUSSIONS

Hematological parameters

It was not detected any statistical alterations between groups according to WBC, lymphocyte (lym), monocyte (mon), and granulocyte (gran) counts in this study ($P > 0.05$). In addition, lym, mon, and gran percentages were not affected from the lactation period in the present study ($P > 0.05$). Conversely, HGB, HCT, and RBC values were higher in lactating donkeys than non-lactating donkeys ($P < 0.05$). Other hematological parameters such as MCV, MCH, MCHC, and PLT also were not influenced by the lactation period in the present study ($P > 0.05$), as shown in TABLE I.

TABLE I
Hematological parameters in different (lactating / non-lactating) donkey groups

Parameters / Groups		n	Mean ± SE
WBC (10^9 / L)	lactation	10	11.14 ± 1.32
	control	10	14.17 ± 1.95
lym (%)	lactation	10	38.22 ± 3.78
	control	10	39.68 ± 3.00
mon (%)	lactation	10	5.18 ± 0.58
	control	10	4.56 ± 0.37
gran (%)	lactation	10	56.60 ± 4.00
	control	10	55.76 ± 3.03
lym (#)	lactation	10	4.30 ± 0.68
	control	10	5.77 ± 1.03
mon (#)	lactation	10	0.54 ± 0.09
	control	10	0.58 ± 0.05
gran (#)	lactation	10	6.30 ± 0.79
	control	10	7.79 ± 1.07
RBC (10^{12} / L)	lactation	10	7.20 ± 1.14**a**
	control	10	5.69 ± 0.63**b**
MCV(fL)	lactation	10	70.25 ± 1.24
	control	10	68.14 ± 1.22
HCT (%)	lactation	10	50.04 ± 7.76**a**
	control	10	39.03 ± 4.78**b**
MCH(pg)	lactation	10	19.13 ± 0.33
	control	10	17.93 ± 0.31
MCHC(pg)	lactation	10	27.32 ± 0.31
	control	10	26.39 ± 0.25
HGB (g / dL)	lactation	10	13.89 ± 2.31**a**
	control	10	10.35 ± 1.30**b**
PLT (10^9 / L)	lactation	10	110.70 ± 13.03
	control	10	150.00 ± 23.23

[a-b]Values within a column with no common superscripts are different, $P < 0.05$. U/L: Units per liter, mg: milligram, dL: deciliter, fL: femtoliters, (g/L): grams per liter, pg: picograms, (%): percentage, (#): count, WBC: White blood cell, Lym: Lymphocyte, Neu: Neutrophil, Mono: Monocyte, Eos: Eosinophil, RBC: Red blood cell, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: Platelet count, SE: Standart Error

Biochemical parameters

In this study, there is no difference was determined when serum ALT values were compared between groups (P > 0.05). On the other hand, lactating animals had statistically increased serum AST and GGT values (P < 0.05). BUN and CREAT values of the donkeys were also not affected by the lactation period in the present study (P > 0.05), as shown in TABLE II.

TABLE II
Biochemical parameters in different (lactating / non-lactating) donkey groups

Parameters / Groups		n	Mean ± SE
ALT (**U / L**)	lactation	10	7.45 ± 0.05
	control	10	7.32 ± 0.29
AST (**U / L**)	lactation	10	176.33 ± 11.97**a**
	control	10	142.60 ± 7.34**b**
GGT (**U / L**)	lactation	10	19.19 ± 1.45**a**
	control	10	14.04 ± 1.41**b**
BUN (**mg / dL**)	lactation	10	37.53 ± 2.01
	control	10	35.51 ± 2.69
CREAT (µmol / L)	lactation	10	58.21 ± 2.87
	control	10	57.78 ± 3.39

[a-b]Values within a column with no common superscripts are different, P < 0.05. ALT: alanine amino transferase, AST: aspartate amino transferase, GGT: Gamma glutamyl transferase, BUN: blood urea nitrogen, CREAT: creatinine, U/L: Units per liter, mg: milligram, dL: deciliter, µmol: micromol, SE: Standart Error

α-Naphthyl acetate esterase and enzymes acid phosphatase positive lymphocyte percentages

Lactating donkeys had elevated enzyme activity, although the percentages of ANAE and AcP-ase-positive lymphocytes were similar among the groups. They were also not influenced by the lactation period in donkeys (P > 0.05), as shown in TABLE III. In addition, ANAE and AcP - ase positive lymphocytes were presented in FIGS. 1, 2, 3, 4.

