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RESEARCH ARTICLE

Investigation of Interleukins and Oxidative Stress Parameters in Cows Naturally Infected with Bovine Viral Diarrhea Virus

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A B S T R A C T

Bovine Viral Diarrhea Virus (BVDV) can evade the immune system by modulating cytokines production, therefore enabling the virus to establish persistent infections or exacerbate the severity of disease in infected cattle. The objective of this research was to investigate interleukins and oxidative stress parameters in cows naturally infected with BVDV. The study comprised of two groups: a naturally infected group of 15 cattle with clinical signs of BVDV infection or a history of abortion and confirmed positive for pestivirus by RT-PCR, and a control group of 15 cattle with no abortion anamnesis or clinical symptoms, confirmed negative by RT-PCR. Anti-inflammatory cytokines (IL-4, IL-20, IL-10) and pro-inflammatory ones (IL-1, IL-1β, IL-6) values were measured in all samples using ELISA method. Additionally, the oxidative stress marker malonaldehyde (MDA) was measured in all samples using the HPLC method. MDA levels were significantly higher in the infected samples compared with the controls. While cytokine levels were elevated in the infected group, however, the differences were not statistically significant. This study found a positive relationship among anti-inflammatory and pro-inflammatory cytokines, as well as a negative relationship among IL-1β and IL-1. It was concluded that oxidative stress occurs in BVDV-infected cattle, and the interleukins measured appear to remain in equilibrium by inhibiting each other. Notably, IL-20 was measured for the first time in BVDV-infected cattle, making it an important finding. Altogether, it may be concluded on the basis of these results that cytokines are important in the evaluation of the disease process.

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INTRODUCTION

Pestivirus infections lead to important financial damage in the cattle industry (Abdelsalam *et al.*, 2020; Dunowska *et al.*, 2024; Riaz *et al.*, 2024). BVDV is classified in the Pestivirus genus within the family Flaviviridae. BVDV is an endemic pestivirus infection that is globally widespread in cattle (Ahmad *et al.*, 2012; Lee *et al.*, 2014; Almeida *et al.*, 2017; Rúa Giraldo *et al.*, 2023). The most cattle herds are threatened by infection (Primawidyawan *et al.*, 2023). The widespread nature of the disease, its transmissibility and lack of treatment have made it globally enzootic and one of the most important cattle diseases (Khodakaram-Tafti and Farjanikish, 2017). The prevalence of this virus in Türkiye is reported to be between 46-86% and the presence of persistently infected animals can change between 0.07-4.9% (Timurkan and Aydın, 2019; Yıldız and Babaoglu, 2022; Simsek *et al.*, 2023). Even though acute BVDV infections typically present with no or mild clinical signs, they have been shown to induce lymphopenia and trigger a cascade of impact on the immune mechanism, setting the stage for the emergence of secondary infections (Risalde *et al.*, 2011).

The link between BVDV and the host's immune system appears to be a key feature in this complexity of infection (Larsson, 2010). Bovine Viral Diarrhea Virus

(BVDV) affects many types of cells but has a particular impact on critical parts of the immune system. It specifically targets monocytes and macrophages, as well as dendritic cells and various lymphocyte populations (Neill *et al.*, 2013). Targeting of these key immune cells causes a significant disruption in the body's defense system (Risalde *et al.*, 2011). Immunosuppression associated with acute infections and permanent damage to the fetus is the most obvious consequence of BVDV's ability to overwhelm the immune system (Larsson, 2010). As a result of the damage caused by BVDV, there are significant effects related to the death of immune cells, the expression of cytokines, and the release of co-stimulatory molecules. Thus, Thus, variations in the profile of cytokines generated by immune or non-immune cells can impact both congenital and specific immunity (Risalde *et al.*, 2011).

