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Evaluation of Neutrophil to Lymphocyte Ratio, Lymphocyte to Monocyte Ratio, and Serum Iron Levels as an Inflammatory Marker in Calves with Diarrhea

Ömer AYDIN [1, a]

[1] Atatürk University, Faculty of Veterinary Medicine, Department of Internal Medicine, Erzurum, TÜRKİYE

[a] ORCİD: 0000-0001-9444-1904

This study aimed to investigate the neutrophil-lymphocyte ratio (NLR), lymphocyte-monocyte ratio (LMR), and serum iron (Fe) levels, which are hematological criteria, in calves with diarrhea, and to determine their relations with each other. The study material consisted of a total of 171 Brown Swiss, Simmental, and their crossbred female and male calves, aged 0-10 days. The study consisted of eight groups: control group (Group-1, n=15), *E. coli* group (Group-2, n=32), Coronavirus group (Group-3, n=27), Rotavirus group (Group-4, n=32), *Giardia* spp. group (Group-5, n=14), *Cryptosporidium* spp. group (Group-6, n=15), Rota-Coronavirus group (Group-7, n=21), and *E. coli*- Rotavirus group (Group-8, n=15). The total leukocyte count, neutrophil count, NLR, and Fe levels were found to be significantly different in the diarrhea groups compared to the control group. The NLR values of the *E. coli* group were higher than the other diarrhea groups and the control group, but the lymphocyte, monocyte, and Fe values were lower. It was concluded that the inflammatory level was higher in the *E. coli*-induced diarrhea group compared to the other diarrhea groups, and NLR, LMR, and Fe levels yielded significant values in determining the inflammatory status in diarrhoeic calves.

Key Words: *Calves diarrhea, lymphocyte/monocyte ratio, neutrophil/lymphocyte ratio, serum iron level*

İshalli Buzağılarda Nötrofil/Lenfosit Oranı, Lenfosit/Monosit Oranı ve Yangısal Marker Olarak Serum Demir Düzeylerinin Değerlendirilmesi

Bu çalışmada ishalli buzağılarda hematolojik kriter olan nötrofil-lenfosit oranı (NLO), lenfosit-monosit oranı (LMO) ve demir (Fe) düzeylerinin araştırılması ve birbirleriyle ilişkilerinin belirlenmesi amaçlandı. Çalışma materyalini 0-10 gün yaş arasında olan toplam 171 adet Brown Swiss, Simmental ve bunların melez dişi ve erkek buzağıları oluşturmuştur. Çalışma kontrol grubu (Grup-1, n=15), *E. coli* grubu (Grup-2, n=32), Coronavirüs grubu (Grup-3, n=27), Rotavirüs grubu (Grup-4, n=32), Giardia spp. grubu (Grup-5, n=14), *Cryptosporidium* spp. grubu (Grup-6, n=15), Rota-Coronavirus grubu (Grup-7, n=21), *E. coli*- Rotavirus grubu (Grup-8, n=15) olmak üzere sekiz gruptan oluştu. Total lökosit sayısı, nötrofil sayısı, NLO ve Fe düzeyleri ishal gruplarında kontrol grubuna göre anlamlı olarak farklı bulundu. *E. coli* grubunun NLO değerleri diğer ishal gruplarına ve kontrol grubuna göre yüksek, lenfosit, monosit ve Fe değerleri ise daha düşüktü. *E. coli* kaynaklı ishal grubunda inflamatuar düzeyin diğer ishal gruplarına göre daha yüksek olduğu ve ishalli buzağılarda inflamatuar durumun belirlenmesinde NLO, LMO ve Fe düzeylerinin anlamlı değerler verdiği sonucuna varıldı.

Anahtar Kelimeler: *Buzağı ishali, lenfosit/monosit oranı, nötrofil/lenfosit oranı, serum demir düzeyi*

Introduction

Neonatal calf diarrhea is a disease that affects calves in the pre-weaning period. This disease can result in death in many cases due to hypovolemia and acidosis in young calves (1). Neonatal calf diarrhea can be infectious and noninfectious. While non-infectious calf diarrhea occurs due to vitamin deficiencies and mistakes in milk-feeding, infectious diarrhea is caused by viral, bacterial, and protozoal pathogens (2).

Inflammation is a reaction to cellular or tissue damage. This reaction manifests itself by reducing or eliminating the effects of agents that damage the organism. Later, this inflammatory response contributes to the restructuring of damaged cells and tissues (3). Acute inflammation is a condition that may develop within hours or even minutes, depending on the intensity and type of tissue damage (4). Chronic inflammation is a type of response that occurs when the organism's response to acute inflammatory responses or autoimmune reactions is insufficient and lasts longer (weeks or even months) than acute inflammatory conditions. This type of inflammation is characterized by the activation of lymphocytes, macrophages, and other inflammatory cells (3).

