Effect of Circulating Calcitriol ($1,25(OH)_2D_3$) Levels on Th1/Th2 Immune Response in Bovine Ephemeral Fever

PELIN FATOŞ POLAT DINÇER[1]*, ADEM ŞAHAN[2], MEHMET BERAT NAYIR[3]

[1] Department of Internal Medicine, Faculty of Veterinary Medicine, Dokuz Eylul University, Izmir, Turkey
[2] Department of Internal Medicine, Faculty of Veterinary Medicine, Harran University, Sanlıurfa, Turkey
[3] Department of Virology, Faculty of Veterinary Medicine, Dokuz Eylul University, Izmir, Turkey

SUMMARY

This study aimed to investigate the changes in Th1, Th2, and Th17 cytokine levels resulting from inflammation in cattle naturally infected with bovine ephemeral fever (BEF) and to explore the relationship between these changes and vitamin D and its metabolites. The study sampled 28 BEF positive cattle, whose samples were diagnosed by polymerase chain reaction (PCR), and 15 healthy cattle as a control group. The BEF group had significantly lower serum $1,25(OH)_2D_3$ levels than the control group (P=0.000) whereas there was no significant difference between the two groups in $25(OH)D_3$ levels (P=0.063). Among the serum cytokines, IL-2 and IL-4 were increased in the BEF group compared to the control (P=0.005, P=0.001). While IL-10 and IL-17 levels were higher in the BEF group than the control group (P=0.000), there was no significant difference between the two groups in IFN-γ levels (P=0.083). BEF group had lower total protein (TP), calcium (Ca) and phosphorus (P) levels (P=0.000, P=0.000, and P=0.002 respectively). In addition, the percentage increases in cytokines in cattle with BEF were determined as IL-17 (37.78%), IL-10 (27.00%), IL-4 (16.66%), IL-2 (15.01%) and IFN-γ (2.93%), respectively. These findings indicate that the Th1 response is partially suppressed, whereas the Th2 and Th17 responses are significantly increased in cattle infected with BEF. In addition, it was determined that extrarenal $1,25(OH)_2D_3$ synthesis was decreased in cattle infected with bovine ephemeral fever virus (BEFV). This suggests that the BEFV suppresses $1,25(OH)_2D_3$ synthesis and causes dysfunction in the steps within the immune system involved in immune response.

KEY WORDS

Cattle; cytokine; interleukin; three-day sickness; vitamin D.

INTRODUCTION

Bovine ephemeral fever (BEF) is a single-stranded RNA virus belonging to the family *Rhabdoviridae*. It is a seasonal, weather dependent, vector-borne disease endemic in Africa, Australia, the Middle East, and tropical regions of Asia (1,2). The disease, which causes high morbidity and significant economic losses, progresses with certain clinical signs, such as transient polyphasic fever, muscle stiffness, paralysis, synovitis, paresis, lameness and/or anorexia (3,4). Due to its inflammatory effects, BEF can also cause vasculitis, tendovaginitis, polyserositis and fibrinous exudate accumulation in the pericardial and joint spaces (3,5). Neutrophils play a serious role in the appearance of clinical signs and the development of the humoral immune response. The pathology occurring in BEF infection may be due to a cytokine storm resulting from altered vascular permeability and the associated inflammatory response (6). Although there are different views about the potential role of proinflammatory cytokines in the disease (3,7,8), anti-inflammatory drugs have been used for years considering the disease's inflammatory characteristics. Inflammation advances as a result of the action of inflammatory cytokines, including interferon-gamma (IFN-γ), interleukin-1 (IL-1) and interleukin-12 (IL-12). Resolution occurs through the use of anti-inflammatory cytokines, such as interleukin-10 (IL-10), interleukin-4 (IL-4), and interferon alpha (IFN-α) (9). Under physiological conditions, cytokine inhibitors function as immunomodulators, mitigating the detrimental effects of inflammatory reactions. In pathological conditions, the effect of anti-inflammatory mediators on proinflammatory activity, especially in diseases affecting the immune system, may be insufficient, suppressive, or excessive (10). In other words, there is a dynamic and constantly changing balance between cytokines in the immune system (11). The clinical approach to inflammation typically centres around inhibiting the production of proinflammatory mediators and suppressing the initiation of the inflammatory response. This involves dampening the positive signalling pathways associated with proinflammatory cytokines (9). Understanding the inflammatory response mechanism in diseases is very important, especially for the development of vaccines and treatment methods.