Oxidative stress can be defined as the balance of oxidants and antioxidants shifting to the oxidant side leading to cell damage. Oxidative stress is known to occur in the pathogenesis of viruses, bacteria and parasites that attack the organism. MDA (malondualdehyde) parameter has been shown to be an important biomarker in determining the oxidative stress process (Ertaş and Kirmizigül, 2021; Simsek *et al.*, 2023).

Cytokines are crucial for orchestrating immune responses and maintaining the homeostatic balance within the central nervous system (Akdoğan and Yöntem, 2018; Ozkan *et al.*, 2015). Cytokines are defined as low molecular weight proteins that aid cell-cell communications. They act by binding to their specific ligands in target cells (Akdoğan and Yöntem, 2018). Interleukins are an important part of the cytokines secreted by the immune system and their function is to stimulate immune system cells (Cui *et al.*, 2023). Cytokines are categorized into various groups according to their function or source and are produce by a diverse range of cells (Akdoğan and Yöntem, 2018). T helper cells are pivotal in this process. Th1 cells produce cytokines such as Interleukin (IL)-2, Interferon (IFN)-γ, and Tumor Necrosis Factor (TNF)-β, while Th2 cells release IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Waldvogel *et al.*, 2000; Anjum *et al.*, 2020).

Several studies have established that the level of interleukins is increased in a number of diseases. For example, Interleukin-6 (IL-6) has been found to be elevated in several inflammatory disturbances, including rheumatoid arthritis (RA) and Crohn's disease (Cronstein, 2007). In addition, it has been recorded that IL-1β levels increase during COVID-19 infection (Mardi *et al.*, 2021). IL-10 has also been reported to be elevated in BVD disease (Risalde *et al.*, 2011). Measurement of cytokines in some diseases is reported to be a guide for response to treatment and prognosis (Akdoğan and Yöntem, 2018). The goal of the present study was to investigate interleukins and oxidative stress parameters in naturally infected cows with BVDV in comparison with non-BVDV infected cows.

MATERIALS AND METHODS

Animals: The study comprised two groups. The naturally infected group included 15 cattle with clinical signs of BVDV infection or a history of abortion, confirmed positive for pestivirus by RT-PCR. The control group consisted of 15 cattle with no history of abortion or clinical symptoms, and they tested negative for pestivirus by RT-PCR. The blood samples were taken from cattle with clinical finding or history of abortion into vacuum tubes (Greiner, Bio-One, Germany) containing ethylene diamine tetraacetic acid (EDTA) and the blood samples were centrifuged at 2000 rpm for 10 minutes at 4°C to separate its components. After centrifugation, the upper plasma layer was discarded. From the remaining sample, 400µl of the peripheral blood mononuclear cell (PBMC) layer was carefully transferred into sterile 1.5 ml Eppendorf tubes. The tubes were then centrifuged a second time at 1500rpm for 5 minutes. The PBMC layer was washed three times with PBS, with each wash followed by centrifugation at 1500rpm for 5 minutes. The leukocytes were then stored at -80°C for future RNA extraction.

Extraction, RT-PCR: The RNA isolation kit (EURX, Poland) was used to extract viral RNA from 30 samples, adhering to the manufacturer's instructions. The extracted genomic RNA (gRNA) was preserved at -80°C until further use. Subsequently, these RNA samples were analyzed using a reverse transcription polymerase chain reaction (RT-PCR) with the OneStep RT-PCR kit (SOFTEC, Türkiye), following the manufacturer's instructions. The RT-PCR assay employed primers specific to the 5'-UTR region of the Panpesti gene, based on the method outlined by Vilcek *et al.* (1994) with some minor adjustments. Forward primer PP-F and reverse primer PP-R were used to amplify a 288 bp fragment of the pestivirus. Positive and negative controls were incorporated with each set of samples as part of the standard protocol.

Biochemical Analysis

Analyzing interleukins: Interleukins (IL-1β, IL-1, IL-6, IL-4, IL-20, IL-10) were measured with commercial enzyme linked immuno sorbent assay (ELISA) test. Samples prepared in accordance with the ELISA commercial kit guidlines and were measured using ELISA reader and the levels were recorded.

