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Evaluation of Some Intestinal Biomarkers in the Determination of Intestinal Damage in Calves with Coccidiosis

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ABSTRACT

This study aimed to assess the usefulness of injury biomarkers specific to the intestines in identifying the presence and degree of intestinal epithelial damage in coccidiosis-infected calves. Forty calves of various breeds and sexes, aged 21 days to 60 days, were used in the study. Of these, 30 were in the experimental group, and 10 were healthy control. The McMaster Oocyte counting technique was used to diagnose *Eimeria* and confirm clinical coccidiosis. Cases with clinical signs and more than 5,000 oocysts in gram feces were included in the study. All calves had blood samples drawn at the 0th hours and 72nd hours. Blood gas measurements were performed with a blood gas analyzer. Hemogram was performed with an automated hematologic analyzer. Bovine-specific enzyme-linked immunosorbent analysis (ELISA) test kits were used to measure the intestinal fatty acid binding protein (I-FABP), trefoil factor-3 (TFF-3), intestinal alkaline phosphatase (IAP), claudin-3 (CLD-3), intestinal smooth muscle actin (ACTG2), and interleukin-8 (IL-8) biomarkers from serum samples. Calves with coccidiosis received a single dose of toltrazuril (15 mg/kg) and supportive care. Before treatment (0th hours) in coccidiosis-infected calves, serum I-FABP and CLD-3 levels were greater than in healthy calves ($p<0.05$), and after treatment (72nd hours), serum TFF-3 and ACTG2 levels were higher than in healthy calves. There was a significant decrease in serum IL-8 levels in coccidiosis-infected calves after treatment (72nd hours) compared to pre-treatment (0th hours) ($p<0.05$). I-FABP, TFF-3, CLD-3, ACTG2, and IL-8 are helpful and reliable biomarkers that can be utilized to assess the presence of intestinal epithelium injury in coccidiosis-infected calves.

Keywords: coccidiosis; intestinal damage biomarker; neonatal calf

INTRODUCTION

Coccidiosis, a parasitic disease caused by *Eimeria*-type protozoa, settles in the gastrointestinal tract and causes enteritis (Bangoura & Bardsley, 2020). The disease causes significant financial losses for farms and poultry enterprises (Ok *et al.*, 2019; Bangoura & Bardsley, 2020). Young animals are particularly vulnerable to coccidiosis, which causes hemorrhagic enteritis, weakness, and weight loss (Sudhakara Reddy *et al.*, 2015; Bangoura & Bardsley, 2020). The formation of coccidiosis is facilitated by stress-inducing factors such as inadequate care and barn hygiene on cattle farms, starvation, transportation, and weaning in calves (Eglenti *et al.*, 2020). Sporozoites from orally ingested oocysts settle in the intestines, multiply in epithelial cells, and cause significant epithelial cell destruction, causing villi loss and malabsorption (Bangoura & Bardsley, 2020). In human medicine, there has been a focus on using intestine-related biomarkers in assessing intestine damage brought on by various diseases.

While some intestine-related biomarkers act only as a signal of the damage that develops in the intestines, they are responsible for repairing the damaged part or preventing damage formation (Lu *et al.*, 2013; Benkoe *et al.*, 2014). Numerous investigations on intestinal injury biomarkers, such as I-FABP, L-FABP, TFF-3, CLD-3, IAP, IL-8, ACTG2, and LP, have been performed in the field of veterinary medicine (Yildiz *et al.*, 2018; Yildiz *et al.*, 2019; Gulersoy *et al.*, 2020; Ok *et al.*, 2020; Yildiz & Ok, 2022).

I-FABP, one of the fatty acid binding proteins, is released into the bloodstream in large amounts in acute ischemic intestinal injury (Guzel *et al.*, 2014), in infants with necrotizing enterocolitis (NEC) (Ng *et al.*, 2013; Coufal *et al.*, 2020; Huo *et al.*, 2021, Howarth *et al.*, 2022), in children infected with Giardia (Cascais-Figueiredo *et al.*, 2020), and in people with inflammatory bowel disease (Sarıkaya *et al.*, 2015). Serum levels of I-FABP and L-FABP were reported to be significantly increased in atresia coli (Yıldız *et al.*, 2018), premature calves with and without respiratory distress syndrome (RDS) (Yıldız *et al.*, 2019), dogs with parvoviral enteritis (Gülersoy *et al.*, 2020) and isosporiasis (Yıldız & Ok, 2022), and calves with neonatal diarrhea (Ok *et al.*, 2020). It has also been suggested that these biomarkers can be

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used as diagnostic biomarkers to determine intestinal epithelial damage.

Intestinal trefoil factor (TFF-3) is released by mucus cells in the intestines. It has been shown that in enterocolitis patients, the TFF-3 concentration rises along with L-FABP and I-FABP following the inflammatory response brought on by intestinal mucosal injury (Ng *et al.,* 2013; Nakov *et al.,* 2019). TFF-3 is a useful diagnostic and prognostic biomarker for colorectal and gastric cancer (Taniguchi *et al.,* 2018, Espinoza *et al.*, 2021). Premature calves with respiratory distress syndrome (Yildiz *et al.,* 2019), dogs with isosporiasis (Yildiz & Ok, 2022), calves with atresia coli (Yildiz *et al.,* 2018), and calves with neonatal diarrhea (Ok *et al.,* 2020) have been shown to have higher levels of TFF-3 than healthy ones.