Although vitamin D plays important roles in calcium metabolism and bone mineralization, it also affects immunity (12). Calcifediol ($25(OH)D_3$) determines the current circulating vi-

Corresponding Author:
Pelin Fatoş Polat Dinçer (pelinfatos.polat@deu.edu.tr)

tamin D status and acts as a substrate for the synthesis of calcitriol ($1,25(OH)_2D_3$) (13). The active form of vitamin D, $1,25(OH)_2D_3$, is synthesized extrarenal in dendritic cells, monocytes, and macrophages. It plays a role in defending against pathogens by binding to vitamin D receptors (VDR) in cells associated with innate immunity (14). VDR is a nuclear steroid hormone. When activated by $1,25(OH)_2D_3$, modified gene expression occurs (15). In acquired immunity, $1,25(OH)_2D_3$ activates pathways that provide self-tolerance. In other words, while Th1/Th2 cytokines are in balance in the physiological process, changes in $1,25(OH)_2D_3$ affect the cytokine balance (16).

In our study, we aimed to determine the cytokine and circulating extrarenal $1,25(OH)_2D_3$ levels related to the immune response in cattle naturally infected with BEF.

MATERIALS AND METHODS

Animal selection and collection of samples

The study material comprised 50 cattle aged 1-4 years old, brought to the Internal Medicine Clinic of Harran University Animal Hospital during the spring and summer of 2021-2022, exhibiting BEF and clinical signs such as lameness, stagnation, and high fever. Polymerase chain reaction (PCR) was performed on the samples taken into EDTA tubes from 50 samples with suspicious clinical symptoms. Of these samples, 28 BEFV (Bovine ephemeral fever virus) positive cattle were detected. The control group was established through clinical examinations of cattle within the same age group from a disease-free farm. Samples were taken from 15 cattle for the control group. After blood samples were collected in tubes without anticoagulant, they were centrifuged at 3000 rpm for 10 minutes and stored at -20°C until tests were performed.

RNA extraction and RT-PCR for the G protein gene

To isolate BEFV RNAs from clinical specimens, the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany) was utilized, following the manufacturer's guidelines. The purified viral RNAs were then preserved at -80°C until subjected to RT-PCR (Reverse Transcription-Polymerase Chain Reaction) analysis. The cDNA synthesis was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA), while PCR was done using 420F (5' AGA GCT TGG TGT GAA TAC 3') and 420R (5' CCA ACC TAC AAC AGC AGA TA 3') primers for detecting the BEFV partial G gene. The PCR reactions were performed using an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 40 sec, at 46°C for 60 sec, at 72°C for 40 sec and a final extension at 72°C for 10 min (17). After electrophoresis using 1% agarose gels, the amplified PCR products were visualized under UV light in a UPV GelSolo System (Analytik Jena, Germany) (Figure 1).

Evaluation of vitamin D metabolites, immunity and biochemistry profile

After the samples had been thawed at room temperature, the cytokine levels were determined using the ELISA reader Multiskan FC Microplate Photometer (Thermo Scientific, USA). The following species-specific ELISA kits were used: $1,25(OH)_2D_3$ (BT Lab, China, No: EA0052Bo), $25(OH)D_3$ (BT Lab, China, No: E0315Bo), interleukin-2 (IL-2) (BT Lab, China, No: E0225Bo), IL-4 (BT Lab, China, No: E0036Bo), IL-10 (BT Lab, China, No: EA0034Bo), interleukin-17 (IL-17) (BT Lab, China, No: E0069Bo), IFN-γ (BT Lab, China, No: E0005Bo). To determine the biochemical parameters, specifically, phosphorus (P) (mg/dL), calcium (Ca) (mg/dL) and total protein (TP) (g/dL) value a Fuji Dri-Chem NX500i (Fujifilm Corporation, Japan) veterinary biochemistry analyzer was used.