The evaluation of hematological data is of great importance for cattle health. With the correct interpretation of hematological data, important information can be obtained both in terms of the early diagnosis of diseases and the prognosis (5). Neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR), mean platelet volume to platelet ratio (MPV/PLT) are obtained from hemogram analysis. It has been shown as a prognostic marker in many diseases such as NLR, PLR, MPV/PLT thromboembolic

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Yazışma Adresi
Correspondence

Ömer AYDIN
Atatürk University, Faculty of Veterinary Medicine, Department of Internal Medicine, Erzurum, TÜRKİYE

aydinomer@atauni.edu.tr

diseases, sepsis in human medicine (6-8). In veterinary medicine, NLR and PLR have mostly been evaluated in certain disease states. Lymphocyte-monocyte ratio (LMR) has been evaluated as a prognostic marker in cancer or lymphoproliferative diseases in human medicine (9). NLR has been extensively investigated in oncology in oropharyngeal tumors (10). In veterinary medicine, it has been stated that LMR shows a poor prognosis in high-grade lymphomas (11).

Iron (Fe) is an essential trace element for living organisms and bacterial pathogens, and also has important functions in many enzymatic reactions (12). Serum Fe concentration decreases rapidly in response to inflammation. It has been reported that this situation is resulted in by the retention of Fe, which is necessary for bacterial virulence and replication, for host defense (13). It has been reported that serum Fe concentration can be used as an acute inflammatory marker in different animal species (13-15).

In this study, it was aimed to determine the levels of NLR, LMR, and Fe in calves with diarrhea caused by different etiological pathogens and also to investigate the relationship between these hematological parameters and Fe.

Materials and Methods

Research and Publication Ethics: The study was approved by the Local Ethics Committee of Atatürk University (Ethics Committee Decision No: 2023/05).

Animals: The study material consisted of calves brought to Atatürk University Faculty of Veterinary Medicine, Animal Hospital with diarrhea and a total of 171 Brown Swiss, Simmental, and their crossbred female and male calves aged 0-10 days. The study consisted of eight groups: control group (Group-1, n=15) and *E. coli* group (Group-2, n=32), Coronavirus group (Group-3, n=27), Rotavirus group (Group-4, n=32), *Giardiasis* spp. group (Group-5, n=14), *Cryptosporidium* group (Group-6, n=15), Rota-Coronavirus group (Group-7, n=21), and *E. coli*- Rotavirus group (Group-8, n=15).

Recording of Clinical Findings: In the inclusion of calves with diarrhea in the study, fecal scoring, and clinical examination findings (body temperature, degree of dehydration) were determined according to the criteria determined by Larson et al. (16). Calves with excessive watery diarrhea, a body temperature above 39.3°C, and a dehydration degree of 8-10% were included in the study due to these parameters. In addition, in determining the severity of advanced dehydration, criteria such as advanced enophthalmos (6 mm and over), decreased cervical skin elasticity (7 s and above), white mucous membranes, cooling in the extremities, remaining in a lying position and inability to stand up were taken into account (17). Health status of the animals in the control group was confirmed by clinical examination, hematological finding based on hematological analysis (Abacus Junior Vet 5®, Hungary).

Sampling: Hematological analyses were performed by taking blood samples into K_2EDTA tubes (Vacutainer, K2E 3.6 mg, BD, UK) from the *vena jugularis* of the animals (Abacus Junior Vet 5®, Hungary), and the blood taken in serum tubes (Vacutainer, BD, UK) was kept at room temperature for 30 minutes. Afterward, serum tubes were +4 °C centrifuged at 3000 rpm for 10 minutes in a refrigerated centrifuge device (Bechman Coulter, USA) to obtain serum samples. The obtained serum samples were transferred to Eppendorf tubes and stored in a refrigerator at -80°C until further processing.

The etiological agents of diarrhea were determined by using a immunochromatographic test (BoviD-5 Ag Test Kit, Korea). This test detects Rotavirus Ag, Coronavirus Ag, *E. coli* K99, Cryptosporidium Ag, and Giardia Ag from the stool samples. Blood and fecal samples were collected only once within 24 hours from the onset of diarrhea. In addition, animals in the diarrhea group were included in the study after being examined for elimination of diseases such as respiratory system diseases, aspiration pneumonia, omphalitis and arthritis.

Hematological Analysis and Biochemical Analysis: Hematological analysis incuding total leukocytes (WBC), lymphocytes (LYM), monocytes (MON), LMR (LMR was calculated the absolute lymphocyte count divided by the absolute monocyte count), neutrophils (NEU), and NLR (NLR was obtained by dividing the absolut neutrophil count by the absolute lymphocyte count) were determined by using a desktop hematology analyzer (Abacus Junior Vet® brand hemogram instrument, Diatron MI Ltd., Hungary). Serum Fe levels were measured using an autoanalyzer device (Randox Monaco, UK) by the colorimetric method.

