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Clinical signs, duodenal histopathological grades, and serum high-mobility group box 1 concentrations in dogs with inflammatory bowel disease

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Abstract

Background: Inflammatory bowel disease (IBD) commonly occurs in dogs, but there is lack of information about potential biomarkers of clinical and histopathologic severity.

Objective: To examine the role of serum C-reactive protein (CRP) and high-mobility group box 1 (HMGB1) concentrations in dogs with IBD.

Animals: Seventeen dogs with IBD and 25 healthy dogs.

Methods: In this prospective study, duodenal histopathologic severity was graded, and the clinical severity of IBD was assessed by the canine IBD assessment index (CIBDAI) score in dogs with IBD. Serum CRP and HMGB1 concentrations were compared between IBD and healthy dogs and analyzed according to histopathologic grade in dogs with IBD. The correlations between serum CRP and HMGB1 concentrations and the CIBDAI score were evaluated.

Results: Dogs with IBD had higher serum CRP (median [range] = 20.39 [1.53-67.69] μg/mL vs 2.31 [0.17-11.49] μg/mL; $P < .001$) and HMGB1 concentrations (0.44 [0.07-1.58] ng/mL vs 0.05 [0.01-0.25] ng/mL; $P < .001$) than healthy dogs. The serum HMGB1 concentration was higher in IBD dogs with a moderate to severe histopathologic grade (0.51 [0.30-1.58] ng/mL, $P = .03$) than in those with a mild histopathologic grade (0.17 [0.07-0.75] ng/mL). Serum CRP concentrations and CIBDAI score were positively correlated in dogs with IBD ($r_s = .49$, $P = .05$).

Conclusions and Clinical Importance: Serum HMGB1 could be a potential biomarker for diagnosing IBD and might be indicative of histopathologic severity in dogs, whereas serum CRP might be an indicator of clinical severity.

KEYWORDS
biomarker, canine, diarrhea, enteropathy, vomiting

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; CI, confidence interval; CIBDAI, canine IBD index; CRP, C-reactive protein; HMGB1, high-mobility group box 1; IBD, inflammatory bowel disease.

Jong-Hwan Lee and Hong-Suk Kim contributed equally to this study.

1 | INTRODUCTION

Inflammatory bowel disease (IBD) is diagnosed in dogs with signs of gastrointestinal disease for more than 2 or 3 weeks, no signs of extraintestinal diseases or intestinal diseases such as parasite infections, failure to respond to dietary and antibiotic treatments, confirmed chronic inflammation and histological changes in the lamina propria on intestinal biopsy, and response to immunosuppressant treatment.[1-4]

Although mucosal histopathology is important for the diagnosis of IBD in dogs, previous studies have not detected convincing associations of mucosal histopathology with clinical signs, inflammatory biomarkers, response to therapy, and outcome.[5] Additionally, in non-hypoproteinemic dogs with IBD, treatment led to clinical and endoscopic improvement, but histopathologic lesions remained unchanged.[6] Therefore, there might be no association between histopathology and clinical activity.[5]

High-mobility group box 1 (HMGB1), a nonhistone chromosomal protein, is passively released from damaged cells or actively released from activated inflammatory cells.[7] HMGB1 is abundantly present in the feces of human children with IBD,[8] and the amount of fecal HMGB1 is correlated with clinical and subclinical mucosal inflammation and healing, indicating the potential usefulness of HMGB1 in monitoring IBD progression and assessing therapeutic outcomes in human IBD patients.[9] Furthermore, humans with IBD had significantly higher serum HMGB1 concentrations than healthy controls.[10] The amino acid sequence of HMGB1 is highly conserved among species, with 100% homology between humans and dogs.[11] Therefore, serum HMGB1 could be a useful biomarker for mucosal inflammation in dogs with IBD. Moreover, because secreted HMGB1 can induce inflammation by stimulating the production of proinflammatory cytokines and activating inflammatory pathways,[12,13] evaluating the associations between serum HMGB1 concentrations and intestinal mucosal lesions could provide new insights into the pathogenesis of IBD and reveal targets for clinical intervention in dogs with IBD.