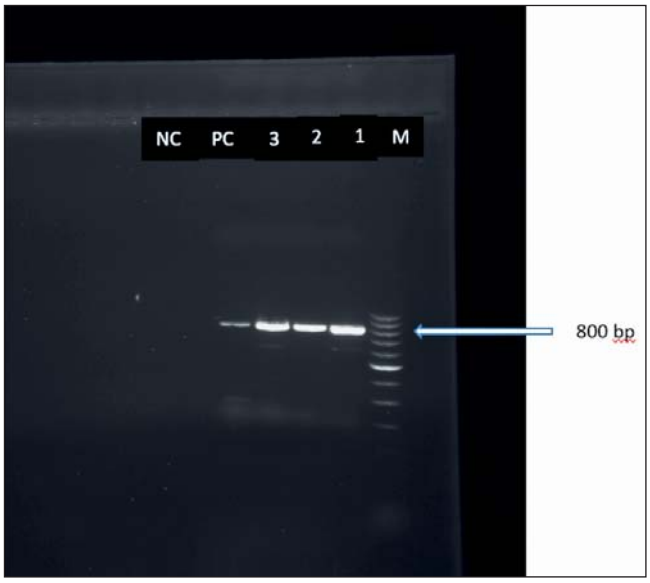

Figure 1 - BEFV RT-PCR gel image. Lanes M: 100 bp DNA ladder. Lane 1,2 and 3: Positive PCR products, PC: Positive Control (800 bp), NC: Negative Control.

Statistical analysis

Histogram and q-q plots were analyzed, and the Shapiro-Wilk test was employed to evaluate the normality of the data. Additionally, the Levene test was utilized to assess the homogeneity of variances. Group differences were compared using the independent groups t-test, a parametric statistical method. The associations between quantitative variables were examined through Pearson correlation analysis. All statistical analyses were conducted using R 4.2.0 software (www.r-project.org).

RESULTS

The PCR analysis confirmed that 28 cattle were BEF detected (Figure 1).

Serum $1,25(OH)_2D_3$ levels were markedly lower in the BEF group compared to the control group (P=0.000), while there was no significant difference in $25(OH)D_3$ levels (P=0.063) (Figure 2).

Among the serum cytokines, IL-2 and IL-4 levels were significantly elevated in the BEF group (P=0.005, P=0.001). Likewise, IL-10 and IL-17 levels were notably higher in the BEF group (P=0.000), while there was no significant difference in IFN-γ levels between the two groups (P=0.083). Additionally, TP, Ca, and P levels were significantly lower in the BEF group (P=0.000, P=0.002) (Table 1).

More specifically, the percentage differences in cytokine levels in BEF-infected cattle were 37.78%, 27%, 16.66%, 15.01% and

