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Research on Hematology, Inflammatory and Antimicrobial Peptide Levels according to Clinical Scoring in Calves with Bovine Respiratory Disease (BRD)

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ABSTRACT Bovine respiratory disease (BRD) is a significant and costly disease in cattle, characterized by various infections with distinct causes and clinical signs. This study focuses on investigating some hematological and inflammatory parameters, as well as cathelicidin antimicrobial peptide (CAMP) parameters, in calves with BRD. Forty-two calves were allocated to three groups based on clinical scoring: Group I (n=10, healthy, score 0), Group II (n=16, BRD, score 3), and Group III (n=16, BRD, score 4). Some hematological (WBC, NEU, LYM, NLR, and PLT), inflammatory (IL-1β, TNF-α, NF-κB, and IL-10), and CAMP parameters were evaluated in all groups. Group III had significantly higher WBC, NEU, and NLR concentrations than Group I, while concentrations of PLT in Group II were higher than Group I ($p<0.05$). NF-κB, TNF-α, and CAMP levels were enhanced in Group III compared to Group I, and CAMP levels were higher in Group II than in Group I $p<0.05$). Strong positive correlations were found between NEU and WBC and NLR. Weak positive correlations existed between WBC, NEU, IL-10, and CAMP, as well as between LYM and IL-1β and NF-κB and CAMP. In conclusion, the most severe inflammation was observed in Group III, aligning with clinical scoring in BRD-affected calves. CAMP was identified as a reliable marker for inflammation assessment. Additionally, NLR, being low-cost and easily measurable, showed promise as an inflammation indicator.

Keywords: *Anti-inflammatory, Bovine Respiratory Disease, Calf, Cathelicidin antimicrobial peptide, Neutrophil-Lymphocyte Ratio, Pro-inflammatory.*

ÖZ

Sığır Solunum Yolu Hastalıklı (BRD) Buzağılarda Klinik Skorlamaya Göre Hematoloji, İnflamatuar ve Antimikrobiyal Peptid Düzeylerinin Araştırılması

Sığır solunum yolu hastalığı (BRD), sığırlarda farklı nedenleri ve klinik belirtileri olan çeşitli enfeksiyonlarla karakterize önemli ve maliyetli bir hastalıktır. Bu çalışma, BRD'li buzağılarda bazı hematolojik ve inflamatuvar parametrelerin yanısıra katelisidin antimikrobiyal peptid (CAMP) parametrelerinin araştırılmasına odaklanmıştır. 42 buzağı klinik skorlamaya gore üç gruba ayrılmıştır: Grup I (n=10, sağlıklı, skor 0), Grup II (n=16, BRD, skor 3) ve Grup III (n=16, BRD, skor 4). Bazı hematolojik (WBC, NEU, LYM, NLR ve PLT), inflamatuvar (IL-1β, TNF-α, NF-κBve IL-10) ve CAMP parametreleri değerlendirilmiştir. Grup III'te WBC, NEU ve NLR konsantrasyonları Grup I'e göre istatistiksel olarak yüksekken, Grup II'de PLT konsantrasyonları Grup I'e göre istatistiksel olarak daha yüksekti ($p<0.05$). NF-κB, TNF-α ve CAMP düzeyleri Grup III'te Grup I'e kıyasla artarken, CAMP düzeyleri Grup II'de Grup I'e kıyasla daha yüksekti ($p<0.05$). NEU ile WBC ve NLR arasında güçlü pozitif korelasyonlar bulundu. WBC, NEU, IL-10 ve CAMP arasında ve ayrıca LYM ile IL-1β ve NF-κB ile CAMP arasında zayıf pozitif korelasyonlar mevcuttu. Sonuç olarak, BRD'den etkilenen buzağılarda klinik skorlama ile uyumlu olarak en şiddetli inflamasyon Grup III'te gözlenmiştir. CAMP, inflamasyon değerlendirmesi için güvenilir bir belirteç olarak tanımlanmıştır. Ayrıca, düşük maliyetli ve kolay ölçülebilir NLR, bir yangı göstergesi olarak umut vaat etmektedir.