We hypothesized that serum HMGB1 concentration would be a biomarker of the histopathologic severity of IBD in dogs. Therefore, the objective was to evaluate associations of serum C-reactive protein (CRP) which is a well-known positive acute phase protein and can be elevated in various inflammatory diseases including IBD in dogs, and HMGB1 concentrations with clinical signs. Furthermore, dogs with IBD were divided into 2 groups according to the duodenal histopathologic grade (mild and moderate to severe), and subgroup analyses were performed.

2 | MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee, and informed consent was obtained from the owners. In this prospective study, we enrolled dogs newly diagnosed with IBD between September 2017 and August 2019.

Inflammatory bowel disease was diagnosed on the presence of clinical signs including 1 or more of vomiting, diarrhea, anorexia, weight loss, or some combination of these signs that lasted at least 3 weeks, the exclusion of other causes of gastrointestinal disease, and the identification of inflammatory infiltrate within the lamina propria on histopathologic evaluation of duodenal samples obtained through endoscopic biopsy.[14] Furthermore, to rule out infectious, endocrine, or neoplastic diseases, we performed a complete clinical evaluation, including complete blood counts, serum biochemistry profile, urinalysis, analysis of trypsin-like and pancreatic lipase immunoreactivity, serum cobalamin, folate, and basal cortisol concentration measurements with post-adrenocorticotrophic hormone stimulated cortisol concentration measurement if necessary, direct (wet mount) and indirect (zinc sulfate flotation) fecal examination for nematode and protozoan parasites, and abdominal radiography and ultrasonography. Before performing endoscopic biopsy, we ruled out food-responsive and antibiotic-responsive diarrhea based on the lack of remission of clinical signs after 2-week treatment of feeding an elimination diet including a hydrolyzed protein diet and administration of metronidazole (10-15 mg/kg, PO, every 12 hours), respectively.[14] Based on the chronicity of clinical signs, the exclusion of underlying infectious, endocrine or neoplastic diseases, food-and antibiotic-responsive diarrhea, and histological inflammatory findings, these dogs were diagnosed as having IBD. Dogs were also excluded if they had received any type of anti-inflammatory/immunosuppressive treatment during the 2 weeks before enrollment and initial sample collection of if the exact history of the treatments was not available. Consequently, 17 dogs with IBD were included. Twenty-five healthy, client-owned dogs were also included as a comparison group. These dogs were recruited from the same veterinary hospital as the IBD dogs. They were recruited when they presented for health examination, and were considered to be clinically healthy based on the findings from physical examination, fecal examination, heartworm antigen testing, complete blood count, serum biochemical analysis, serum electrolyte analysis, urinalysis, adrenocorticotropic hormone response testing, thyroid function testing and diagnostic imaging (survey radiography and abdominal ultrasonography).

2.1 | Assessment of clinical signs of IBD in dogs

The clinical signs before and after treatment of all the dogs with IBD were assessed by the canine IBD assessment index (CIBDAI) scoring system,[15] which considers general attitude/activity, appetite, frequency of vomiting, stool consistency, stool frequency, and weight loss. The total score was determined by summating each variable score.

2.2 | Histopathologic scoring of duodenal mucosal biopsy samples

Endoscopic duodenal biopsies were performed in all the dogs with IBD. For histopathologic assessment, biopsy samples were fixed in neutral-buffered 10% formalin until they were processed. All samples were processed, and histopathologic examinations were performed by 1 of 9 American College of Veterinary Pathologists board-certified

pathologist according to the World Small Animal Veterinary Association International Gastrointestinal Standardization Group guidelines.[3] At least 10 endoscopic duodenal biopsy samples were obtained,[2] and only tissue sections of adequate diagnostic quality were used.[16] Morphological indicators of inflammation such as epithelial injury, crypt distension, lacteal dilatation, and mucosal fibrosis and histological indicators of inflammation such as the number of plasma cells, laminal propria lymphocytes, eosinophils, and neutrophils were scored, and IBD was histologically graded as mild (total score = 1-6), moderate (total score = 7-12), and severe (total score >13) based on the total score. For correlation analysis, the individual morphological and histological indicators of inflammation in duodenal biopsy samples were used.