Oxidative stress analysis (HPLC Analysis); In a falcon centrifuge tube, 50 µL sample serum + 750 µL 0.44 µM H_3PO_4 + 250 µl TBA + 450 µl distilled water was added and placed in a boiling water bath for 60 minutes, then cooled on ice or in tap water. Then 1.5 ml of alkaline methanol prepared in a 1:1 ratio was added and centrifuged at 2500 rpm for 3 minutes. Malondialdehyde (MDA) quantification was then performed by HPLC analysis using 1260 Infinity liquid chromatograph systems (Agilent, USA). A reversed phase-HPLC column (Ace Generix 5C18 25 cm × 4.6 mm I.D., 5 µm particle size) was used for analysis. Chromatographic separation was performed using mobile phase A containing 25mM dipotassium phosphate (K_2HPO_4) in water and mobile phase B containing 100% methanol. The flow rate of the thermostatically controlled column at 25°C was set to 0.8 mL/min and the injection volume was 20 µL. The isochartic working system was used to determine 40% (Mobile phase

A) and 60% (Mobile phase C). Finally, the samples prepared according to the method were analyzed using a fluorescence detector (FLD) with excitation at 527 nm and emission at 551 nm.

Statistical analysis: The study assessed whether continuous measurements followed a normal distribution by analyzing skewness and kurtosis coefficients. To compare the groups, we employed the Mann-Whitney U test and the independent samples t-test. Pearson correlation coefficients were computed to evaluate the associations between the variables. A significance level of 5% (α) was used for the analyses. These analyses were conducted using IBM SPSS Statistics for Windows, version 26.

RESULTS

A total of 15 cattle showing positive results by RT-PCR for the presence of BVDV were selected for the study. Fig. 1 showed RT-PCR results revealing that out of 10 potentially diseased samples six samples (1, 3, 5, 6, 7, 9) were found genotypically positive for BVDV while four of these (2, 4, 8, 10) didn't show the bands and were considered negative. The blood samples from 15 positive animals along the RT-PCR negative control group (15-animals) were taken and processed for the presence of interleukins using ELISA kits.

Malondialdehyde (MDA) levels were compared among both control and patient groups which gave a mean MDA level of 153.06 and 264.51 for control and patient group, respectively. The standard error in the mean value was observed to be 8.15 and 51.12 for the control and patient group mean MDA levels, respectively (Table 1). The p-value of 0.048 suggests a significant level of increase in oxidative stress parameters for the patient group compared to the control group.

Table 1: Analysis of oxidative stress parameters show that there is an important statistical variety among the patient, control groups via MDA (p<0.05).

	Groups	N	Mean	Std. Error	Minimum	Maximum	p
MDA	Control	15	153.06	8.15	106.16	198.20	0.048*
	Patient	15	264.51	51.12	44.90	583.03	
	Total	30	250.73	48.10	44.90	583.03	

* Independent sample t-test

Interleukin parameters were analyzed using Mann Whitney U test, the mean value for IL-1, IL-1β, IL-4, IL-6, IL-10, and IL-20 was 25.17±4.30, 577.90±89.27, 94.20±28.83, 35.67±2.59, 145.62±32.01, and 110.50±9.12 respectively for control group while for patients it was estimated to be 29.62±7.51, 862.32±186.68, 136.29±28.83, 58.50±11.40, 225.24±74.41, and 143.98±24.83 respectively (Table 2 & Fig. 2). However, the p-value inferred no significant increase in any of the tested types of interleukins in the patient group.