Anahtar Kelimeler: *Anti-inflamatuar, Sığır Solunum Hastalığı, Buzağı, Cathelicidin antimikrobiyal peptid, Nötrofil-Lenfosit Oranı, Pro-inflamatuar.*

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INTRODUCTION

Respiratory tract infections in livestock farming cause serious economic losses such as mortality, loss of yield, and growth retardation. Bovine respiratory disease (BRD) of calves is defined as a multi-etiological disease condition in which many bacterial and viral agents play a role (Edwards 2010). The etiology of this complex is predominantly caused by bacterial and viral agents (Panciera and Confer 2010). During the initial stages of the disease, rapid and shallow breathing (hyperventilation) is evident. Dyspnea occurs when the disease progresses and most of the lung tissue loses its function. Another important finding is cough. Nasal and ocular discharges, fever, anorexia, depression, and increased respiratory rate may accompany these symptoms. As the disease worsens, dyspnea, severe lacrimation, and closed nostrils are observed. Hoarse sounds are heard on lung auscultation. The animal shows the posture of extending its neck forward and moving its elbows away from its body (Guterbock 2014). The clinical severity of the disease can also be scored by using the above-mentioned clinical signs (Perino and Apley 1998).

The neutrophil-lymphocyte ratio (NLR) is a cost-effective and easily accessible metric that provides information on both the cellular immunological and systemic inflammatory response. It is calculated by dividing the number of neutrophils (NEU) by the number of lymphocytes (LYM). NLR represents the equilibrium between NEU, the inherent immune response, and LYM, the acquired immune response, during inflammatory and diseased conditions. It stands as a crucial parameter for identifying sepsis, inflammation, and infections. NLR monitoring has been shown to be beneficial, especially in severe pneumonia cases (Buonacera et al. 2022).

Cytokines are small proteins that provide intercellular communication. These proteins contain anti-inflammatory cytokines, as well as pro-inflammatory cytokines. Proinflammatory cytokines are mostly produced by activated macrophages. While these increase in case of inflammatory reaction, anti-inflammatory cytokines provide an immunoregulatory effect regulating pro-inflammatory cytokines (Winter et al. 2007). NF-κB plays a pivotal function in tissue inflammation and the activation of the immune system. NF-κB selectively attaches to the exclusive DNA-binding sites of genes associated with pro-inflammatory cytokines, including TNF-α and IL-1β (Gloire et al. 2006). The activation of NF-κB is stimulated by various factors, potentially including pro-inflammatory cytokines like TNF-α and IL-1β. TNF-α is the foremost cytokine in bacterial sepsis or endotoxemia (Batra et al. 2011). Because of the increased TNF-α level in bacterial sepsis in previous animal models (Hesse et al. 1988), it was thought that there may be a relationship between the severity of the disease and TNF-α level in calves with BRD. IL-1β plays a crucial function in stimulating the immune system during inflammation associated with fever infectious diseases (Zhang and An 2007). IL-10 is a cytokine with anti-inflammatory properties and important functions in the immune response against pathogenic factors, providing homeostasis and preventing damage to the host (Iyer and Cheng 2012). Cathelicidins are small cationic peptides synthesized by innate immune cells and epithelial cells, and they serve crucial functions in defending the airway's epithelial surfaces. Cathelicidins can prevent infection directly by stimulating the recruitment of immune cells in the respiratory tract. They exhibit extensive antimicrobial activity against bacteria, viruses, and parasites (Tecle et al. 2010).

Identifying the causative agent of respiratory tract infections in calves and routinely diagnosing the disease remain formidable challenges (de Carvalho et al. 2016). The objective of this research was to examine the levels of pro-inflammatory and anti-inflammatory cytokines, antimicrobial peptide concentrations, and NLR, along with clinical scoring, as a supplementary diagnostic method for evaluating the extent of inflammation in BRD-affected calves.

MATERIAL AND METHODS

The experimental study was carried out with the authorization of the Animal Experiments Local Ethics Committee of Atatürk University on January 26, 2023, under the reference number 2023/23.

Animals

The animal material consists of calves aged between 30 and 45 days of different breeds and genders.