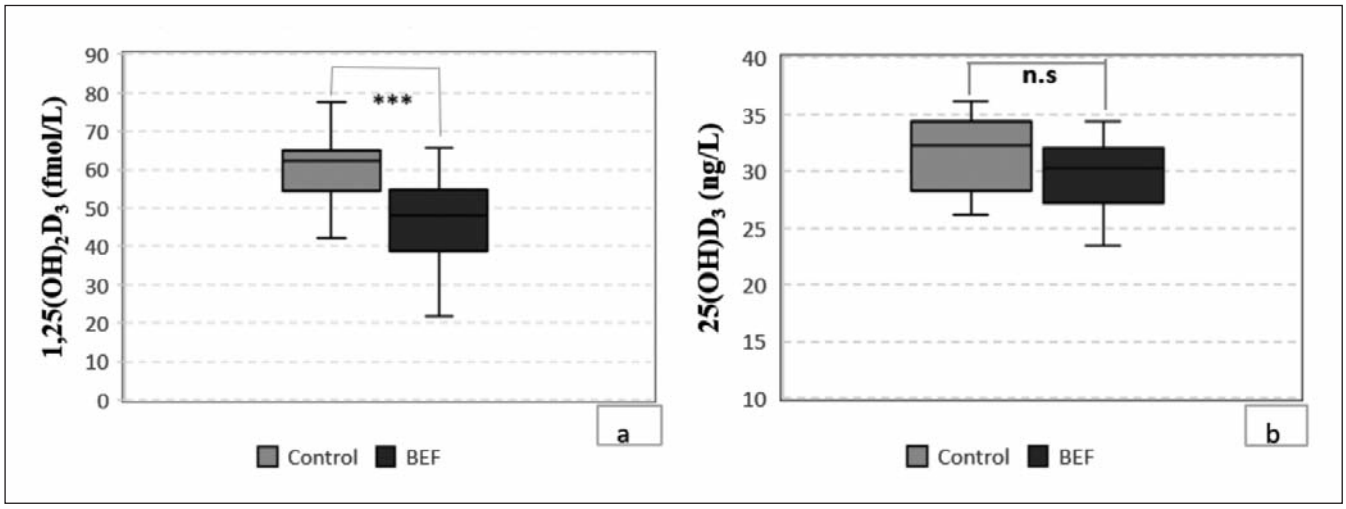

Figure 2 - (a) Difference and statistical significance of serum 1,25 $(OH)_2D_3$ level of BEF and control group (b) Difference and statistical significance of serum 25$(OH)D_3$ level of BEF and control group. ***: P<0.001, ns: non significant.

2.93% for IL-17, IL-10, IL-4, IL-2 and IFN-γ, respectively (Figure 3).

Figure 4 shows the correlations, and r and p values for the relationships between cytokines, vitamin D, and the biochemical variables in the BEF group. Serum 1,25$(OH)_2D_3$ levels showed positive correlations with IL-2, IL-4, IL-10, 25$(OH)D_3$, TP, and Ca values, while exhibiting negative correlations with IFN-γ, IL-17, and P values (Figure 4).

DISCUSSION

BEF is an inflammatory disease characterized by acute, high fever with a complex pathogenesis. Infection causes many different signs and increases the number of young neutrophils in the circulation and levels of plasma fibrinogen while decreasing calcium levels (3,4). Physiologically, decreased serum calcium levels result in elevated parathyroid hormone concentrations, increasing the production of 1,25$(OH)_2D_3$ and intestinal calcium absorption (18). The current study aimed to investigate alterations in proinflammatory, Th1, Th2, and Th17 cytokine levels due to hypocalcemia and inflammation in cattle naturally infected with BEFV and to explore the association with vitamin D and its metabolites.

Many studies have investigated the immunomodulatory effect stimulated by 1,25$(OH)_2D_3$ (19,20,21) and its relationship with autoimmunity (22) and infectious diseases. Dabak *et al.* reported an important increase in extrarenal 1,25$(OH)_2D_3$ in the circulation in malignant catarrhal fever disease cases, independently of calcium homeostasis (21). In their experimental study of tuberculosis in cattle, Rhodes *et al.* found that extrarenal 1,25$(OH)_2D_3$ synthesis in macrophages increased serum 1,25$(OH)_2D_3$ levels. Although vitamins A and D use is recommended to prevent hypocalcemia in postpartum cows, they detected a temporary increase in 1,25$(OH)_2D_3$ levels simultaneously with increased susceptibility to other infections, such as mastitis, metritis and paratuberculosis, that may be caused by hypocalcemia (20). Consequently, understanding the impact of recommended supplements for disease treatment on immunity and immune related vitamin D levels are crucial for prognosis and assessing potential complications. In this study,

Table 1 - The arithmetic mean, standard deviation of the 25$(OH)D_3$, 1,25$(OH)_2D_3$, IL-2, IL-4, IL-10, IFN-γ, IL-17, TP, P and Ca values of the cattle in the BEF and control groups and the importance of the difference between the groups.