2.3 | IBD treatment

After diagnosis of IBD, all dogs were treated with immunosuppressive drugs (1 mg/kg of prednisolone, PO, every 12 hours, with or without 5 mg/kg of cyclosporine, PO, every 12 hours or 10 mg/kg of mycophenolate, PO, every 12 hours). Some dogs were also administered antibiotics (10-15 mg/kg of metronidazole, PO, every 12 hours) as well as an elimination diet and received cobalamin (25 mcg/kg, subcutaneous, once per week for 6 weeks followed by once per month for 6 months) during immunosuppressive treatment at the attending clinician's discretion. All dogs also were treated with famotidine (0.5-1 mg/kg, PO, every 12 hours).

2.4 | Serum sample analysis

Blood samples were collected from dogs with IBD and controls on admission and from dogs with IBD after improvement in clinical signs. Blood was collected from the jugular or a peripheral vein, serum was separated from clotted whole blood by centrifugation at 1200*g* for 10 minutes, within 1 hour of blood collection, and the sera were stored at −80°C until assay.

The serum CRP concentration was measured with a canine-specific ELISA kit (Canine C-Reactive Protein ELISA Kit, BD Biosciences, San Jose, California), according to the manufacturer's instructions; the intraassay variability was <5%, interassay variability was <10%, and the detection limit was 0.015 μg/mL. The serum HMGB1 concentration was measured with a canine-specific ELISA kit (Dog HMGB1 ELISA Kit, CUSABIO, Wuhan, China); the intra- and interassay variability were <8% and <10%, respectively. The detection limit was 0.02 ng/mL. All samples, standards, and controls were assayed in duplicate. The optical density was determined at 450 nm by an automated microplate reader (ELx 808; BioTek Instruments Inc, Winooski, Vermont).

2.5 | Evaluation of dogs with IBD after treatment

Each dog with IBD was reevaluated at the same veterinary hospital ~30 days after the initiation of treatment. In the follow-up evaluation, the dogs with IBD were reassigned a CIBDAI score by the same procedures as the initial evaluation. A repeat blood sample was obtained to determine serum CRP and HMGB1 concentrations.

2.6 | Statistical analyses

Data were analyzed by commercially available statistical software (Prism 6.01; GraphPad Software Inc, La Jolla, California). Data are expressed as medians and ranges. *P* values were calculated for 2-tailed tests, and 95% confidence intervals (CIs) were determined for the differences between medians. The D'Agostino-Pearson omnibus test was performed to determine whether data were normally distributed. The following statistical analyses were performed by nonparametric tests because the data of many subgroups were not normally distributed. The Mann-Whitney *U* test was used for comparison between the IBD and control dogs or between IBD dogs with mild histopathologic severity and those with moderate to severe histopathologic severity. The Wilcoxon signed rank sum test was used to compare pre- and posttreatment data. The area under the receiver operating characteristic curve (AUC) was calculated to determine the optimal cutoff value of the serum HMGB1 concentration for differentiating between IBD dogs and healthy dogs. The correlations between variables were evaluated by Spearman's correlation analysis. Statistical significance was set at $P < .05$.

3 | RESULTS

3.1 | Study sample

Seventeen dogs with IBD (intact female/spayed female/intact male/castrated male, 3/6/2/6; median [range] age, 8.0 [3-14] years; weight, 3.4 [1.7-28] kg; body condition score, 3 [2-6] /9] and 25 healthy dogs (intact female/spayed female/intact male/castrated male, 5/9/4/7; median [range] age, 8 [4-13] years; weight, 4.1 [2.1-25.5] kg; body condition score, 4 [3-6] / 9] were included in this study. Five dogs with IBD were Maltese, 4 were crossed breed, 3 were Yorkshire Terrier, 2 were Miniature Poodle, and the remainder were 1 each of Shih Tzu, Dachshund and German Shepherd. In the healthy dogs, 8 were Maltese, 5 were cross breed, 4 were Shih Tzu, 3 were Miniature Poodle, 2 were Beagle, 2 were Yorkshire Terrier and 1 was Jindo. No statistically significant differences were identified in terms of age, body weight or body condition score between the IBD and healthy dogs.