Experimental Groups

To create the calf groups included in the study, the "Severity Score Criteria For Undifferentiated Respiratory Disease" protocol determined by Perino and Apley (1998) was used (Table 1).

The study consisted of 3 groups:

Group I (n=10): This is the healthy control group. Calves with a clinical score of 0 were included.

Group II (n=16): Calves with a clinical score of 3 were included in this group.

Group III (n=16): Calves with a clinical score of 4 were included in this group.

Table 1: Severity score criteria for undifferentiated respiratory disease.

Score	Clinical Signs
0	Normal, no signs of disease
1	Noticeable depression, signs of weakness are usually not apparent
2	Marked depression, moderate signs of weakness may be apparent but without significantly altered gait
3	Severe depression accompanied by signs of weakness such as altered gait or lowered head
4	Moribund, unable to rise

Clinical Examination

The calves' respiratory and heart rate, and rectal temperature were assessed, additionally clinical outcomes were documented using the scoring parameters listed in Table 1.

Blood Sample Collection

Blood samples were obtained from *v. jugularis externa* of all the calves involved in the study and collected in tubes with EDTA(EDTA K3, Pty Ltd., Adelaide, SA, Australia) as well as serum (Vacutainer, Becton Dickinson Co. USA) tubes. A series of blood samples were obtained in serum tubes, left to coagulate for 30 minutes at ambient temperature, subjected to centrifugal force at 3000

revolutions per minute for 10 minutes, and then stored at a temperature of -80 °C until analysis.

Hematological Analysis

White blood cell (WBC), lymphocyte (LYM), neutrophil (NEU), NLR (NEU/LYM ratio) and platelet (PLT) parameters in blood samples collected in EDTA tubes were analysed using a haemogram device (Abacus Junior Vet 5, Diatron, Hungary).

Biochemical Analysis

Serum concentrations of NF-κB[a], TNF-α[b], IL-1β[c], IL-10[d], and Cathelicidin[e] levels were determined by using bovine-specific ELISA kits (BT-LAB Bioassay Technology Laboratory) according to manufacturer's recommen dations ([a]Cat. No. E0314Bo, [b]Cat. No. E0019Bo, [c]Cat. No. E0197Bo, [d]Cat. No. E0252Bo, and [e]Cat. No. E0400Bo).

Statistical Analysis

Analysis of the study data was conducted using SPSS software (version 25.0, IBM Software, Inc. in Chicago, United States). Prior to analyzing the data of the study, the Shapiro-Wilk test was used to ascertain the normal distribution of the data. An analysis of variance (ANOVA) was conducted to identify the differences in the parameters among the groups. Subsequently, the *post hoc* Tukey test was used. A Pearson correlation analysis was conducted to determine the correlation among the values in the provided data. A significance level of 0.05 was used to determine statistical significance, and the results were reported as the mean ± standard deviation (SD).

RESULTS

Clinical Findings

Respiratory rate, heart rate and rectal temperature of all calves are shown in Table 2. Respiratory rate and rectal temperature were higher in group II and group III than in group I ($p<0.001$).

Table 2: Clinical findings of the groups.

Clinical Findings	Group	Mean ± SD	p - Values
Respiration Rate (min)	Group I*	35.1±3.25[a]	< 0.001
	Group II**	55.2±5.01[b]	
	Group III***	57.4±4.73[b]	
Heart Rate (min)	Group I*	97.2±3.97	> 0.05
	Group II**	99.1±10.7	
	Group III***	97.9±8.65	
Rectal Temperature (°C)	Group I*	38.6±0.35[a]	< 0.001
	Group II**	39.6±0.29[b]	
	Group III***	39.7±0.25[b]	

* score 0 (Normal, no signs of disease), **score 3 (Severe depression accompanied by signs of weakness such as altered gait or lowered head), ***score 4 (Moribund, unable to rise).

Haematological Findings

Haematological results for animals are summarized in Table 3 in all groups. Group III showed higher WBC, LYM, NEU and NLR levels than group I, while group II had higher PLT levels ($p<0.05$).

Table 3: Hematological findings of the groups.