Variables	BEF group (*n*=28)	Control group (*n*=15)	*P*	
25$(OH)D_3$ (ng/mL)	29.82±2.98	31.73±3.41	0.063	ns
1,25$(OH)_2D_3$ (pg/mL)	46.36±11.14	60.05±9.39	**<0.001**	***
IL-2 (ng/L)	26.82±4.87	23.33±2.76	**0.005**	*
IL-4 (ng/L)	88.59±9.86	75.93±12.91	**0.001**	**
IL-10 (ng/L)	200.32±9.71	157.73±11.09	**<0.001**	***
IFN-γ (pg/mL)	177.08±8.52	172.05±9.48	0.083	ns
IL-17 (pg/mL)	404.08±11.69	293.28±8.76	**<0.001**	***
TP (g/dL)	6.11±0.37	6.97±0.16	**<0.001**	***
P (mg/dL)	6.58±0.38	6.95±0.30	**0.002**	**
Ca (mg/dL)	6.81±0.29	10.17±0.65	**<0.001**	***

Values are expressed as mean±SD. ns: non significant (P>0,05), *:P<0.05, **: P<0,005, ***: P<0,001

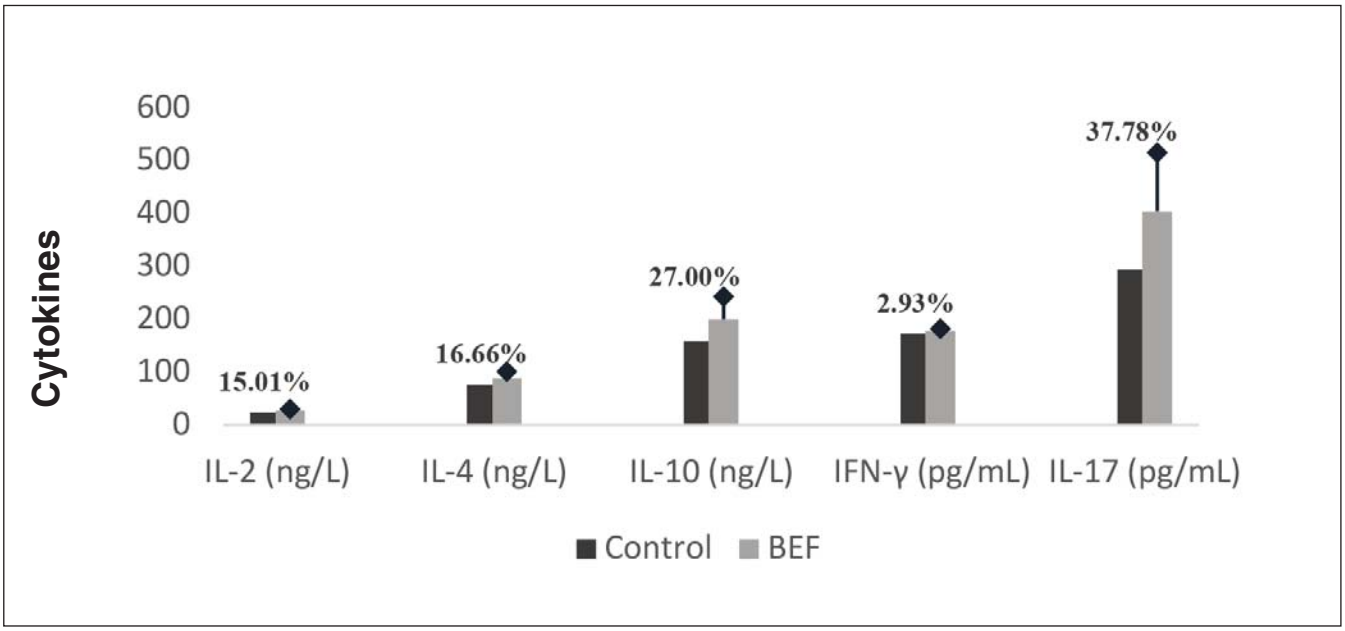

Figure 3 - Percentage cytokine increases in BEF cattle.

we observed no elevation in 1,25(OH)$_2$D$_3$ levels in BEF-infected cattle; conversely, these levels were lower than in healthy cattle, while 25(OH)D$_3$ levels remained consistent in both groups. Haug *et al.* indicated that 25(OH)D$_3$ levels were within the normal range, while 1,25(OH)$_2$D$_3$ levels were lower in patients infected with human immunodeficiency virus (HIV). Under typical conditions, 25(OH)D$_3$ exhibits a concentration 1,000 times higher than that of 1,25(OH)$_2$D$_3$. They concluded that for 25(OH)D$_3$ to affect 1,25(OH)$_2$D$_3$ levels, it should be almost absent in circulation, so the resulting decrease could be due to many reasons, including a lack of precursor and binding protein (19).