Statistical Analysis: The analysis of the data was conducted using IBM SPSS Statistics software version 27.0.1. Normality measures of numerical values were obtained by determining the Shapiro-Wilk normality test. A homogeneity test was applied for normally distributed data (WBC, LYM, MON, NEU, NLR, Temperature, Pulsation ratio, Respiration ratio). For data showing normal distribution but not homogeneity (WBC, LYM, MON, NEU, NLR), the Brown-Forsythe test was used, and the Gamel-Howell test was used as the post hoc test. One-way analysis of variance was used for normally and homogeneously distributed data (Temperature, Respiration ratio and Pulsation ratio), and LSD (for temperature) and Tukey HSD (for respiration ratio and pulsation ratio) tests were applied as post hoc tests. Since LMR and Fe values did not show a normal distribution, the Kruskal-Wallis test was performed, and the Dunn-Bonferroni test was applied as a post hoc test. Spearman's Rank Correlation Coefficient was applied to the correlation between the data. As stated by Chan (18), Spearman's correlation coefficients <0.3 were considered as a weak correlation, 0.3-0.5 as a moderately strong correlation, 0.6-0.8 as a strong correlation, and a value of at least 0.8 and above as a very strong correlation. Linear regression analysis was applied to normally distributed data. $P<0.05$ was considered to be as statistically significant.

Results

Clinical Findings: When the groups were examined clinically, it was determined that the body temperature and respiratory frequency were within the normal reference range, and there were no signs of dehydration in the calves in the control group. In calves with diarrhea, T>39.3 was determined, and the degree of dehydration was 8-10% (16). Clinical findings between the groups are shown in Table 1. When evaluated in terms of clinical findings, it was determined that the highest value among the groups in terms of temperature was in group-2 (*E. coli group*) (P<0.001). It was observed that the other diarrheal group values were higher than the control group values (P<0.001). It was determined that the lowest value for pulsation rate was in the control group and was significantly higher in all other diarrheal groups than in the control group (P<0.001). It was determined that the lowest value for respiration rate was in the control group and that the values of all other diarrheal groups were higher than the control group (P<0.05). It was determined that the values of groups 3, 6 and 7 were significantly higher than the values of group 4 and group 8 (P<0.05).

Hematological Findings and Biochemical Findings: The mean data of the hematological and serum Fe values between the groups are shown in Table 2.

In the evaluation between groups in terms of WBC values, it was determined that all other diarrhea group data were significantly higher than the Group-1 value (P<0.001). In addition, Group-3 and Group-6 values were found to be significantly higher than Group-4 values (P<0.05). It was determined that the Group-5 value was higher than the Group-2 value, which has the lowest numerical value in terms of LYM values (P<0.05). In the comparison between groups in terms of MON values, Group-5 and Group-7 values were found to be statistically higher than Group-2 values (P<0.05). It was determined that the Group-5 value was higher than the Group-3, 6, and 8 values, and this increase was significant (P<0.05). In terms of NEU values, it was observed that all diarrhea group values had a significant difference compared to the control group (P<0.001). In terms of NLR value, it was determined that the Group-1 value was the lowest value and all diarrhea group data were significantly higher (P<0.05). Similarly, for the NLR value, Group-2 data was found to be significantly higher than all other diarrhea group data, except for Group-8 data (P<0.05). In terms of LMR values, it was noticed that the Group-5 value was significantly lower than all diarrheal group values except for the value of Group-7 (P<0.05). For Fe values, the Group-1 value was found to be significantly higher than all diarrheal group values except the Group-5 group (P<0.01).

Correlation Findings: Data obtained from the whole blood and serum of animals are shown in Table 3. There was a moderate positive correlation between WBC data and LYM values (*rho*= 0.533; P<0.001), and a weak positive correlation between WBC and MON values (*rho*=0.191; P<0.05). A very strong correlation was obtained between the WBC and NEU value (*rho*=0.815; P<0.001), while a weak correlation was observed between the WBC and NLR (*rho*=0.169; P<0.05) and LMR (*rho*=0.176; P<0.05) values. There was a moderate correlation between LYM and MON value (*rho*=0.393; P<0.001), while a weak correlation was obtained between LYM and LMR (*rho*=0.285; P<0.001) and Fe (*rho*=0.287; P<0.001). A strong correlation was observed between LYM and NLR (*rho*=-0.676; P<0.001). There was a weak correlation between MON and NLR values (*rho*=-0.289; P<0.001) and a strong correlation between MON and LMR values (*rho*=-0.723; P<0.001). A strong correlation was found between NEU and NLR (*rho*=0.626; P<0.001), while a weak correlation was obtained between NEU and Fe (*rho*=-0.165; P<0.05). A weak correlation was observed between NLR and LMR (*rho*=-0.194; P<0.05) and a moderately strong correlation was observed between NLR and Fe (*rho*=-0.390; P<0.001). The correlation between the values is shown in Figure 1.