The results of correlation analysis carried out on selected types of interleukins and MDA suggested a negative correlation between IL-1 and IL-1β with an r-value (correlation coefficient) of -0.643. However, significant positive correlations were observed between IL-1β and IL-4, IL-10, and IL-20 with a r-value of +0.837, +0.785, and +0.911, respectively (Table 3). Similarly, IL-4 shared a positive correlation with IL-10 and IL-20 with r-values of +0.782 and +0.908, respectively; while IL-10 and IL-20 showed a significant correlation of +0.937. Except the negative correlation between IL-1 and IL-1β which was significant at the level of 0.05, all other positive correlations were found to be highly significant at the level of 0.01 emphasising a direct proportionality between the parameters.

Table 2: Analysis of Interleukins parameters among the patient and control groups in terms of IL -1β, IL -1, IL -6, IL -20, IL -4, IL -10 (p>0.05).

Parameters	Groups	N	Mean	Std. Error	Minimum	Maximum	p
IL -1	Control	15	27.17	4.30	12.36	65.46	0.983#
	Patient	15	29.62	7.51	8.82	130.25	
	Total	30	28.40	4.26	8.82	130.25	
IL -1β	Control	15	577.90	89.27	249.31	1498.04	0.384#
	Patient	15	862.32	186.68	273.20	2490.14	
	Total	30	720.11	105.04	249.31	2490.14	
IL-4	Control	15	94.20	12.24	46.92	210.54	0.520#
	Patient	15	136.29	28.83	51.18	382.03	
	Total	30	115.24	15.87	46.92	382.03	
IL -6	Control	15	35.67	2.59	20.71	57.94	0.152#
	Patient	15	58.50	11.40	16.86	194.66	
	Total	30	47.09	6.12	16.86	194.66	
IL -10	Control	15	145.62	32.01	72.14	550.85	0.384#
	Patient	15	225.24	74.41	59.49	1180.91	
	Total	30	185.43	40.48	59.49	1180.91	
IL-20	Control	15	110.50	9.12	73.89	213.14	0.520#
	Patient	15	143.98	24.83	62.35	428.63	
	Total	30	127.24	13.37	62.35	428.63	

Mann Whitney U test

Table 3: Interleukins and Oxidative Stress Parameters Correlations Between Control and Patient Groups.

		Correlations						
Control Patient		IL -1	IL -1β	IL -4	IL -6	IL -10	IL -20	MDA
IL -1	r	-0.330	-0.630*	-0.388	0.007	-0.337	-0.285	0.231
	p	0.230	0.012	0.153	0.981	0.219	0.304	0.408
IL -1β	r	0.055	0.116	0.837**	-0.191	0.785**	0.911**	0.366
	p	0.846	0.681	0.000	0.495	0.001	0.000	0.180
IL -4	r	0.051	0.105	0.834**	-0.238	0.782**	0.908**	0.389
	p	0.858	0.709	0.000	0.393	0.001	0.000	0.152
IL -6	r	0.119	-0.002	-0.041	-0.001	-0.157	-0.163	0.061
	p	0.672	0.994	0.884	0.996	0.575	0.562	0.829
IL -10	r	-0.039	0.119	0.839**	-0.360	0.976**	0.937**	0.435
	p	0.892	0.673	0.000	0.187	0.000	0.000	0.105
IL -20	r	0.031	0.138	0.842**	-0.269	0.941**	0.987**	0.374
	p	0.912	0.625	0.000	0.332	0.000	0.000	0.169
MDA	r	0.339	0.103	-0.105	0.222	-0.180	-0.149	-0.063
	p	0.217	0.715	0.710	0.427	0.520	0.597	0.824

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed). r. correlation coefficient p. *p*-value.

Correlation analysis was also conducted between the interleukin parameters of the control group and the patient group which gave significant correlation results for IL-4, IL-10, and IL-20. IL-4 showed a significantly strong positive correlation of +0.834, IL-10 also presented a strong correlation of +0.976, and IL-20 apprehended a correlation of +0.987 (Fig. 3). Other interleukin parameters were however, not found significantly sharing any correlation between the patient and control group.