The 17 dogs with IBD were divided into 2 subgroups according to histopathologic severity: mild and moderate to severe. Eight dogs with IBD had mild histopathologic severity (intact female/spayed female/intact male/castrated male, 1/4/1/2; median [range] age, 10 [3-13] years; weight, 2.9 [1.7-4.6] kg; body condition score, 3 [2-4]/9) and 9 dogs with IBD had moderate to severe histopathologic severity (intact female/spayed female/intact male/castrated

male, 2/2/1/4; median [range] age, 7 [3-14] years; weight, 3.6 [1.7-28] years; body condition score, 4 [3-6]/9). Four dogs with IBD with mild histopathologic severity were cross breed, 2 were Maltese, and the remainder were 1 each of Dachshund and Yorkshire Terrier. In the group of dogs with IBD with moderate to severe histopathologic severity, 3 were Maltese, 2 were Shih Tzu, 2 were Yorkshire Terrier, and the remainder were 1 each of Miniature Poodle and German Shepherd. No statistically significant differences were identified in terms of age, body weight, or body condition score between these dogs with IBD.

3.2 | Comparison of blood analysis results of dogs with IBD according to histopathologic grade

There were no differences in complete blood counts, biochemistry profiles, and electrolyte concentrations between dogs with IBD with mild histopathologic severity and those with moderate to severe histopathologic severity on admission; however, there were differences in serum total cholesterol concentration and alkaline phosphatase (ALP) activity (Table 1). Additionally, of the all blood analysis results, only the total cholesterol concentration was significantly correlated with the lacteal dilatation grade of the morphological indicators ($r_s = -.55$, $P = .01$), whereas serum ALP activity was not correlated with any histological or morphological variable.

3.3 | Serum CRP and HMGB1 concentrations of dogs with IBD before treatment and healthy dogs

The median (range) of the serum CRP concentration of dogs with IBD was significantly higher than that of healthy dogs (20.39 [1.53-67.69] μg/mL vs 2.31 [0.17-11.49] μg/mL, $P < .001$; Figure 1). Additionally, the median (ranges) of the serum HMGB1 concentration of dogs with IBD was significantly higher than that of healthy dogs (0.44 [0.07-1.58] ng/mL vs 0.05 [0.01-0.25] ng/mL; $P < .001$).

3.4 | AUC of the serum HMGB1 concentration in dogs with IBD

The AUC of the serum HMGB1 concentration was 0.93 (95% CI, 0.86-1; Figure 2). The corresponding optimal cutoff values to distinguish between dogs with IBD and healthy dogs was 0.18 ng/mL; the sensitivity and specificity were 96% (95% CI, 77-100%) and 76% (95% CI, 50-93%), respectively.

TABLE 1 Complete blood counts, blood chemistry profiles, and serum electrolyte concentrations between dogs with mild (n = 8) or with moderate to severe histopathologic severity (n = 9)

Variables	Reference interval	Mild IBD	Moderate to severe IBD	*P* value
Complete blood counts				
Whole blood count ($\times 10^3/\mu L$)	5.1-16.8	16.2 (4.2-53.7)	11.1 (8.4-11.8)	.13
Neutrophils ($\times 10^3/\mu L$)	3-11.6	8.23 (3.5-46.8)	6.9 (5.2-10.4)	.97
Lymphocytes ($\times 10^3/\mu L$)	1.1-5.1	3.5 (0.2-5.6)	1.5 (0.3-2.9)	.1
Monocytes ($\times 10^3/\mu L$)	0.2-1.1	0.5 (0.2-1.5)	0.6 (0.4-1.2)	.87
Eosinophils ($\times 10^3/\mu L$)	0.1-1.2	0.5 (0.1-4.2)	0.2 (0.1-0.9)	.15
Basophils ($\times 10^3/\mu L$)	0-0.1	0 (0-0.2)	0 (0-0.1)	.36
PCV (%)	37.3-61.7	37.2 (20.5-43.6)	41 (31.4-57.1)	.13
Platelets ($\times 10^3/\mu L$)	148-484	410 (238-671)	378 (195-681)	.78
Chemistry				
Total protein (g/dL)	5.4-7.1	6.6 (3.7-9.5)	5.3 (3.7-7.3)	.53
Albumin (g/dL)	2.6-3.3	2.6 (1-3.9)	2.4 (1.4-3.4)	.56
Globulin (g/dL)	2.7-4.4	2.9 (1.1-7)	2.9 (1.5-4.6)	.8
Cholesterol (mg/dL)	135-270	178 (154-209)	94 (46-212)	.03
ALT (IU/L)	21-102	37 (3-50)	98 (15-308)	.07
ALP (IU/L)	29-97	81 (61-113)	463 (73-915)	.03
Electrolytes				
Na (mmol/L)	141-152	151 (139-156)	144 (135-149)	.12
K (mmol/L)	3.6-5.8	4.2 (3.7-5)	4.8 (4.4-5.3)	.09
Cl (mmol/L)	105-115	116 (112-124)	114 (97-117)	.09
Ca (mg/dL)	9-11.3	8.1 (6.8-9.9)	8.4 (7.2-10)	.52
P (mg/dL)	2.6-6.2	4 (2.8-6.6)	4.4 (2.5-6.2)	.46