Intestinal alkaline phosphatase (IAP) is believed to be one of the mucosal defense components necessary to maintain intestinal microbiota homeostasis (Malo *et al.,* 2014, Fawley & Gourlay, 2016; Bilski *et al.,* 2017; Santos *et al.,* 2022). Crohn's disease patients had low serum IAP levels (Park *et al*., 2018). Calves with atresia coli were found to have higher serum IAP levels than healthy calves (Yıldız *et al*., 2018). In addition, serum IAP concentrations of calves with neonatal enteritis were significantly higher than healthy calves (Ok *et al*., 2020). According to research by Yildiz & Ok (2022), dogs with isosporiasis had considerably higher serum IAP levels than healthy dogs.

Claudins are integral membrane proteins that range in size from 20 to 27 kDa. They have two extracellular loops, N and C terminal cytoplasmic regions, and four hydrophobic transmembrane domains (Koval, 2013). It has also been reported that Claudin-3 release is severely reduced in people with inflammatory bowel disease (Zeissig *et al.,* 2007). Claudin-3 levels are elevated in patients with psoriasis and have been considered to indicate intestinal barrier malfunction and damage (Sikora *et al.,* 2019; Ahmed *et al.,* 2021). It has been reported that Claudin-3 levels are lower in calves with neonatal enteritis (Ok *et al.,* 2020) and respiratory distress syndrome (Yildiz *et al.,* 2019) compared to healthy ones. It has been reported that while Claudin secretion decreases in acute colitis (Mennigen *et al.,* 2009), it increases in patients with celiac disease (Adriaanse *et al.,* 2017).

The ACTG2 gene in humans encodes the protein known as intestinal smooth muscle actin (Actin, gamma-enteric smooth muscle, or ACTG). Actins are proteins that help different types of cells move and repair their cytoskeletons (Lattanzi *et al.,* 2003). According to studies, newborn enterocolitis has elevated levels of ACTG2, and this biomarker may be a helpful tool for identifying intestinal muscle injury (Evennett *et al.,* 2014). It has been demonstrated that ACTG2 levels were elevated in calves with enteritis caused by *E. coli* (Ok *et al.,* 2020), respiratory distress syndrome (Yildiz *et al.,* 2019), and dogs with isosporiasis (Yildiz & Ok, 2022).

Interleukin-8, a chemokine family member, is an essential chemotactic and activating peptide for neutrophils (Daig *et al.,* 1996). Eckmann *et al.* (1993) reported that intestinal cells produce IL-8 under typical circumstances. A study of people with Crohn's disease and ulcerative colitis showed that IL-8 concentration increased (Daig *et al.,* 1996) and may function as a specific biomarker for enterocolitis (Benkoe *et al.,* 2014). According to reports, IL-8 is a helpful marker for determining the need for surgery in newborns with NEC (Seo *et al.,* 2021). IL-8 levels were reported to be significantly higher in calves with neonatal diarrhea caused by various etiologies (Ok *et al.,* 2020).

Determination of intestinal damage in coccidiosis is currently performed by biopsy, which is an invasive method. Non-invasive determination of intestinal damage in animals with coccidiosis by biomarkers and determination of the changes in biomarker levels before and after treatment may contribute to disease follow-up and treatment monitoring in the herd in the future. The study aimed to determine whether intestine-related injury biomarkers might be used to assess the presence of intestinal epithelium damage in coccidiosis-infected calves.

MATERIALS AND METHODS

Animal Material

Thirty calves (17 females, 13 males; age range: 21–60 days; breed: 19 Holstein, 11 Simmental) brought to Selcuk University Veterinary Faculty, Large Animal Internal Medicine Clinic with the complaint of bloody diarrhea were used as animal material. The animal material in the control group (10 calves of 4 males and 6 females, between the ages of 21-60 days; breed: 10 Holstein) were provided by Prof. Dr. Humeyra Ozgen Research and Application Farm, Selcuk University Veterinary Faculty. The body weights of the calves included in the study were between 50-55 kg. The Ethics Committee of the Selcuk University, Faculty of Veterinary Medicine (No. 2019/85) approved the use of animals for this study and all study protocols.

Calves with Coccidiosis

For every calf included in the study, the breed, age, and gender were noted. The calves were subjected to standard clinical examinations. Calves with complaints of straining and bloody diarrhea, the feces samples were collected and forwarded to the parasitology laboratory.

Coccidiosis was diagnosed by applying the Fülleborn flotation technique and McMaster Egg Counting Technique to determine Oocyte per gram (OpG). In the initial diagnosis, animals with Oocytes per gram (OpG) values above 5.000 (Bangoura *et al.,* 2012) and showing clinical symptoms were diagnosed with coccidiosis. Fecal samples obtained from calves with coccidiosis were tested for *E. coli* K 99, rotavirus, coronavirus, and cryptosporidium by fecal rapid antigen tests (BoviD-5 Ag ®, BIONOTE, Sweden) and calves with negative results were included in the study.

Reduced skin elasticity (>4 seconds), enophthalmia (>3 mm), pale mucous membranes, dry mouth, breast, cold extremities, inability to stand or lie in the sternal position, a moderate and higher degree of dehydration

in calves with diarrhea acknowledged as an inclusion criterion (Sen *et al.*, 2009).

Healthy Calves

Calves whose fecal samples did not contain oocysts, routine clinical examinations, and laboratory tests (hemogram and blood gas analysis) were normal, and fecal rapid antigen tests (BoviD-5 Ag ®, BIONOTE, Sweden) negative were considered healthy and included in the control group.