Parameters /Groups	Group	Mean ± SD	p - Values
WBC (10^{-3}/µl)	Group I*	11.26±3.01[a]	< 0.01
	Group II**	13.76±6.94[ab]	
	Group III***	20.23±8.65[b]	
LYM (10^{-3}/µl)	Group I*	6.19±2.07	> 0.05
	Group II**	6.08±1.58	
	Group III***	7.01±4.29	
NEU (10^{-3}/µl)	Group I*	4.77±2.32[a]	< 0.01
	Group II**	7.2±5.7[ab]	
	Group III***	12.82±8.03[b]	
NLR	Group I*	0.84±0.55[a]	< 0.01
	Group II**	1.17±0.81[a]	
	Group III***	2.10±1.01[b]	
PLT (10^{-3}/µl)	Group I*	272±75[a]	< 0.01
	Group II**	608±297[b]	
	Group III***	449±205[ab]	

* score 0 (Normal, no signs of disease), **score 3 (Severe depression accompanied by signs of weakness such as altered gait or lowered head), ***score 4 (Moribund, unable to rise). WBC: Leukocyte, LYM: Lymphocyte, NEU: Neutrophil, NLR: Neutrophil-lymphocyte ratio, PLT: Platelet. Data are presented as the mean ± SD, SD: standard deviation. [a, b]: The means shown in different lowercase letters between the groups (on the line) are statistically significant ($p<0.05$).

Biochemical Findings

The calf samples from all the groups are summarised in Table 4, which shows that the levels of NF-kB, TNF-α and cathelicidin were higher in the III group than in the I group. In addition, the levels of cathelicidin were higher in group II compared to group I ($p<0.05$). Our correlation results, represented in Table 5, indicate a strong positive correlation between NEU and both WBC ($r=0.924$, $p<0.05$) and NLR ($r=0.872$, $p<0.05$), and a strong positive correlation between WBC and NLR ($r=0.706$, $p<0.05$). Moderate positive associations were found between WBC and LYM and between TNF-α, NLR and NF-kB. Additionally, weak positive correlations were discovered between WBC and IL-10 and CAMP and between NEU and IL-10 and CAMP. Lastly, weak positive correlations were observed between LYM and IL-B, as well as between NF-κB and CAMP.

Table 4: Biochemical findings of the groups.

Parameters/Groups	Group	Mean ± SD	p - Values
NF-kB (ng/mL)	Group I*	1.26±0.40[a]	< 0.01
	Group II**	1.59±0.33[ab]	
	Group III***	1.83±0.33[b]	
TNF-α (ng/L)	Group I*	120±38.9[a]	< 0.05
	Group II**	142±31.91[ab]	
	Group III***	156±28.09[b]	
IL-1β (ng/L)	Group I*	6.01±1.52	> 0.05
	Group II**	6.17±1.62	
	Group III***	6.97±2.76	
IL-10 (ng/L)	Group I*	135±16.49	> 0.05
	Group II**	145±27.8	
	Group III***	155±28.39	
CAMP (nmol/mL)	Group I*	16.41±9.64[a]	< 0.01
	Group II**	23.86±5.35[b]	
	Group III***	25.28±4.62[b]	

* score 0 (Normal, no signs of disease), **score 3 (Severe depression accompanied by signs of weakness such as altered gait or lowered head), ***score 4 (Moribund, unable to rise). NF-kB = Nuclear Factor Kappa B, TNF-a = Tumor Necrosis Factor Alpha, IL-1B = Interleukin-1 Beta, IL-10 = Interleukin-10, CAMP: Cathelicidin Antimicrobial Peptide. Data are presented as the mean ± SD, SD: standard deviation. Different letters in the same line are statistically significant ($p<0.05$).

Table 5: Correlation of parameters between groups (Pearson Correlation).