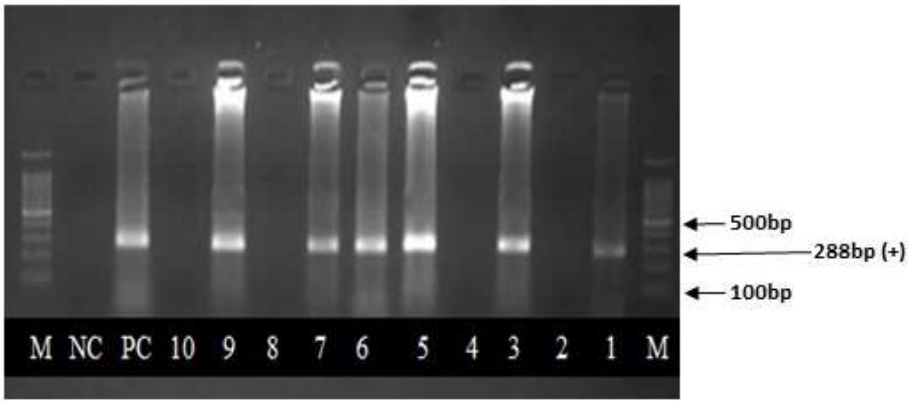

Fig. 1: RT-PCR results for Pestivirus 5'UTR nucleic acid detection (288bp). M: Marker (100 bp); NC: negative control; PC: positive control; positive samples: 1, 3, 5, 6, 7, 9; negative samples: 2, 4, 8, 10.