TABLE III
ANAE and AcP - ase enzyme activities in different (lactating / non - lactating) donkey groups

Parameters / Groups		n	Mean ± SE
ANAE	lactation	10	74.17 ± 1.05
	control	10	73.04 ± 1.18
AcP - ase	lactation	10	65.13 ± 1,59
	control	10	62.91 ± 1.89

ANAE: α - naphthyl acetate esterase, AcP – ase: acid phosphatase enzyme, SE: Standart error

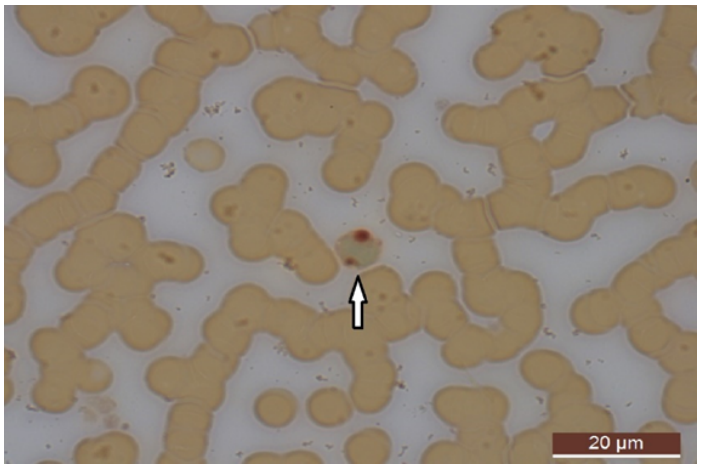

FIGURE 1. α - Naphthyl acetate esterase positive lymphocyte in non - lactating donkeys. Arrow: ANAE positive lymphocyte

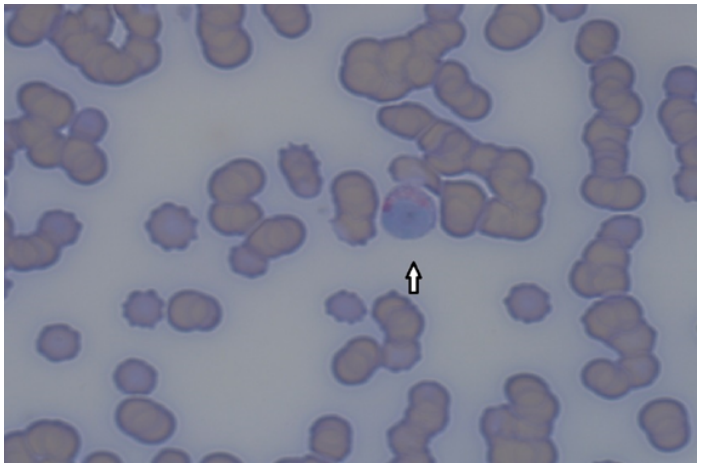

FIGURE 2. Acid phosphatase positive lymphocyte in non - lactating donkeys. Arrow: AcP positive lymphocyte

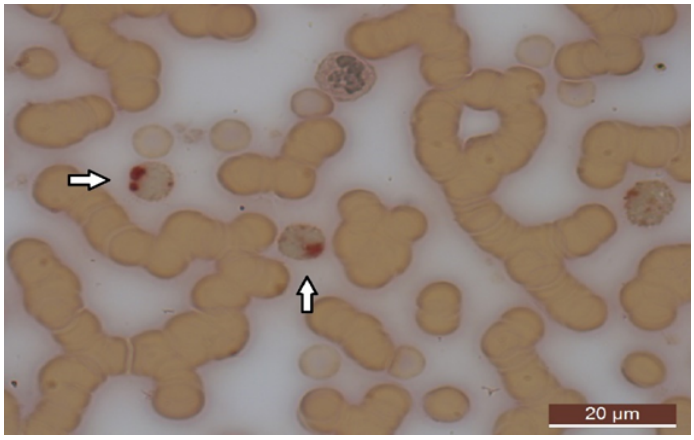

FIGURE 3. α - Naphthyl acetate esterase positive lymphocytes in lactating donkeys. Arrow: ANAE positive lymphocytes