Regression Analysis: Since P<0.05 was obtained in all numerical parameters investigated for linear regression, the established regression model was found to be significant. Data obtained from whole blood are shown in Table 4. According to the results of the regression analysis, it was found that 28% of the WBC value was shaped by LYM and the effect was positive (R=0.53; R^2=0.280; P<0.001). It has been shown that 0.05% of WBC is formed by MON and this effect is positive (R=0.22; R^2=0.05; P<0.01). It was found that 78% of WBC was caused by NEU and the effect between the two parameters was positive (R=0.53; R^2=0.78; P<0.001) (Figure 2). A negative effect was observed between NLR and LYM (R=0.65; R^2=0.43; P<0.001) (Figure 3). A positive effect was observed between NLR and NEU (R=0.59; R^2=0.35; P<0.001) (Figure 4).

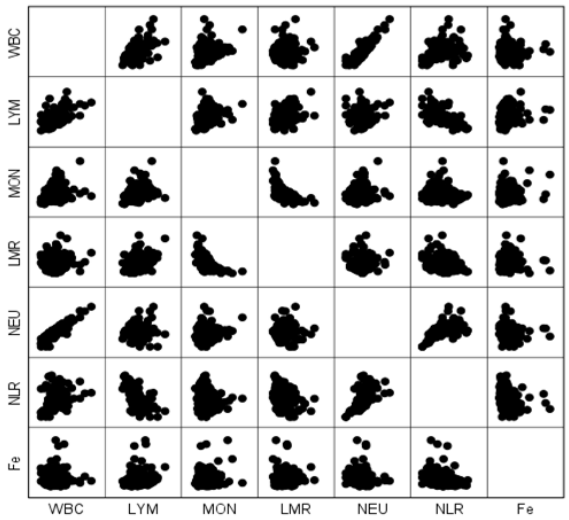

Figure 1. Correlation graph of the parameters. WBC:Total leukocyte count; LMY: Lymhocyte; MON: Monocyte; NEU: Neutrophil; NLR: Neutrophil to lymphocyte ratioe; LMR: Lymphocyte to monocyte ratio; Fe: Iron

Table 1. Clinical findings in diarrheal and control group calves

Parameters	Group-1 (n=15) $\bar{x}$ ± sd	Group-2 (n=32) $\bar{x}$ ± sd	Group-3 (n=27) $\bar{x}$ ± sd	Group-4 (n=32) $\bar{x}$ ± sd	Group-5 (n=14) $\bar{x}$ ± sd	Group-6 (n=15) $\bar{x}$ ± sd	Group-7 (n=21) $\bar{x}$ ± sd	Group-8 (n=15) $\bar{x}$ ± sd	P Value
Rectal Temperature (°C)	38.5 ± 0.37^{a}	39.8 ± 0.19^{c}	39.6 ± 0.18^{b}	39.6 ± 0.14^{b}	39.6 ± 0.23^{b}	39.6 ± 0.18^{b}	39.6 ± 0.24^{b}	39.6 ± 0.23^{b}	<0.001
Pulsation ratio (pulse wave\min)	85.60 ± 2.64^{a}	120.87 ± 8.24^{b}	117.81 ± 9.55^{b}	117.78 ± 8.86^{b}	117.92 ± 8.97^{b}	116.73 ± 9.29^{b}	123.47 ± 6.53^{b}	123.00 ± 7.26^{b}	<0.001
Respiration ratio (cycle\min)	23.13 ± 2.44^{a}	34.84 ± 3.28^{bc}	35.66 ± 1.61^{c}	32.56 ± 3.76^{b}	33.64 ± 3.60^{bc}	35.80 ± 2.04^{c}	36.38 ± 1.68^{c}	31.93 ± 4.21^{b}	<0.05

The significiantly differences has been showed that the lower cases between group that at the same line. P<0.05 is statically significant.