Collecting of Blood Samples

Anticoagulant and non-anticoagulant blood samples were collected from the *vena jugularis* of all calves before (0^{th} hours) and after treatment (72^{nd} hours). As much as 2.5 mL Heparin injector for blood gases, 5 mL tube with K_3 EDTA for hemogram analysis, and 8 mL vacuum tube were used for biomarker measurements. Analyses of hemograms and venous blood gases were performed between 15 minutes and 1 hour following blood collection. Blood collected in vacuum tubes for biomarker measurement was centrifuged at 20× *g* for 10 min after clotting. Sera were removed and stored at -80 °C.

Collecting of Feces Samples

Before and after treatment (0^{th} hours and 72^{nd} hours), feces were collected by rectal touching in feces sample receptacles. Feces samples were collected and forwarded to the parasitology laboratory and were examined for oocysts within an hour using the Fülleborn Flotation technique at the 0^{th} hours and 72^{nd} hours. In addition, the McMaster oocyte counting technique was initially applied to confirm clinical coccidiosis. The examination of feces by flotation and the McMaster method was performed based on Reinecke (1983).

Hemogram Analysis

Complete blood count (WBC, LYM, MON, GRA, RBC, MCV, HTC, MCH, MCHC, RDW, HB, and PLT) from K_3 EDTA venous blood samples collected from all calves at 0^{th} and 72^{nd} hours on MS4e device (CFE 279 hematology Analyzer, Melet Schlosing Laboratories, France) was made.

Blood Gas Analysis

pH, pCO_2, pO_2, sO_2, K, Na, Ca, Glu, Lac, and HCO_3 parameters from the heparinized venous blood samples drawn from all calves at 0^{th} hours and 72^{nd} hours were measured in the blood gas device (ABL 90 FLEX, Blood Gas/Electrolyte analyzer, radiometer Medical ApS, Denmark).

Intestinal Biomarkers Measurement

Serum I-FABP, TFF-3, IAP, Claudin-3, ACTG2, and IL-8 biomarker concentrations were measured from blood samples drawn from all calves at 0^{th} hours and 72^{nd} hours, bovine - specific ELISA test kits (Shanghai coraine Biotech Co, Ltd) following the manufacturer's instructions. Concentrations were measured using a microplate reader (ELx800 Absorbance Microplate Reader, United States). Intra-assay (within-assay) and inter-assay (between-study) CV reported for I-FABP (Cat. No. E0066Bo) <8% and <10%, respectively, while MDC Intra-assay and inter-assay CV reported for 0.28 ng/mL, detection range 0.5 ng/mL-150 ng/mL, TFF-3 (Cat. No. E0369Bo) <8% and <10%, MDC 0.017 ng/mL, detection range 0.05 ng/mL-15 ng/mL, intra-assay (within-run) and inter -assay reported for IAP (Cat. No. E0371Bo) -assay (inter-run) CV <8% and <10%, respectively, MDC 0.28 ng/mL, detection range 0.5 ng/mL-150 ng/mL for CLD-3 (Cat. No. E0370Bo) reported intra-assay and inter-assay CV <8% and <10%, respectively, and MDC 3.62 ng/L detection range 7 ng/L-2500 ng/L, ACTG2 (Cat Intra-assay (within-run) and inter-assay (inter-run) CV reported for .No: E0363Bo) <8% and <10%, respectively, MDC 0.028 ng/mL, detection range 0.05 ng/mL-30 ng/mL, intra-assay (within-run) and inter-assay (between-run) CV reported for IL-8 (Cat. No. E0002Bo) <8% and <10%, respectively, and MDC 2.39 ng/L the detection range was 5 ng/L-1000 ng/L.

Treatment Protocol

Calves with coccidiosis received standard supportive care, fluid-electrolyte therapy and anticoccidial therapy. As anticoccidial therapy, toltrazuril (Coc-Cide® Alke, Istanbul) was administered once at a dose of 15 mg/kg (Sultana *et al.*, 2017; Ok *et al.*, 2019). According to the degree of dehydration (body weight x dehydration %), the fluid volume to be administered during fluid-electrolyte treatment was estimated and planned. Due to blood gas measurement, isotonic 0.9% NaCl was given to calves without acidemia. Isotonic (1.3%) $NaHCO_3$ (Carbotek ®, Teknovet, Istanbul) was administered in instances with metabolic acidosis (6 cases) by the base deficit determined in blood gases (body weight x 0.6 x base deficit). In addition to these therapies, a 0.5g/kg serum containing 5% dextrose (Dextrosol ®, Vilsan, Istanbul) was given as needed. Tranexamic acid (Transamin ®, Actavis, Istanbul) was parenterally supplied for hemorrhagic enteritis at 20 mg/kg. 3 mL of vitamin C (Vita -C Vetoquinol ®, Novakim, Kocaeli) and vitamins A, D, and E (Ademin®, Ceva, Istanbul) were given parenterally once a day as a supportive treatment. All calves were fed fresh milk (10% of body weight) three times a day by the bottle.

Statistical Analysis

The data were evaluated using the Statistical software SPSS 25 (IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY: IBM Corp). One to indicate whether all data are parametric or non -parametric, Samle Kolmogorov-Smirnov test was applied. One for Student's t-test and variance analysis of parametric data Way ANOVA was evaluated as mean ±SD with Tukey test, and median (min/max) with Kruskal - Wallis test

for non-parametric ones. The spearman correlation test was used to determine the correlation between variables. The significance level of the tests was accepted as $p<0.05$ and $p<0.01$.