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; IBD, inflammatory bowel disease.

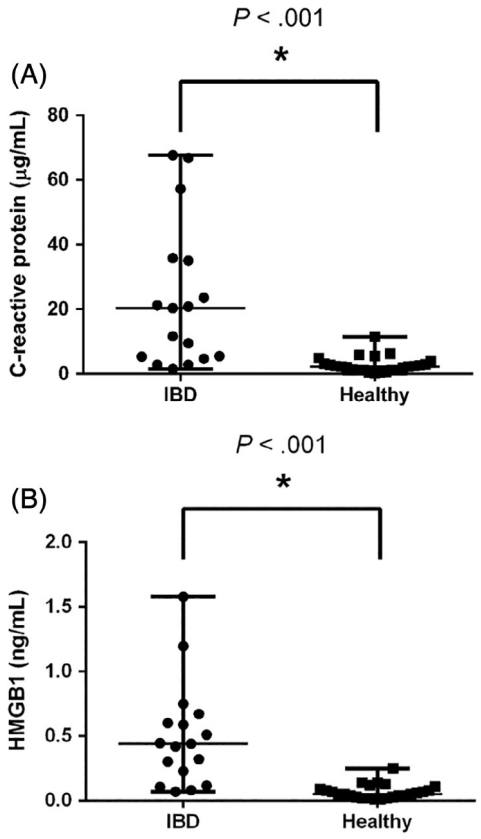

FIGURE 1 Comparison of serum CRP (A) and HMGB1 (B) concentrations between dogs with IBD (n = 17) and healthy dogs (n = 25). *P < .05 (Mann-Whitney U test). CRP, C-reactive protein; HMGB1, high-mobility group box 1; IBD, inflammatory bowel disease

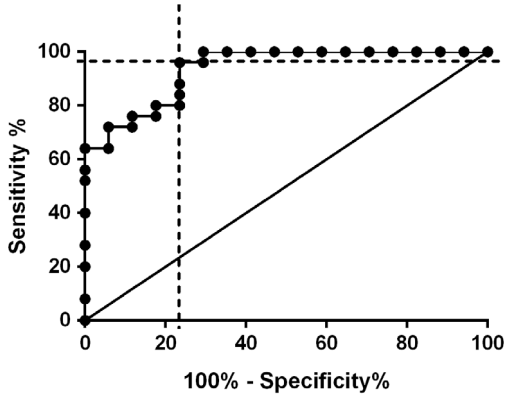

FIGURE 2 ROC curve predicting IBD based on the serum HMGB1 concentration in dogs. The thick diagonal line shows a 50% chance. The AUC of the serum HMGB1 concentration was 0.93 (95% CI = 0.86-1). The point of intersection represents the optimal cutoff value of 0.18 ng/mL for the differentiation of IBD dogs from healthy dogs, with a sensitivity and specificity of 96% (95% CI = 80-100%) and 76% (95% CI = 50-93%), respectively. AUC, area under the receiver operating characteristic curve; CI, confidence interval; HMGB1, high-mobility group box 1; IBD, inflammatory bowel disease; ROC, receiver operating characteristic

3.5 | Changes in serum CRP and HMGB1 concentrations and CIBDAI score in dogs with IBD after treatment