Table 2. Hematological index values among groups

	Groups								
	Group-1 (n=15) $\bar{x}$ ± sd	Group-2 (n=32) $\bar{x}$ ± sd	Group-3 (n=27) $\bar{x}$ ± sd	Group-4 (n=32) $\bar{x}$ ± sd	Group-5 (n=14) $\bar{x}$ ± sd	Group-6 (n=15) $\bar{x}$ ± sd	Group-7 (n=21) $\bar{x}$ ± sd	Group-8 (n=15) $\bar{x}$ ± sd	P Value
WBC (×10^{3}/µL)	8.36 ± 1.52^{a}	13.22 ± 2.55^{bc}	14.90 ± 5.75^{c}	12.40 ± 2.93^{b}	13.81 ± 3.67^{bc}	14.75 ± 1.54^{c}	13.20 ± 1.55^{bc}	13.28 ± 2.36^{bc}	<0.001
LYM (×10^{3}/µL)	4.67 ± 0.78^{ab}	3.77 ± 1.47^{a}	5.26 ± 1.74^{ab}	5.17 ± 2.02^{ab}	5.58 ± 1.39^{b}	5.06 ± 1.46^{ab}	5.01 ± 1.50^{ab}	4.20 ± 0.47^{ab}	<0.05
MON (×10^{3}/µL)	0.20 ± 0.07^{abc}	0.15 ± 0.03^{a}	0.19 ± 0.06^{ab}	0.21 ± 0.12^{abc}	0.37 ± 0.17^{c}	0.19 ± 0.08^{ab}	0.24 ± 0.09^{bc}	0.16 ± 0.05^{ab}	<0.05
NEU (×10^{3}/µL)	3.27 ± 1.16^{a}	9.24 ± 1.66^{b}	9.40 ± 5.22^{b}	6.95 ± 1.75^{b}	7.81 ± 2.95^{b}	9.42 ± 0.02^{b}	7.90 ± 0.04^{b}	8.86 ± 2.13^{b}	<0.001
NLR	0.70 ± 0.25^{a}	2.75 ± 0.89^{c}	1.94 ± 0.94^{b}	1.53 ± 0.65^{b}	1.48 ± 0.63^{b}	1.99 ± 0.53^{b}	1.71 ± 0.51^{b}	2.12 ± 0.52^{bc}	<0.05
LMR	Median (Q1-Q3) 22.42 (18.88-28.42)^{ab}	Median (Q1-Q3) 27.35 (16.21-30.04)^{b}	Median (Q1-Q3) 27.44 (19.59-37.65)^{b}	Median (Q1-Q3) 28.14 (15.18-44.17)^{b}	Median (Q1-Q3) 15.33 (12.26-20.75)^{a}	Median (Q1-Q3) 25.11 (18.85-33.05)^{b}	Median (Q1-Q3) 18.67 (15.13-28.46)^{ab}	Median (Q1-Q3) 26.82 (22.26-30.69)^{b}	<0.05
Fe (µg/dL)	Median (Q1-Q3) 123.43 (98.75-153.12^{bc}	Median (Q1-Q3) 30.09 (17.41-56.80)^{a}	Median (Q1-Q3) 49.81 (29.34-59.19)^{a}	Median (Q1-Q3) 50.63 (28.28-86.06)^{a}	Median (Q1-Q3) 89.71 (32.11-(121.13) ^{ab}	Median (Q1-Q3) 56.98 (17.61-116.33)^{a}	Median (Q1-Q3) 50.15 (33.39-56.41)^{a}	Median (Q1-Q3) 57.47 (25.35-106.74)^{a}	<0.01

WBC: Total leukocyte count; LMY: Lymhocyte; MON: Monocyte; NEU: Neutrophil; NLR: Neutrophil to lymphocyte ratio; LMR: Lymphocyte to monocyte ratio; Fe: Iron. The significiantly differences has been showed that the lower cases between group that at the same line. P<0.05 is statically significant.

Table 3. Results of the correlation analyses among hematological parameters

Parameters	WBC	LYM	MON	NEU	NLR	LMR	Fe
WBC *rho*	1.000	0.533**	0.191*	0.815**	0.169*	0.176*	0.013
LYM *rho*		1.000	0.393**	0.039	-0.676**	0.285**	0.287**
MON *rho*			1.000	-0.030	-0.289**	-0.723**	0.092
NEU *rho*				1.000	0.626**	0.042	-0.165*
NLR *rho*					1.000	-0.194*	-0.390**
LMR *rho*						1.000	0.135
Fe *rho*							1.000

WBC: Total leukocyte count; LMY: Lymhocyte; MON: Monocyte; NEU: Neutrophil; NLR: Neutrophil to lymphocyte ratioe; LMR: Lymphocyte to monocyte ratio; Fe: Iron. *Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level. P<0.05 is statically significant.

Table 4. Linear regression among hematological parameters

Values Dependent*Independent	B	Standart Error	Beta	Confidence Interval (95 %)	*R*	R^2
WBC*LYM	1.17***	0.14	0.53	(0.88-1.45)	0.53	0.28
WBC*MON	7.59**	2.54	0.22	(2.57-12.61)	0.22	0.05
WBC*NEU	1.03***	0.04	0.88	(0.95-1.11)	0.88	0.78
NLR*NEU	0.17***	0.01	0.59	(0.13-0.20)	0.59	0.35
NLR*LYM	-0.35***	0.03	-0.65	[(-0.42- (-0.29)]	0.65	0.43

WBC: Total leukocyte count; LMY: Lymhocyte; MON: Monocyte; NEU: Neutrophil; NLR: Neutrophil to lymphocyte ratioe; LMR: Lymphocyte to monocyte ratio; Fe: Iron. *Correlation is significant at the 0.05 level. **Correlation is significant at the <0.01 level. *** Correlation is significant at the <0.001 level.