RESULTS

Clinical Findings

The experimental group of calves included in the study showed bad-smelling, mild to severe bloody diarrhea that lasted for a few days, and feces with blood in the perineum and tail region. Moderate and severe dehydration and increased capillary refill time (>2 seconds) were observed in all calves, while body temperature was within the normal reference range in all the calves. While there were varying degrees of decrease in the sucking reflex and loss of appetite in 22 cases, the appetite was normal in 8 of them. Significant tenesmus was observed in 11 cases with coccidiosis. All the calves with coccidiosis responded to the treatment, recovered, and were discharged.

Blood Gas Findings

Blood pH, pO_2, and sO_2 levels of the calves with coccidiosis at 0^{th} hours were statistically significantly lower than the control group ($p<0.05$) (Table 1). It was observed that sO_2 increased significantly after the treatment (72^{nd} hours) compared to the 0^{th} hours ($p<0.05$) (Table 1). Na levels of calves with coccidiosis at the 0^{th} hours were statistically significantly lower than the control group ($p<0.05$) (Table 1). A statistically significant decrease in HCO_3 level was determined in the experimental group calves at the 0^{th} hours and 72^{nd} hours compared to the control group. While the lactate level was found to be statistically significantly higher in calves with coccidiosis compared to the control group at the 0^{th} hour ($p<0.05$), there was no statistical difference compared to the control group at the 72^{nd} hour (Table 1).

Hemogram Findings

Compared to the control group, the number of WBCs in coccidiosis-infected calves increased statistically significantly ($p<0.05$). At the 0^{th} and 72^{nd} hours,

Table 1. Venous blood gas findings of calves with coccidiosis and healthy

Variables	Control group (n:10)	Calves with coccidiosis	
		0^{th} hours (n:30)	72^{nd} hours (n:30)
pH	7.37 ± 0.02[a]	7.32 ± 0.07[b]	7.38 ± 0.03[a]
pCO_2 (mmHg)	46.01 ± 3.34	44.41 ± 6.21	43.43 ± 6.29
pO_2 (mmHg)	43.32 ± 15.6[a]	33.83 ± 7.96[b]	38.64 ± 8.41[ab]
sO_2 (%)	73.42 ± 16.48[a]	59.95 ± 16.55[b]	71.76 ± 10.52[a]
K (mmol/L)	4.32 ± 0.36	4.64 ± 0.64	4.26 ± 0.56
Na (mmol/L)	149.90 ± 3.69[a]	131.56 ± 9.66[b]	135.93 ± 9.37[b]
Ica (mmol/L) (Median/Min-Max)	0.95 (0.80-1.10)	1.00 (0.70-1.20)	1.00 (0.70-1.20)
Cl (mmol/L)	105.60 ± 3.74	103.36 ± 7.24	101.23 ± 5.67
Glucose (mg/dL)	76.90 ± 7.26	79.70 ± 15.74	77.20 ± 12.29
HCO_3 (mmol/L)	26.37 ± 1.26[a]	21.7267 ± 4.01[b]	25.63 ± 1.91[a]
Lactate (mmol/L)	1.18 ± 0.67[a]	2.49 ± 1.51[b]	1.52 ± 0.82[a]

Note: pCO_2= Partial carbon dioxide pressure, pO_2= Partial oxygen pressure, sO_2= Oxygen saturation, K= Potassium, Na= Sodium, ICa= Ionized calcium, Cl= Chlorine, HCO_3= Bicarbonate. Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 2. Hemogram findings of calves with coccidiosis and healthy

Variables	Control group (n:10)	Calves with coccidiosis	
		0^{th} hours (n:30)	72^{nd} hours (n:30)
WBC (m/mm^3)	11.42 ± 3.03[a]	17.59 ± 9.28[b]	13.99 ± 5.20[ab]
LYM (m/mm^3) (Median/Min-Max)	7.27 (3.69-14.90)	7.66 (2.82-42.48)	6.84 (2.89-19.63)
MON (m/mm^3) (Median/Min-Max)	0.51 (0.15-0.96)[a]	0.78 (0.52-1.15)[b]	0.77 (0.42-9.00)[b]
GRA (m/mm^3) (Median/Min-Max)	2.84 (0.90-6.67)[a]	6.58 (2.55-13.45)[b]	5.00 (2.25-9.25)[b]
RBC (m/mm^3)	9.75 ± 0.86[ab]	10.78 ± 2.18[a]	9.00 ± 1.63[b]
MCV (fl)	31.83 ± 2.91[a]	35.05 ± 2.59[b]	34.04 ± 2.51[a]
HCT (%)	33.04 ± 4.18[a]	38.60 ± 7.36[b]	36.36 ± 5.25[ab]
MCHC (g/dL)	31.47 ± 0.99[a]	32.66 ± 1.12[b]	33.11 ± 1.44[a]
HB (g/dL)	10.35 ± 1.08	10.94 ± 2.26	10.82 ± 1.39
PLT (m/mm^3)	344.80 ±183.66	391.53 ±145.26	423.16 ±105.47

Note: WBC= Total leukocyte, LYM= Lymphocyte, MON= Monocyte, RBC= Erythrocyte, MCV= Mean erythrocyte volume, HCT= Hematocrit, MCHC= Mean erythrocyte hemoglobin concentration, HB= Hemoglobin, PLT= Platelet. Means in the same row with different superscripts differ significantly ($p<0.05$).

the monocyte (MON) and granulocyte (GRA) counts were statistically higher compared to the control group ($p<0.05$) (Table 2). In coccidiosis-infected calves, the RBC count was considerably lower 72nd hours after treatment than it was at the start (0th hours) ($p<0.05$) (Table 2). At 0th hours, compared to the control group, the mean erythrocyte volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were statistically higher ($p<0.05$) (Table 2). At the 0th hour, it was discovered that the HCT of the calves in the experimental group was statistically substantially greater than that of the control group ($p<0.05$) (Table 2).