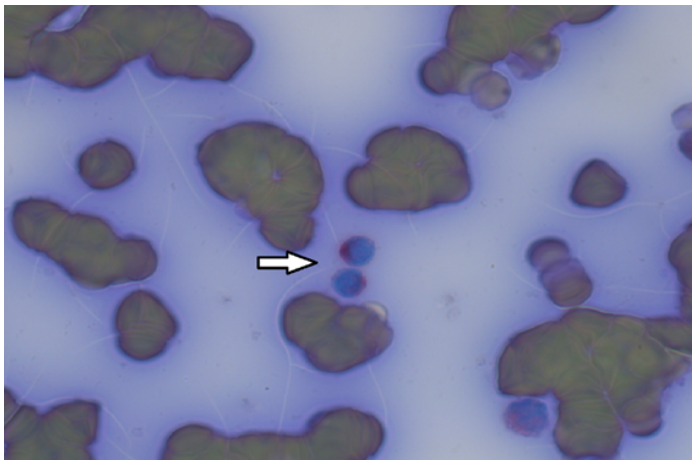

FIGURE 4. Acid phosphatase positive lymphocyte in lactating donkeys. Arrow : AcP positive lymphocyte

Assessing hematological parameters is a useful step toward figuring out a living being's overall health. In the present study, WBC counts were not influenced by the middle lactation period in donkeys. Besides**,** the number and percentages of WBC subgroups (lym, mon, and gran) were also found to be similar between both lactating and non-lactating donkeys.

On the other hand, Gul *et al*. [6] suggested that WBC counts increased due to the lactation period in donkeys. In addition, neutrophil and eosinophil percentages decreased, however mon, lym, and basophil percentages increased in non-lactating donkeys compared to lactating donkeys in the same research. Dezzutto *et al*. [8] also wanted to evaluate the changes in the hematological, electro-phoretic, and hemato-chemical parameters in different lactating periods of donkeys. As a result, they revealed that the WBC count was higher in early lactation than in middle and late lactation, and the rate of WBC subgroups (lym, mon, and gran) was not affected by different lactation periods which was consistent with this study's results.

Following earlier research on mares, Bonelli *et al.* [9] also found that donkeys had a higher WBC count at foaling than throughout late pregnancy and lactation [22 , 23]. Salari *et al*. [7] suggested that the three phases of the lactation period (early, middle, and late) did not affect the WBC counts in lactating donkeys. Hormonal fluctuations (especially the release of cortisol and catecholamine) occurring during different periods of lactation may have caused these different results [24].

Although the RBC count was found to be high in lactating donkeys, no change was observed between the groups regarding MCV, MCHC, and MCH values in this study. Gul *et al*. [6] demonstrated the decreased RBC counts in lactating donkeys when compared to non-lactating. In a previous study, Dezzutto *et al*. [8] determined that RBC and MCHC values were not affected by different lactation periods in donkeys, but MCH values were found the highest in the early lactation period. Salari *et al*. [7] also reported that RBC and MCV values were not influenced by the three phases of the lactation, but MCH and MCHC values tended to increase in the second phase (middle) of lactation in donkeys.

In another study, results for MCHC, MCV, and MCH showed a constant trend over different periods of lactation in donkeys, as reported for mares [25]. Furthermore, HGB values were determined higher in lactating donkeys than non-lactating ones in this study. These results were consistent with a previous study which was conducted by Gul *et al*. [6].

In the previously conducted limited studies on this subject, it was determined that HGB values did not alter at any stage of lactation in donkeys [7 , 8 , 9]. Different environmental conditions (altitude), ration, or lactation period may have caused different results. PTL values also did not change due to the lactation period in donkeys in the present study. Dezzutto *et al*. [8] also stated that PLT values were not affected by any type of lactation period in donkeys.

According to Bonelli *et al*. [9] and Salari *et al*. [7], the PLT values were higher in the middle of lactation than in other periods. These results were not compatible with previous studies on mares where PLT was constant over lactation time. Furthermore, Gul *et al*. [6] found higher fibrinogen values in non-lactating donkeys than lactating in a previous study. Prolactin is one of the main hormones that increases during pregnancy and lactation [26].