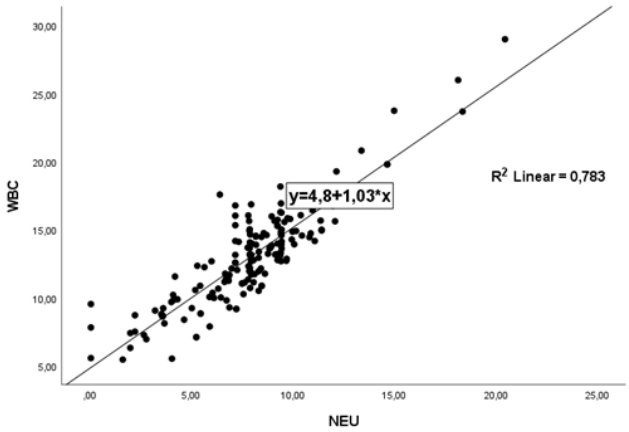

Figure 2. Relations between total leukocyte count and neutrophil count. NEU: Neutrophil; WBC: Total leukocyte count

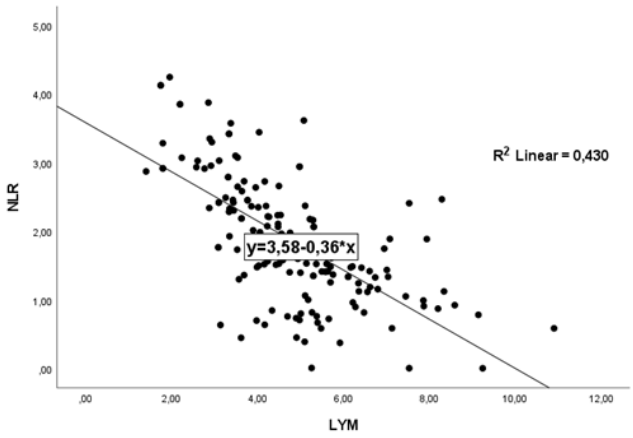

Figure 3. Relations between neutrophil-lymphocyte ratio and lymphocyte count. LMY: Lymhocyte; NLR: Neutrophil to lymphocyte ratio

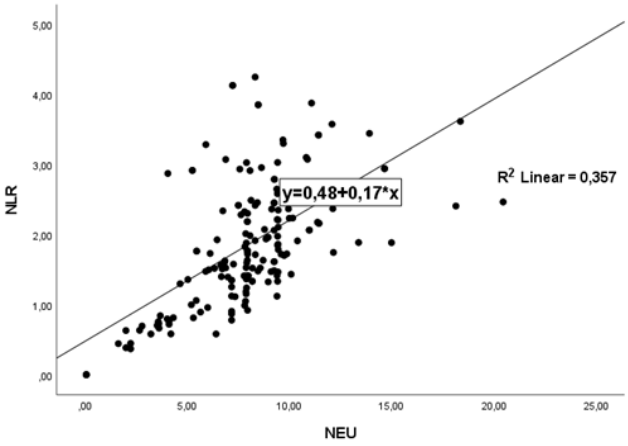

Figure 4. Relations between neutrophil-lymphocyte ratio and neutrophil count. NEU: Neutrophil; NLR: Neutrophil to lymphocyte ratio

Discussion

In this study, it was aimed to investigate NLR, LMR, and serum Fe levels and the relationship between these data in calves with different diarrhea etiologies. Many inflammatory biomarkers have been investigated in calf diarrhea, but there are barely any studies in which the levels of NLR and LMR were investigated in calves with diarrhea.

It has been reported that depending on the severity of the infection, clinical symptoms such as reluctance to move, anorexia, increased body temperature, increased respiratory rate and skin fold test time, and decreased sucking reflex may develop in calves with diarrhea (19). In this study, higher body temperature, respiration and pulsation rates were obtained in calves in the diarrheal groups than in the control group calves. It is thought that the reason for the increase in body temperature in calves with diarrhea may be due to the effects of infectious pathogens and the severity of dehydration (20). It is thought that the reason for high respiration in calves with diarrhea may be due to a compensatory polypnea to return the pH to normal levels, and tachycardia may occur due to faster heart working to ensure more intense on pumping of blood to the periphery in case of hypovolemia caused by dehydration (21).

The main factors of infectious causes of calf diarrhea can be listed as viruses (rotavirus, bovine viral diarrhea virus, and coronavirus), bacteria (*Escherichia coli* and *Salmonella* spp.), and protozoa (like *Eimeria zuernii*), as well as non-infectious factors such as stress, sudden ration changes, and imbalances in ambient humidity (22, 23).