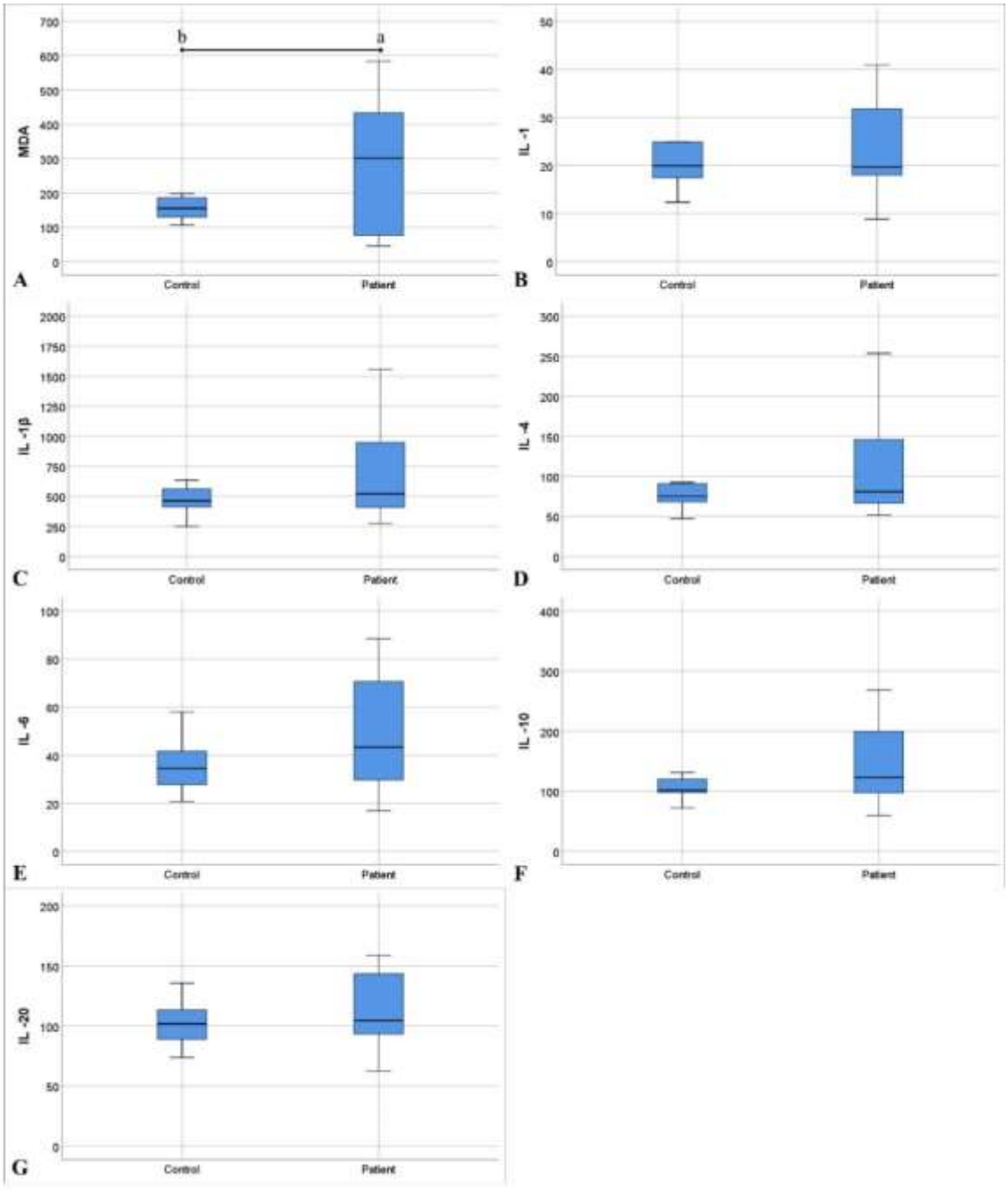

Fig. 2: Interleukins and Oxidative Stress Parameters Correlations (A: MDA, B:IL-1, C: IL-1β, D:IL-4, E:IL-6, F:IL-10, G:IL-20).