Furthermore, prolactin was acknowledged as a platelet coactivator in earlier research on the topic, where it improved ADP - induced platelet activation [27]. It is well known that until the final two weeks of pregnancy, mares' plasma prolactin concentrations are generally low. However, at this time, levels rise significantly as the mare's mammary gland develops and lactogenesis begins.

The last two weeks of pregnancy see an increase in plasma prolactin concentration, which stays high during the early stages of nursing and returns to normal 1 - 2 months following delivery [28]. It is believed that, among other things, variations in outcomes are especially influenced by this hormone's amount. In the literature studies, a limited number of studies were found on the relationships between PLT numbers and different periods of lactation in donkeys [13].

In the present study, while ALT values, better known as liver tissue enzymes, were not affected by lactation, AST and GGT values considerably increased in female donkeys. These obtained results were corresponding with Gul *et al.* [6] according to ALT and AST values. Dezzutto *et al.* [8] detected similar AST and GGT values in the different periods of lactation in donkeys.

Additionally, it was proposed by Bonelli *et al.* [9] that the GGT activity trend in donkeys was lower during lactation and at birth than the late gestation. Conversely, the AST activity trend was observed an increase near to parturition and early lactation in donkeys in the same research. These findings could be explained by the fact that the liver responds differently to various animal species, dietary regimens, and stages of lactation.

In this study, BUN and CREAT values, which are mostly used as kidney function tests, were not affected by the lactation period in donkeys. Also, Dezzutto *et al.* [8] showed that BUN and CREAT values were not affected by any lactation period in donkeys. Bonelli *et al.* [9] suggested that the amount of BUN increased near birth (2 weeks) and then remained constant during the birth and breastfeeding period in donkeys. Moreover,

CREAT values were determined stable, within normal ranges for adult species [22], throughout the study period in above mentioned research. The evaluation of this study's findings along with those of other research leads to the conclusion that the energy requirements associated with lactation do not affect on the BUN and CREAT levels of nursing female donkeys [22].

ANAE and AcP - ase enzyme activities (Two of the enzyme histochemical techniques) were found to be similar in both lactating and non-lactating donkeys in the present study. In a previous study, the mean ANAE - positive lymphocyte percentage of Kyrgyz donkeys was determined as 42.90 ± 1.18 % [11].

Besides, Krumrych *et al.* [29] detected the ANAE and AcP - ase enzyme activities in horses as 67 % and 27 %, respectively. Furthermore, Ozaydın *et al.* [30] demonstrated the mean ANAE positive peripheral blood lymphocytes (PBL) as 67.70, 73.10, and 49.20 % in female horses; 64.00, 70.53, and 50.60 % in males 1 d, 3 d, and 1 year old, respectively.

They also suggested the AcP - ase positive PBL as 27.33, 32.83, and 37.40 % in female horses; 29.67, 31.67, and 38.40% in males 1 d, 3 d, and 1 year old, respectively. In addition, Oruç *et al.* [31] determined the ANAE - positive lymphocyte rates (%) before and also after the race in jumping horses as 54.90 ± 1.60 % and 38.90 ± 1.20 %, respectively. Moreover, mean percentages of ANAE - positive PBL were found as 76 %, whereas mean percentages of AcP - positive PBL were detected as 28.46 % in Arabian horses by Aydin *et al.* [12].

Although ANAE - positive lymphocyte rates of donkeys have been found in literature studies, no information on the AcP activities of donkeys both in lactating and non - lactating has been found. Accordingly, we can express it as the first report. After analyzing the data, it may be suggested that the middle - lactation phase does not affect the activities of ANAE and AcP in female donkeys in particular.

CONCLUSION

In female donkeys, the middle lactation phase was observed to affect a number of blood parameters, but not the activity of enzymes (AcP and ANAE). Large-scale studies on this subject are required.

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Conflict of interest

The authors declared that there is no conflict of interest.

Ethics

The Balikesir University Experimental Animal Ethics Committee's Experimental Medicine Research and Application Center (Approval no: 2022 / 10 - 6) authorized all animal handling and procedures.

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