Early confirmation of inflammatory conditions is of utmost importance for cattle herd health, welfare, and early diagnosis of diseases. Nowadays, WBC, differential WBC counts, and acute phase protein levels are mostly used for the detection of inflammatory conditions in cattle (24). WBC counts are higher in calves less than 6 months old as compared to adult cattle. However, this level returns to normal levels after 3 years of age (25). Neutrophilia occurs primarily during the recovery period of intense inflammatory conditions or during mild to moderate inflammatory conditions. Neutrophilia has also been reported to occur in infectious diseases, neoplastic conditions, non-inflammatory conditions, or tissue damage. Neutropenia occurs in cattle during acute, intense inflammatory conditions such as sepsis, mastitis, metritis, salmonella infections, pneumonia, and peritonitis (5). In a study in which Fe levels were investigated for the confirmation of inflammatory status in cattle, it was reported that band NEU levels increased significantly in mastitis and RPT disease compared to cattle in the control group (14). In a study in which a bovine respiratory disease model was established for viral and bacterial infections, it was reported that WBC and NEU counts were significantly increased after experimental intratracheal exposure of *Mannheimia haemolytica* (*M. haemolytica*) agent compared to the group that did not receive the *M. haemolytica* exposure (26). The reason for this situation was attributed to the fact that intratracheal *M. haemolytica* can rapidly initiate acute reactions within 24 hours after exposure, as stated by Corrigan et al. (27). In study conducted on calf diarrhea, it was reported that higher WBC and NEU values were obtained in the diarrhea groups compared to the control group (28).

In this study, it was observed that the WBC and NEU values in the diarrhea group were significantly higher than those in the control group ($P<0.001$). This is believed to be due to the rapid onset of acute inflammatory reactions, as stated by Corrigan et al. (27). In addition, the strong correlation (*rho*=0.815; $P<0.01$) and regression findings ($R=0.88$; $R^2=0.78$) between WBC and NEU support this finding.

In human medicine, it is stated that NLR is a useful biomarker in determining sepsis (29). In the field of veterinary medicine, studies on the NLR ratio are mostly investigated in tumoral diseases and different disease states in dogs (10, 30, 31). It has been understood in the literature review that studies on NLR are less common in cattle. It has been reported that an increase in the level of WBC, NEU, LYM and NLR in calves infected with *M. haemolytica* infection is caused by an increase in the infection load that triggers an inflammatory response (32). It has been reported that the higher NLR level in cattle with acute toxic mastitis compared to the control group may be due to the increased number of neutrophils with the effect of increased acute inflammatory response (33).

In this study, similar to the above studies, higher WBC, NEU, and NLR levels were found in the diarrhea groups compared to the control group. This may be due to the increase in the number of NEUs as a result of the increase in the acute inflammatory response caused by infectious agents as mentioned by Braun et al. (33). The highest NLR value was found in group-2 (*E. coli* group). In addition, the strong correlation between WBC and NEU (*rho*=0.815; $P<0.01$) and the strong degree regression data between WBC and NEU ($R=0.88$;

R^2=0.78) proved that acute inflammation was more severe in group-2 compared to the other groups.

Lymphocytosis is not commonly seen in cattle, but it has been reported to occur under conditions such as chronic viral and pyogenic infections. It has been stated that lymphopenia occurs in corticosteroid administration and stress or diseases and pathological conditions such as acute viral and bacterial infections, and rarely immunodeficiency (34). Circulating leukocytes show an increase in the number of neutrophils in the face of stressful situations, while the number of lymphocytes decreases (35). LMR is an important marker of systemic inflammatory response (36). To the best of the author's knowledge, there have been very few studies investigating LMR in cattle. It has been reported that NLR was decreased and LMR was increased with the administration of hydrolyzed tannin extract in a study on calf health and this may be attributed to the anti-inflammatory and immunomodulatory properties of tannin extract (37). In inflammatory bowel disease, low level of LMR has been reported to be positively correlated with advanced disease (38). Similarly, low level of LMR has been reported to be associated with poor prognosis in lymphomas in dogs and cats (39, 40). It has been reported that the presence of moderately pronounced lymphopenia haematologically is directly proportional to the severity of the disease and the damage caused by this severity (41). It has been reported that monocytes have an active role in the control of infection as well as in the pathogenesis of inflammatory diseases (42). In a study on breast cancer in human medicine, it was reported that elevated Fe-Monocyte-Lymphocyte ratio and low LMR ratio associated with low Fe in individuals with Coronavirus-19 (COVID-19) disease indicate poor prognosis (43, 44). Different studies on giardiosis have reported that increased activation of macrophages plays an active role in the digestion of giardia trophozoites (45, 46).

In this study, it was determined that group-5 (*Giardia* spp.) had the highest value in terms of MON numbers (P<0.05). It was observed that the group with the lowest LMR values was formed in group-5, and this value created a significant difference (P<0.05). This may be due to the highly active role of macrophages (hence monocytes) against *Giardia* spp. infections as stated by Hill and Pohl (45). In addition, the statistically insignificant correlation between the level of LMR, which is an inflammatory marker, and Fe also supports this finding (*rho*=0.135; P>0.05).