Biomarker Findings

It was determined that the serum I-FABP concentrations of calves with coccidiosis at the 0th hours and 72nd hours were significantly higher than the control group ($p<0.05$) (Table 3). Calves with coccidiosis had statistically higher serum TFF-3 levels after treatment (72nd hours) compared to the control group ($p<0.05$) (Table 3). When compared to the control group, there was no statistically significant difference in the serum IAP level of the coccidiosis-infected calves before (0th hours) and after (72nd hours) treatment ($p>0.05$) (Table 3). Calves with coccidiosis had significantly higher serum Claudin-3 concentrations than the control group before treatment (0th hours) ($p<0.05$) (Table 3). At 72nd hours, there were statistically significantly higher serum ACTG2 levels in coccidiosis calves compared to the control group ($p<0.05$) (Table 3). Serum IL-8 concentrations in coccidiosis calves decreased statistically significantly after treatment (72nd hours) compared to pre-treatment (0th hours) ($p<0.05$) (Table 3).

Correlation Analysis Findings

Biomarker concentrations, blood gases, and hemogram parameters of the calves included in the study are presented in Table 4. There were positive correlations between serum I-FABP concentration and IAP ($p<0.05$), CLD-3 ($p<0.01$), and ACTG2 ($p<0.01$) concentrations, but a negative correlation between serum TFF-3 concentration and IL-8 concentration ($p<0.05$). The positive correlations ($p<0.05$) were found between serum IAP levels and CLD-3 and IL-8 levels. There were also positive correlations between serum CLD-3 concentration and ACTG2 ($p<0.01$) and IL-8 ($p<0.05$). Serum I-FABP and lactate concentrations positively correlate ($p<0.01$). Serum IL-8 concentrations had positive correlations with WBC ($p<0.01$) and RBC ($p<0.05$) but a negative correlation with HCO_3 ($p<0.01$). Serum TFF-3 concentra-

Table 3. Intestine-related biomarker findings in calves with coccidiosis and healthy

Variables	Control group (n:10)	Calves with coccidiosis	
		0th hours (n:30)	72nd hours (n:30)
I-FABP (ng/mL)	3.50 (3.00-5.00)[a]	4.96 (3.30-7.84)[b]	4.76 (3.00-9.65)[b]
TFF-3 (ng/mL)	0.19 (0.06-1.07)[a]	0.46 (0.03-4.53)[ab]	0.86 (0.03-6.52)[b]
IAP (ng/mL)	0.40 (0.00-1.10)	0.45 (0.09-1.27)	0.34 (0.07-2.62)
CLD-3 (ng/L)	31.73 (19.39-118.15)[a]	51.84 (11.09-82.48)[b]	33.09 (10.24-70.18)[a]
ACTG2 (ng/mL)	0.63 (0.28-0.74)[a]	0.70 (0.01-3.07)[ab]	0.93 (0.01-2.19)[b]
IL-8 (ng/L)	65.09 (38.57-82.61)[ab]	71.82 (16.00-116.77)[b]	47.84 (5.52-9.65)[a]

Note: I-FABP= Intestinal fatty acid binding protein, TFF-3= Intestinal trefoil factor-3, IAP= Intestinal alkaline phosphatase, CLD-3= Claudin-3, ACTG2= Intestinal smooth muscle actin gamma 2, IL-8= Interleukin-8. Means in the same row with different superscripts differ significantly ($p<0.05$).

Table 4. Correlation results between biomarker concentrations and blood gases and hemogram findings (Spearman correlation analysis) (* $p<0.05$, ** $p<0.01$)

Variables	I-FABP	TFF-3	IAP	CLD-3	ACTG2	IL-8	pO_2	sO_2	Lactate	HCO_3	WBC	RBC
I-FABP	1.000	0.112	.304 *	.463 **	.423 **	0.060	-0.002	0.038	.306 **	-0.133	0.133	0.120
TFF-3		1.000	0.119	-0.038	-0.037	-.301 *	-0.055	0.030	-0.198	0.035	-.279*	-0.179
IAP			1.000	.263*	0.147	.254*	.312**	.296*	0.086	-0.075	-0.108	-0.023
CLD-3				1.000	.425**	.279*	0.072	0.013	0.230	-0.178	0.219	0.176
ACTG2					1.000	-0.189	.245*	0.130	0.148	0.151	-0.006	-0.075
IL-8						1.000	-0.166	-0.194	0.091	-310**	.384**	.282*
pO_2							1.000	.806**	-0.200	0.045	-0.128	-0.187
sO_2								1.000	-.245*	0.088	-0.195	-0.106
Lactate									1.000	-360**	0.173	0.108
HCO_3										1.000	-0.221	-0.179
WBC											1.000	.325**
RBC												1.000

Note: I-FABP= Intestinal fatty acid binding protein, TFF-3= Intestinal trefoil factor-3, IAP= Intestinal alkaline phosphatase, CLD-3= Claudin-3, ACTG2= Intestinal smooth muscle actin gamma 2, IL-8= Interleukin-8, pO_2= Partial oxygen pressure, sO_2= Oxygen saturation, HCO_3= Bicarbonate, WBC= Total leukocyte count, RBC= Erythrocyte count.

tions were found to have a negative correlation with WBC ($p<0.05$). There was a positive correlation found between serum ACTG2 concentration and pO_2 ($p<0.05$) (Table 4).