Parameters	WBC	NEU	LYM	NLR	PLT	NF-κB	TNF-α	IL-1β	IL-10	CAMP
WBC	1.000	0.924**	0.437**	0.706*	0.088	0.175	0.247	0.305	0.352*	0.361*
NEU		1.000	0.064	0.872**	0.090	0.059	0.291	0.207	0.375*	0.344*
LYM			1.000	-0.209	-0.006	0.302	-0.048	0.346*	0.071	0.116
NLR				1.000	0.069	0.008	0.412*	0.189	0.286	0.280
PLT					1.000	-0.007	-0.031	0.028	-0.018	0.142
NF-κB						1.000	0.417**	0.192	0.094	0.368*
TNF-α							1.000	0.214	0.153	0.250
IL-1β								1.000	-0.049	0.203
IL-10									1.000	0.149
CAMP										1.000

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION AND CONCLUSION

Bovine respiratory disease (BRD) is a significant ailment that leads to substantial mortality rates among beef and dairy calves globally. Although there are many factors that cause BRD, this disease is caused by the interaction of bacteria and viruses (Panciera and Confer 2010). This study purposed to assess pro-inflammatory and anti-inflammatory cytokines, antimicrobial peptide concentrations and NLR according to clinical scoring as an auxiliary diagnostic tool in determining the severity of inflammation in BRD in calves.

Symptoms such as high fever and tachypnea can be seen in animals with BRD. The formed fever causes the calf to tachypnea. At the same time, the cause of tachypnea is explained by the increased respiratory rate due to the non-functionality of a large part of the infected lung tissue (Guterbock 2014). In the present study, in line with the

above information, it was found that both respiratory rates and rectal temperatures of calves in group I were lower than in the other groups. This could be due to a fever caused by an infection, as well as a major portion of the lung tissue becoming dysfunctional.

It was reported that the leukocyte counts of the groups with BRD were higher than the control group in calves (Howard et al. 1976). Martin and Lumsden (1987) reported that a table of leukocytosis was formed in their study in calves with BRD and this was probably due to acute respiratory tract inflammation due to infection. In this study, both WBC and NEU counts were higher in infected calves than in healthy calves. Compared to group I, this increase was found to be significant in group III ($p<0.01$). The possible cause of the high leukocyte elevation is thought to be neutrophilic leukocytosis formed due to acute inflammation. At the same time, the very strong positive correlation between WBC and NEU supports this.

According to one study, NEUs in cattle can boost the production of pro-inflammatory cytokines such as TNF- α and IL-1β (Sohn et al. 2007). According to research comparing M. bovis, which causes respiratory disease, and NEUs, the count of NEUs increased. This is explained by the fact that M. bovis stimulates TNF-α production by promoting NEU apoptosis (Howard et al. 1976). In this study, we found that concentrations of NEUs and TNF-α were higher in calves of groups II and III in proportion to I. This aligns with previous research. This can be explained by pathogen agents enhancing TNF-α production by stimulating NEU death, as previously indicated.

Recent research has highlighted the significance of NLR as a marker for identifying inflammation and predicting the course of the disease (Zahorec 2021). It has been reported that NLR levels are increased in goats with pneumonia (Jarikre et al. 2016). In this study, group II and III had greater NLR levels than group I. group III had a statistically significant rise compared to group I. Consistent with the aforementioned reports, it was discovered that the NLR level increased as BRD severity increased, in this study. This could be attributed to an increase in WBC, NEU and TNF-α. In addition, the high positive association between NLR level and WBC and NEU as well as the moderate positive correlation between NLR level and TNF-α provide support for this assumption. Primary thrombocytosis is a condition caused by adrenaline-induced splenic contraction. Seconder (reactive) thrombocytosis is triggered by the release of cytokines in the body and is seen in conditions such as stress and inflammation (Jones and Allison 2007). In the study, according to clinical scoring, PLT levels in group II and III animals were shown to be greater than in group I.

Pro-inflammatory cytokines have a significant impact on initiating the activation of acute-phase proteins (APPs) throughout the body. Each of these cytokine groups induces a different APP (Baumann and Gauldie 1994). Therefore, the study focused on different pro-inflammatory cytokines, such as NF-κB, TNF-α, and IL-1β. NF-κB facilitates the activation of pro-inflammatory cytokines like IL-1β and TNF-α, and anti-inflammatory cytokines such as IL-10, by binding to the nucleic regions that are crucial for cytokine initiation. Pro-inflammatory cytokines also cause NEU and PLT adhesion and binding to endothelial cells (Celik et al. 2013). Studies conducted on humans have reported that the level of NF-κB increases in case of pneumonia (Wahl et al. 2003; Devaney et al. 2013). In this study, NF-κB levels in the calves of group II and III were greater than those in group I. In fact, this elevation was shown to be statistically significant in group III compared to group I. It is thought that the possible reason for this elevation is macrophage activation in infected lymphoid cells, causing an increase in both NF-κB and TNF-α production. The moderate positive correlation between NF-κB and TNF-α supports this hypothesis.