Since acute phase proteins such as haptoglobin or serum amyloid A require special equipment for use in clinical practice, it is extremely important to be able to determine the inflammation status more quickly in cattle health and to use alternative biomarkers with less cost, since it is time consuming and impractical. Fibrinogen is an acute phase protein that can be easily measured manually in blood (14). The levels of Fe, which is one of the trace elements, decrease rapidly in inflammatory diseases in cattle. Moreover, Fe can be easily measured together with other parameters with standard biochemical devices (47). Fe stimulates immune system activation by affecting the function of immune system cells. This effect is manifested by activation of NEU, LYM and macrophages in the presence of sufficient levels of Fe. In inflammatory conditions, the storage of Fe in reticuloendothelial cells increases with the effect of acute phase proteins such as hepcidin, alpha-1 antitrypsin and cytokines and blood Fe levels decrease accordingly. This is a protective mechanism that inhibits the proliferation of pathogenic microorganisms that utilize Fe (48). Serum Fe concentration has been used to confirm inflammatory status in horses (13). However, in terms of cattle health, it is seen that relatively limited data are obtained for the confirmation of inflammatory conditions of Fe. It has been reported that serum level of Fe is significantly decreased in various inflammatory diseases (47), cattle with traumatic peritonitis and mastitis (14, 49), bovine respiratory disease (BRD) and Fe can be used as an inflammatory marker (50). Tsukano et al. (50) also reported that the number of experiments was not sufficient for Fe to be used as an inflammatory marker and the etiology and severity of BRD were not determined. In contrary to these studies, it was stated that there was no significant change in serum levels of Fe within 24 hours in cattle with endotoxemia model and its use as a prognostic marker was not significant. The reason for this situation has been shown as not knowing the beginning of the infection period in animals with endotoxemia and the small number of animals used in the research (51).

In this study, although similar results were obtained to the studies mentioned above, different results were also observed. First of all, the reason for this difference may be the differences in experimental design between the above-mentioned studies and this study, the inability to create a possible sample in terms of disease severity in the experimental group animals of the above-mentioned studies, and the differences in the immune status of the animals depending on their age. In addition, one of the most important difference is that the animals in the experimental group in this study were a suitable sample in accordance with the diarrhea protocol and the number of animals used in the study was sufficient. The formation of significant data between the diarrhea and control groups in terms of serum Fe levels in this study supports the importance of the number of subjects used in the study as stated by Tsukano et al. (50).

Boyuk et al. (52) reported that the NLR rate was higher and Fe levels were lower in the patient groups in *Helicobacter pylori* infection and a negative correlation was observed between NLR and Fe. This negative correlation has been suggested to occur since Fe increases the proliferation of immune system cells (53). In severe COVID-19 infection, low level of Fe, which negatively correlates with high NLR with excessive increase in inflammatory response, has been reported to be indicators of increased rate of mortality (54). In the present study, the levels of Fe were found to be significantly lower in all diarrhea groups compared to the control group (P<0.01). The lowest Fe level was observed in group-2 (*E. coli* group). In addition, when the correlation findings were examined, a negative

correlation (*rho*=-0.390; P<0.01) was obtained between the NLR and Fe levels. Therefore, it was concluded that the inflammatory response was significantly higher in group-2, which had the highest NLR level and the lowest Fe level, compared to the other diarrhea groups. This may be explained by the enhancing effect of Fe on the proliferation of immune system cells (53) or by the severity of the inflammation (high NLR level) in the *E. coli* group (54).

The present study has several limitations. First, although the groups were selected according to broadly different aetiologies, it is evident that more sensitive analyses are needed to determine the etiology with certainty. However, Rotavirus, Coronavirus, *E. coli* (K99), and *Cryptosporidium* spp. are reported as the causative agents of calf diarrhea in the first 30 days. The prevalence of these agents was reported to be 6.69% for *Cryptosporidium* spp., 2.84% for bovine coronavirus, and 1.64% for Enterotoxigenic *E. coli* (55). Therefore, in the current study, a rapid diagnosis kit was used to determine the most common factors in neonatal calf diarrhea. Secondly, blood and stool samples were taken from the calves only once. It is seen that the prognostic evaluation of the study with repeated measurements can yield meaningful results. Finally, it is predicted that valuable results can be obtained with studies investigating hematological inflammatory markers (NLR, LMR), Fe, inflammatory cytokines, and acute phase biomarkers together.

In this study, it was observed that NLR, LMR, and Fe levels may be found at different levels according to the etiological agents in calves with diarrhea and may explain the inflammatory state. It was determined that NLR levels in calves with *E. coli* diarrhea were higher than in other calves with diarrhea, but LYM, MON, and Fe levels were lower. Therefore, it was concluded that the inflammatory response was more intense in calves with *E. coli* diarrhea according to NLR and Fe levels used as inflammatory markers. However, in order to verify these data, it is extremely important to investigate the reality of this situation with repeated measurements and experimental studies by forming patient groups on larger scales.

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