DISCUSSION

The use of biomarkers in determining the size and extent of intestinal damage caused by various etiological agents in humans and animals is becoming more common. The damage and extent of *Eimeria* species in the intestinal mucosal barrier in calves with coccidiosis were revealed for the first time in this study using intestine-related damage biomarkers, a non-invasive method.

Intestinal fatty acid binding proteins are protein molecules only synthesized by enterocytes (Zenger *et al.*, 2021). It has been reported that the level of I-FABP is significantly increased in acute mesenteric ischemia (Guzel *et al.*, 2014; Khadaroo *et al.*, 2014; Zenger *et al.*, 2021), Celiac disease (Oldenburger *et al.*, 2018), giardiasis (Cascais-Figueiredo *et al.*, 2020), and necrotizing enterocolitis (Ng *et al.*, 2013; Shaaban *et al.*, 2021; Huo *et al.*, 2021). Serum I-FABP and L-FABP concentrations are higher in calves with atresia coli than in the healthy calves (Yıldız *et al.*, 2018). It has been demonstrated that I-FABP concentrations measured from plasma and peritoneal fluid in horses with colic can be used for survival and the necessity for abdominal surgical intervention (Nieto *et al.*, 2005). Serum I-FABP and L-FABP concentrations are higher in calves with atresia coli than in the healthy calves (Yıldız *et al.*, 2018). I-FABP concentrations measured in plasma and peritoneal fluid in horses with colic have been shown to predict survival and the need for abdominal surgical intervention (Nieto *et al.*, 2005). It was reported that serum I-FABP and L-FABP concentrations increased significantly in calves with neonatal diarrhea, and these biomarkers were important and reliable in revealing intestinal epithelial injury (Ok *et al.*, 2020). It has been reported that the serum level of I-FABP in dogs with parvoviral enteritis is higher than in healthy dogs (Gulersoy *et al.*, 2020; Ay *et al.*, 2022). Yıldız & Ok