TNF-α and IL-1β play a crucial role in the activation of the acute phase response. Under conditions of inflammation, the pro-inflammatory cytokines TNF-α and IL-1β, which are secreted by macrophages and monocytes, act as triggers for the production of anti-inflammatory proteins (APPs) by liver cells (Baumann and Gauldie 1994). A study by Zhang et al. (2019) reported the detection of pro-inflammatory cytokines, including TNF-α and IL-1β, being released by inflammatory cells in the alveoli during bacterial lung infections. An explanation for this rise was provided by the fact that pathogenic agents stimulated the activation of monocytes and macrophages, leading to an increase in TNF-α synthesis (Thacker 2006). Corroborating other studies, the calves in groups II and III had much higher levels of TNF-a compared to the calves in group I. The observed increase was determined to be statistically significant in group III as compared to group I. With increasing illness severity, the numerical values of IL-1β were seen to be greater in groups II and III compared to group I, but this difference was not statistically significant. The aforementioned outcome is believed to be caused by the stimulation of monocytes and macrophages by infectious pathogens in pneumonias. Furthermore, when the intensity of the infection becomes more severe, the levels of pro-inflammatory cytokines likewise rise. This notion is supported by the substantial positive connection between TNF-α and NLR.

IL-10 helps regulate pro-inflammatory cytokines and is produced by immune system cells to control tissue damage (Zhang and An 2007). However, with increasing clinical severity, IL-10 levels were higher for groups II and III. One potential explanation for this increase is believed to be the increased production of IL-10 in infected cells, which inhibits macrophages and T cells, as reported by Brown et al. (2008). This is supported by the fact that both WBC and NEU concentrations of calves in groups II and III were statistically higher than those in group I. The positive correlation between IL-10 and WBC and NEU in this study supports this hypothesis.

Cathelicidins are related components of inherited immunity that are responsible for activating cells responsible for inflammation, such as macrophages, NEUs, and B lymphocytes (Tecle et al. 2010). There are very few studies evaluating CAMP in calves with BRD. CAMP levels were reported to be higher in the patient group than in the healthy group in a study of calves with BRD (Koçer 2022). In an in vitro study, it has been documented that CAMP possesses antiviral activity against respiratory syncytial virus. Additionally, in both in vivo studies conducted in mice and humans, CAMP was found to serve a protective role (Leite et al. 2002). Tomasinsig et al. (2010) reported that cathelicidins trigger the release of TNF-α in bovine mammary cells. In this study, in parallel with the above studies, TNF-α levels were found to be higher in infected calves compared to healthy calves. This increase was found to be significant in group III compared to I. Lippolis and Reinhardt (2005) reported that cathelicidins are of NEU origin and that bovine NEUs produce cathelicidin. In our study, both NEU and WBC were positively correlated with CAMP, and in group II and III, NEU and WBC concentration of calves were increased compared with

group I. This elevation was found to be statistically significant in group III. Moreover, CAMP concentration of group II and III were also enhanced compared with group I. The observed rise is believed to be caused by the loss of epithelial cells and infection in the respiratory tract of ill calves, increased protection against respiratory infections, increased expression of Toll-like receptors 3, 7, and 9, and activation of immune systems (Martin and Lumsden 1987).

As a result, elevated WBC, NEU, NLR, NF-κB, TNF-α, and CAMP levels were found in group III, which consisted of animals with the highest severity score according to clinical scoring in BRD calves. NLR is seen as a potential marker since it is a low-cost, easily quantifiable, and repeatable assay for detecting/assessing inflammation.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: MSA
Supervision / Consultancy: MSA
Data Collection and / or Processing: MSE, Mİ
Analysis and / or Interpretation: ÖA, SK
Writing the Article: EE, KEY
Critical Review: ŞD

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