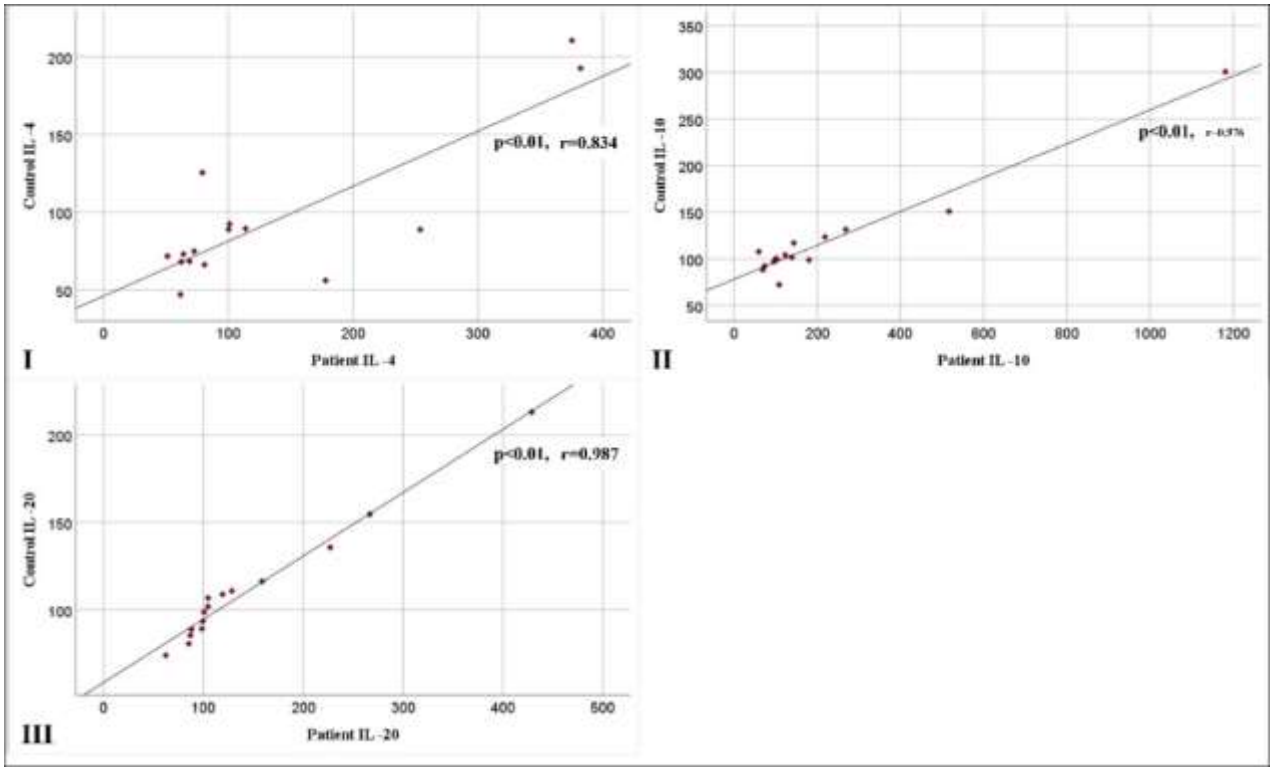

Fig. 3: Correlation graphs with significant (I: Control IL-4 and Patient IL-4; II: Control IL-10 and Patient IL-10; III: Control IL-20 and Patient IL-20).

DISCUSSION

BVDV is a significant pathogen affecting cattle herds and poses a major economic threat (Yıldız and Babaoglu, 2022; Wu *et al.*, 2023). Abortion is the most commonly observed symptom in cattle infected with the BVDV (Yang *et al.*, 2023). Similarly, nucleic acid from pestiviruses was detected in samples from animals that had either experienced abortion or had a history of abortion.

MDA level is an important marker used for oxidative stress. In studies, increased MDA levels were observed in some parasitic and viral diseases, cirrhosis, tumor, trace element deficiency and malnutrition. Increased serum MDA level indicates oxidative stress and cell damage (Ertaş and Kirmizigül, 2021). Şimşek *et al.* (2023) measured the MDA marker in two groups, BVDV and healthy, and reported that it was higher in the patient group. Likewise, in the present study, BVD was detected to be importantly higher in the naturally infected patient group. The reason for this is the belief that oxidative stress occurs.

Among the molecules that play important roles, cytokines constitute a key model in the design of the inflammation and immune system in the organism. The majority of cytokines are interleukins secreted by the immune system with a key role to stimulate the immune mechanisms (Akdoğan and Yöntem, 2018; Corrêa *et al.*, 2020). Interleukin levels are an important biomarker for the existence of the disease. Interleukins, which have been selected as immunotherapeutic agents, shape the immune response. Knowledge of interleukin responses may be fundamental for understanding the degree of infection at the clinical stage (Mingala *et al.*, 2009). The biological value of the polarized interleukin response in infectious diseases appears to correlate with the manifestation of T helper cells (Corrêa *et al.*, 2020). T helper (Th) cells are classified into several subgroups, namely Th1 and Th2 (Wagner *et al.*, 2010).

Palomares *et al.* (2014) experimentally infected calves with BVD virus and compared mRNA expression of pro-inflammatory (IL-2, L-1β) and anti-inflammatory (IL-10, IL-4) cytokines. It was found that mRNA values of IL-4, IL-2, IL-10 and IL-1β cytokines in tracheobronchial lymph node and spleen samples were higher in the high virulence group but lower compared with the control group. Similarly, Mingala *et al.* (2009) determined that IL-2 and IL-4 expressions were high in animals infected with BVDV. Moreover, Risalde *et al.* (2011) compared BHV-1.1-infected calves with healthy animals and observed a Th1 immune reply depended on reduced IL-10 production in BVDV-infected calves and no important modifications IL-4 production in two groups of BHV-1-infected calves. Risalde *et al.* (2011) also reported small increases in IL-6 in the livers of calves vaccinated with BVDV. In parallel, Seong *et al.*, (2016) reported that (IL)-6 was higher in mouse models infected with BVD2 than BVD1. Table 2 indicates that there were no statistically important differences in IL-1β, IL-1, IL-6, IL-4, IL-20, and IL-10, values among patient groups and the control ($p>0.05$). Unlike these studies Abdelselam *et al.* (2020) observed no significant difference in the mRNA levels of apoptosis-related cytokines (IL-6, TNF-α and IL-1β) in BVD-infected macrophages derived from bovine monocytes. Likewise, Dursun *et al.* (2021) measured IL-6 and IL-1β values in their study and stated that they did not find any statistical differences.