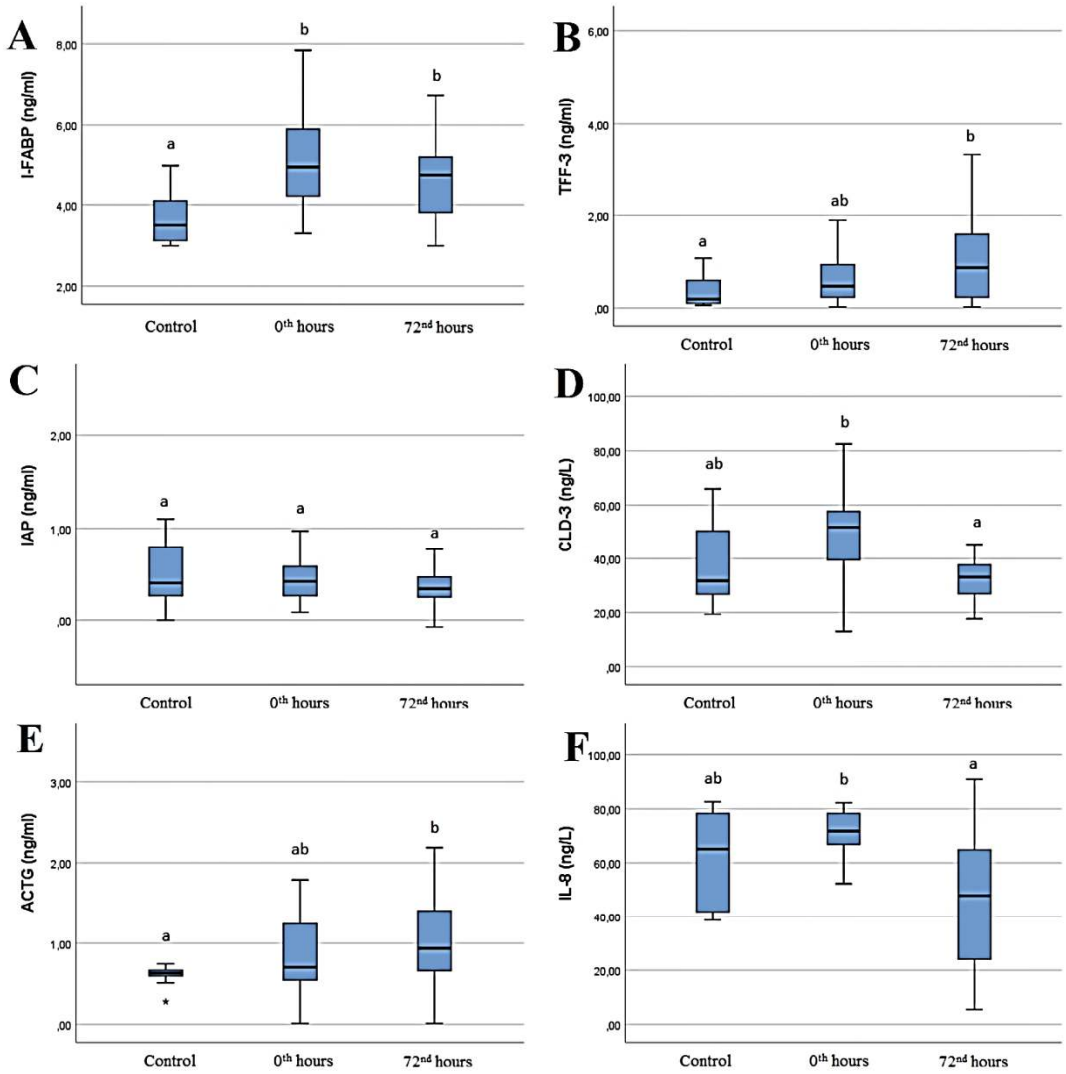

Figure 1. Intestine-related biomarker concentrations in calves with coccidiosis and healthy controls. I-FABP= Intestinal fatty acid binding protein, TFF-3= Intestinal trefoil factor-3, IAP= intestinal alkaline phosphatase, CLD-3= Claudin-3, ACTG2= Intestinal smooth muscle actin gamma 2, IL- 8= Interleukin-8. Different letters (a, b, c) on the treatments are statistically significant ($p<0.05$).

(2022) reported that serum I-FABP levels in dogs with isosporiasis were higher than in healthy dogs, and I-FABP levels were reduced following therapy.

The present study determined that the serum I-FABP level before (0^{th} hours) and after (72^{nd} hours) treatment in calves with coccidiosis was statistically significantly higher than in healthy calves. As reported by many researchers (Ng *et al.,* 2013; Guzel *et al.,* 2014; Yildiz *et al.,* 2018; Yildiz *et al.,* 2019; Cascais-Figueiredo *et al.,* 2020; Gülersoy *et al.,* 2020; Ok *et al.,* 2020; Huo *et al.,* 2021; Shaaban *et al.,* 2021; Yıldız & Ok, 2022), serum I-FABP concentration was significantly increased in the current study. Therefore, it was believed that I- FABP is a useful and reliable marker for detecting intestinal damage due to different causes of the coccidia agents. (Table 3, Figure 1A). The positive correlations of I-FABP with ACTG2, CLND-3, IAP, IL-8, and lactate further enhance the usefulness and reliability in assessing intestinal injury (Table 3). An increased lactate level may indicate that the intestinal microcirculation is compromised, contributing to intestinal damage. It was evaluated that the increase in serum I-FABP concentration in calves with coccidiosis was related to the conversion of fatty acid-binding proteins into circulation due to damage to the enterocytes during the life cycle of the intestines. According to several investigators' studies (Gulersoy *et al.,* 2020; Ok *et al.,* 2020; Ay *et al.,* 2022; Yıldız & Ok, 2022), the persistence of elevated serum I-FABP levels after therapy (72^{nd} hours) is likely the outcome of intestinal damage in coccidiosis in a short period. It was considered as unable to be corrected, and the damage persisted in the current study.

In the present study, it was determined that serum TFF-3 levels in calves with coccidiosis did not show statistical significance before treatment (0^{th} hours) compared to the control group but showed a significant increase after treatment (72^{nd} hours) (Table 3, Figure 1B). This finding was consistent with previously published research (Ng *et al.,* 2013; Srivastava *et al.,* 2015; Gulersoy *et al.,* 2020; Yıldız *et al.,* 2018; Ok *et al.,* 2020). The possible reason for the lack of pre-treatment increase is that TFF-3 is associated with repair and damage. This situation may be related to the lack of rapid release from goblet cells, such as another biomarker. The reason for the increase in serum TFF-3 level after treatment is epithelial destruction by *Eimeria*. It can also be interpreted that the intense release of oocysts from goblet cells to repair the epithelial injury created in the intestines may be related. On the other hand, Yıldız & Ok (2022) found that there was no statistically significant rise in serum TFF-3 levels in dogs with isosporiasis compared to the control group before therapy. It was shown to be compatible, confirming the conclusions of our study that this marker's potential significance in the healing of intestinal injury may account for the considerable rise after therapy. The negative correlations of TFF-3 with IL-8 and WBC suggest that TFF-3 is associated with the repair, and IL-8 and WBC are associated with an inflammatory response.

Studies conducted in mice lacking the IAP gene induced by experimental colitis revealed that intestinal inflammation was more severe than in normal mice (Kühn *et al.,* 2020). IAP has been reported to be higher in patients with necrotizing enterocolitis (Kampanatkosol *et al.,* 2014). IAP levels are also low in people with Chron's disease (Park *et al.,* 2018). It has been reported that serum IAP concentration is significantly higher in calves with Atresia coli (Yıldız *et al.,* 2018), dogs with isosporiasis (Yıldız & Ok, 2022), and calves with neonatal diarrhea (Ok *et al.,* 2020). In the present study, there was no statistically significant difference in serum IAP concentration before (0^{th} hours) and after (72^{nd} hours) treatment in calves with coccidiosis compared to the control group (Table 3, Figure 1C). *Eimeria* agents mostly cause damage to the caecum and colon, but IAP is released much less in these parts of the intestines (Malo *et al.,* 2014). This condition is most likely the cause of the lack of a rise in serum IAP levels seen in coccidiosis-infected calves in the current investigation. We think oocysts may cause intestinal damage, mainly in the jejunum, caecum, and colon, because IAP release capacity is low in this part of the intestine. High serum IAP levels were detected in many studies (Kühn *et al.,* 2020; Ok *et al.,* 2020) and are primarily detected in small intestinal inflammation supports our view.