Comprehending the immune mechanisms in diseases constitutes a pivotal step in developing of various preventive measures, including diagnostic markers (23). Uren presented the first study of the immune response in BEF at a conference. They found that plasma IL-1 and TNF-α expression increased in BEF-infected cattle (7). Barigye also examined the plasma kinetics of cytokines by comparing the plasma expression of four cytokines. He reported that IL-1 levels were higher than those of TNF-α, IL-10, and interleukin-6 (IL-6) (3). Abo-Sakaya reported that BEFV affects the immune system and raises se-

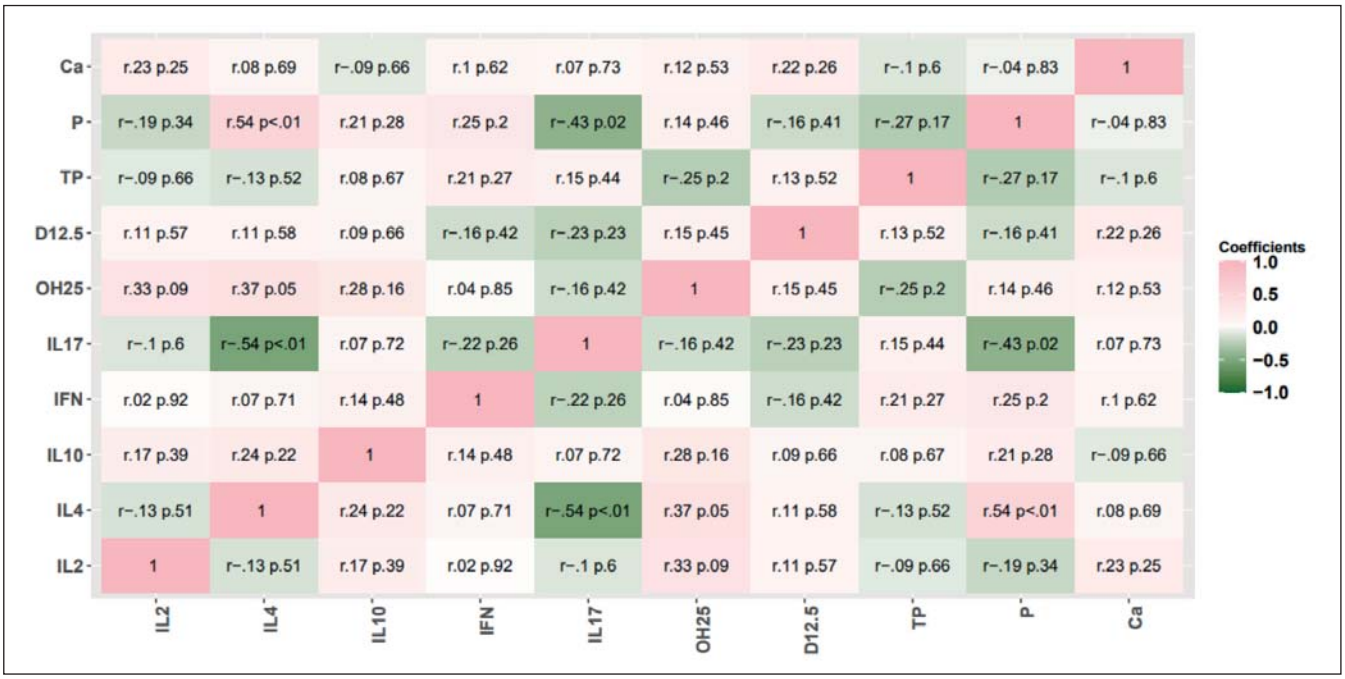

Figure 4 - The Pearson correlation between cytokines, vitamin D, and biochemical variables in the BEF group was depicted in the graph. Positive correlations are indicated in pink, while negative correlations are represented in green, as illustrated in the scaled gradient on the right side of the graph. The colour intensity of the boxes corresponds to the correlation coefficients. The correlation coefficient and p-values (r=.23, p=.25) are displayed inside each box.