Researches on the levels of interleukins have found different values. As can be seen, some studies showed high levels while others found no differences (Dursun *et al.*, 2021). This may indicate host immune system is maintaining a balance between local and systemic pro-

inflammatory responses and equally robust anti-inflammatory mechanisms protecting against the worsening of inflammation. It is argued that if the anti-inflammatory response is too high, this will lead to a decrease in immune competence, so-called "second-strike" infections. Therefore, the local and systemic disproportion among pro- and anti-inflammation is a critical cause of multi-organ defects and the overall inflammatory response (Gerlach, 2016). In the light of this information, it was concluded that the probable reason for the lack of statistical significance of the elevated proinflammatory and anti-inflammatory cytokines in the present study is that these two reactions are in balance.

According to the researches, no study measuring interleukin 20 was found in the studies on cytokines measurements and BVD virus. This study presented here stands out by addressing the measurement of the interleukin 20 parameter for the first timeIL-20 is a key cytokine within the IL-10 family, known for its anti-inflammatory properties. TNF-α and IL-1β have been reported to induce IL-20 secretion, especially in macrophages (Hsu *et al.*, 2017; Caparrós and Francés, 2018;Yemenoglu *et al.*, 2024).

Yemenoğlu *et al.* (2024) measured the IL-20 parameter in periodontric patients and stated that it was found to be high. In another study, Encecik *et al.* (2018) measured IL-20 in Behçet's disease and stated that it was high in patients. Unlike these studies, our study is not statistically significant, although it is high in BVD infected.

When the research on correlation is examined, Yemenoglu *et al.* (2024) revealed a positive correlation among IL-20 and TNF-α, IL-1β. Schindler *et al.* (1990) stated that they found a positive correlation among IL-6 and IL-lβ in their study. Analogically among IL-1β with IL-4, IL-6, IL-10, IL-20 and IL-4 with IL-4, IL-10, IL-20 and IL-10 with IL-4, IL-10, IL-20 as well as IL-20 with IL4, IL-10, IL-20 have a positive correlation in this work reports. In addition, a negative correlation was discovered among IL-1β and IL-1. According to these results, this study proves that anti-inflammatory and pro-inflammatory ctokines are in a state of balance.

As a result, the present study revealed that MDA levels were importantly higher in infected samples compared to controls. While cytokine levels were elevated in the infected group, the increase was not statistically important. A positive correlation was observed between anti-inflammatory and pro-inflammatory cytokines, while a negative correlation was revealed among IL-1 and IL-1β. The study also indicated the presence of oxidative stress and demonstrated that anti-inflammatory and pro-inflammatory cytokines are balanced by inhibiting each other. Notably, IL-20 was measured for the first time in BVDV-infected cattle, highlighting its importance. Overall, cytokines can play a crucial role in understanding the disease process in animals.

Ethical Statement: This study was authorized by Animal Research Local Ethic Committee of Van Yuzuncu Yil University (approval date: 26/10/2023, decision no. 2023-12-02)

Conflict of Interest: There is no conflict of interest.

Authors contributions: FEO: Planned, collected samples, checked and finalized the manuscript. ARB: analyzed the virus by PCR, read and checked the manuscript. AFD: made statistical analysis, FT and EO measured interleukins by ELISA method. and checked the manuscript. NP and EO: measured MDA patrameter by HPLC method.

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