It has been determined that CLD -1, -3, -4, and -5 releases are significantly reduced in acute colitis in humans compared to healthy subjects (Mennigen *et al.,* 2009). Zeissig *et al.* (2007) reported that CLD-3 concentration was lower in people with Crohn's disease, indicating intestinal barrier dysfunction. However, it has been reported that CLD-3 level is higher in Psoriasis patients than in healthy people (Sikora *et al.,* 2019; Ahmed *et al.,* 2021), CLD-2 and CLD-3 levels are significantly increased in Celiac patients (Adriaanse *et al.,* 2017). It has been reported that CLD -3 concentrations are lower in premature calves with respiratory distress syndrome (Yıldız *et al.,* 2019) and calves with neonatal diarrhoea (Ok *et al.,* 2020) than in healthy calves. In the present study, serum CLD-3 level was significantly higher than the control group before treatment (0^{th} hours) (Table 3, Figure 1D). We believe that the potential cause of the rise before therapy may be connected to the rise in CLD-3 release as a preventative measure to reinforce the intestinal tight junctions during the early stage of intestinal injury, as reported by other researchers (Sikora *et al.,* 2019; Ahmed *et al.,* 2021). In addition, there are positive correlations between CLD-3 and I-FABP ($p<0.01$), IL-8 ($p<0.05$), ACTG2 ($p<0.01$), and IAP ($p<0.05$) levels (Table 4) showing that these biomarkers have essential relationships between the body's inflammatory response, damage, and repair to the pathological condition in the intestines due to different causes.

Unlike other biomarkers, the ACTG2 is used to detect damage in the deep muscle layer of the intestine rather than in mucosal barrier damage (Pontell *et al.,* 2011; Ok *et al.,* 2020; Yıldız & Ok, 2022). It was reported that ACTG2 and I-FABP levels were significantly increased in experimentally induced ischemic intestinal damage in rats and newborn infants with necrotizing enterocolitis (Evennet *et al.,* 2014). It has been reported that serum ACTG2 levels are higher in calves with diarrhea caused by *E. coli* (Ok *et al.,* 2020) and in premature

calves with respiratory distress syndrome (Yıldız *et al.*, 2019) compared to healthy ones. There was a statistically significant increase in ACTG2 levels in dogs with isosporiasis after treatment compared to healthy dogs (Yıldız & Ok, 2022). In the present study, there was no significant difference in serum ACTG2 concentrations of calves with coccidiosis before treatment (0^{th} hours) with the control group. In contrast, a significant increase was detected after treatment (72^{nd} hours) compared to the control group (Table 3, Figure 1E). The severe intestinal damage, including the intestinal muscle layer, in enterocolitis caused by *Eimeria* species in calves (Bangoura & Bardsley, 2020), the repair of the muscle layer taking a more extended period, and the need for more ACTG2 release for repair are possible explanations for the observed increase in serum ACTG2 level after treatment. Yıldız & Ok (2022) supported our results with the high post-treatment serum ACTG2 concentration in isosporiasis of dogs with similar intestinal pathogenesis. Additionally, our findings are consistent with the high course observed following therapy in premature calves with RDS (Yıldız *et al.*, 2019) and calves with enteritis caused by *E. coli* (Ok *et al.*, 2020). In addition, we determined a positive correlation which further strengthened the availability and reliability of this biomarker, between ACTG2 biomarker and I-FABP ($p<0.01$), CLD-3 ($p<0.01$), and pCO_2 ($p<0.05$) concentrations in calves with coccidiosis (Table 4).

While the release of interleukin-8 occurs in healthy intestinal cells, IL-8 release increases intestinal damage (Eckmann *et al.*, 1993). In humans, significantly increased IL-8 concentrations have been reported in Crohn's disease, ulcerative colitis (Daig *et al.*, 1996), and necrotizing enterocolitis (Benkoe *et al.*, 2014; Seo *et al.*, 2021). Ok *et al.* (2020) reported that IL-8 serum levels increased significantly in calves with neonatal diarrhea compared to healthy calves. The present study showed no statistically significant difference in serum IL-8 levels in calves with coccidiosis before (0^{th} hours) and after (72^{nd} hours) treatment compared to the healty calves. However, a statistically significant decrease was observed in serum IL-8 levels after treatment (72^{nd} hours) compared to pre-treatment (0^{th} hours) (Table 3, Figure 1F).

While various investigators detected an increase in IL-8 levels (Daig *et al.*, 1996; Benkoe *et al.*, 2014; Ok *et al.*, 2020; Seo *et al.*, 2021) in different diseases with intestinal damage, it was not observed in the present study. The probable reason for this situation may be that protozoan agents cannot activate neutrophils as much as bacteria and viruses, and do not release large amounts of IL-8 as a severe inflammatory response. Ok *et al.* (2020) found that IL-8 release was lower in enteritis caused by cryptosporidium species compared to virus and bacteria-infected groups, which supports our view. The decrease in IL-8 levels after treatment (72^{nd} hours) may be related to the fact that coccidiosis was cured, the inflammatory response to *Eimeria*-induced damage was reduced, and intestinal mucosal damage began to heal.

CONCLUSION

In conclusion, serum concentrations of I-FABP, CLD-3, TFF-3, and ACTG2 were shown to be considerably higher in coccidiosis calves in this investigation. These indicators were shown to be important in evaluating the presence of damage in the intestines.

CONFLICT OF INTEREST

The authors disclose no conflict of interest.

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