rum IL-2 and IL-4 levels (8). Barigye *et al*. followed four heifer patients for 9 days, including the viremia period, finding a significant increase in IL-10 compared to the negative control, while IL-6, IFN-γ, and IL-2 levels also increased. The viremia stage lasted longer in those animals with the lowest IFN-γ and IL-2 levels (24). Th1 cells (IFNγ, IL-2) are necessary mediators for Tc cell formation and NK cell activation (25). In the present study, IL-2 levels were higher in patients than in control cattle, whereas IFN-γ levels were not important different. Although these results suggest a Th1 response in terms of IL-2, the IFN-γ response is disrupted. Th2 cells (IL-4 and IL-10) are effective against extracellular pathogens and stimulate the proliferation of B cells and the synthesis of immunoglobulins from plasma cells (26). In our study, patients had raised levels of IL-4, which is important for the development and functionalization of Th2 cells. The BEF group also had higher levels than the control group of IL-10, which plays an important role in responding to infections. More specifically, it has anti-inflammatory properties, inhibits macrophages, and helps minimise organismal damage by stimulating B lymphocytes (27). Another effector cell with different properties is Th17, which produces IL-17. The latter provides strong stimulation in acute and chronic inflammations, and may even cause inflammatory tissue damage up to autoimmunity (28). In our study, IL-17 levels were significantly higher in patients, probably due to the enhancement of the decreased innate immunity and thereby improved protection against infection. The levels of TP, P, and Ca were all lower in patients than in controls. Hypophosphatemia may be caused by prolonged low intake, decreased absorption, or increased renal tubular losses (29). The resulting hypocalcemia may be based on hypoproteinemia (30). In addition, hypocalcemia, which is a known effect of BEF, raises parathyroid hormone levels in the parathyroid glands, which in turn increases $1,25(OH)_2D_3$ levels (4,31). However, we found that lower $1,25(OH)_2D_3$ levels were related to low Ca levels. This suppression of $1,25(OH)_2D_3$ may be related to many variables that vary with the disease, such as phosphate, calcitonin, prolactin, glomerular filtration, and parathyroid hormone (PTH) that affect 1a-hydroxylation enzyme levels (32). Kamr *et al*. reported lower $1,25(OH)_2D_3$ levels in conjunction with hypocalcemia in newborn septic foals and suggested that PTH receptor resistance may contribute to the pathogenesis of calcium, phosphorus, and vitamin D homeostasis (33).

One limitation of the study is that PTH levels were not measured due to financial constraints. Another limitation is that measurements were not taken daily. In particular, serum cytokine and $1,25(OH)_2D_3$ levels may vary over the course of the disease. However, since our study sampled cattle naturally infected with BEF and there was a flow of patients from different districts of the city to our hospital, sampling could only take place during the active period when the cattle had observable signs. We therefore suggest that future studies in which measurements are taken at different stages from disease onset to the recovery period will improve our understanding of immunopathogenesis. In addition, there are many factors affecting the organism in vivo studies. This type of study can be performed with isolated immune cells, and the results can be evaluated more illuminating.

CONCLUSIONS

In conclusion, the findings of the present study indicate that in cattle infected with BEFV, the Th1 response is partially suppressed, while the Th2 and Th17 responses are significantly increased. Contrary to previous findings, extrarenal synthesis of $1,25(OH)_2D_3$ in inflammatory diseases is decreased in cattle infected with BEF. Although $25(OH)D_3$ levels do not change, lower $1,25(OH)_2D_3$ levels appear to be the reason why BEFV suppresses $1,25(OH)_2D_3$ synthesis and causes dysfunction in certain steps in the immune system.

Ethical approval

This study was granted ethical approval permission of the Harran University Local Ethics Committee of Experimental Animals (11.12.2020 and Decision No: 01-17).

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Authors' contributions

PFPD: conceptualization and supervision; PFPD: Funding acquisition; PFPD, A : sample collection and ELISA analysis; PFPD, A : statistical analysis; PFPD, A : writing, review and editing.

Conflict of interest statement

The authors declare no conflict of interest.

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