Evaluation after treatment was not performed in 7 of the 17 dogs with IBD because their owners refused permission. In the remaining 10 dogs, reevaluation was performed a median (range) of 30 (27-32) days after treatment, and the median (range) of serum CRP concentration significantly decreased from 16.24 (1.53-66.83) to 4.09 (0.04-10.22; 95% CI for the difference between medians = −25.63 to 0.03; P = .01) and serum HMGB1 concentration significantly decreased from 0.45 (0.12-1.20) to 0.31 (0.06-0.55; 95% CI for the

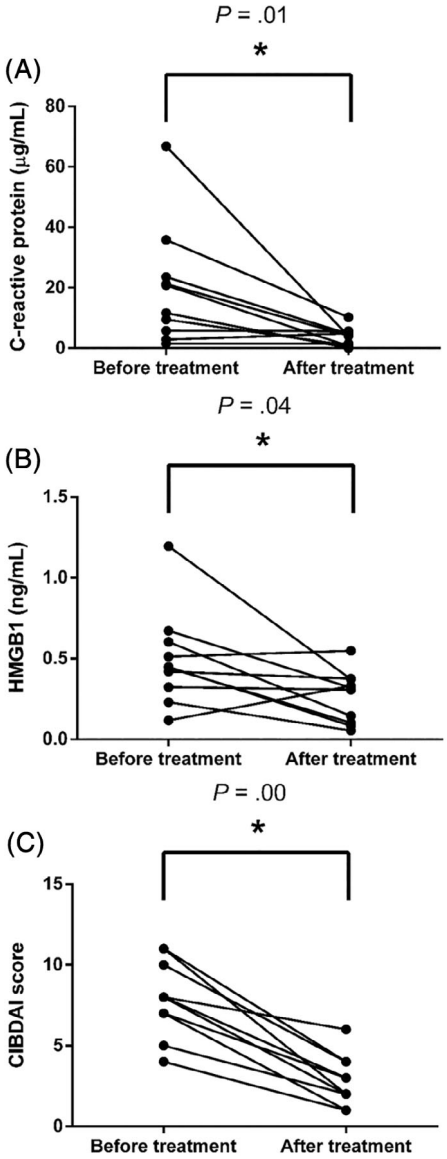

FIGURE 3 Comparison of serum CRP (A) and HMGB1 (B) concentrations and CIBDAI (C) scores (n = 10) before and after treatment. *P < .05 (Wilcoxon signed rank sum test). CRP, C-reactive protein; CIBDAI, canine IBD activity index; HMGB1, high-mobility group box 1; IBD, inflammatory bowel disease

difference between medians = −0.46 to 0.04; $P = .04$) after treatment (Figure 3). The median (range) of the CIBDAI score of these dogs was also significantly reduced from 8 (4-11) to 3 (1-6; 95% CI for the difference between medians = −7 to −3, $P = .00$) after treatment.

3.6 | Comparison of serum CRP and HMGB1 concentrations and CIBDAI scores of dogs with IBD according to the duodenal histopathologic grade

There was no difference in the median (range) serum CRP concentration between dogs with IBD with mild histopathologic severity and those with moderate to severe histopathologic severity (20.61 [2.84-67.69] μg/mL vs 9.48 [1.53-35.85] μg/mL, $P = .38$; Figure 4). The median (range) serum HMGB1 concentration of dogs with IBD with mild histopathologic severity (0.17 [0.07-0.75] ng/mL) was significantly lower than that of dogs with IBD with moderate to severe histopathologic severity (0.51 [0.30-1.58] ng/mL, $P = .03$). There was no difference between the median (range) CIBDAI scores of dogs with IBD with mild histopathologic severity and those with moderate to severe histopathologic severity (10 [6-18] vs 7 [4-10], $P = .11$). Additionally, all the dogs with IBD were classified into 2 subgroups according to the presence of hypoalbuminemia (serum albumin concentration < reference interval), but there was no difference in the median (range) serum HMGB1 between dogs with IBD with and without hypoalbuminemia (0.38 [0.07-0.67] ng/mL vs 0.59 [0.08-1.58] ng/mL, $P = .36$).

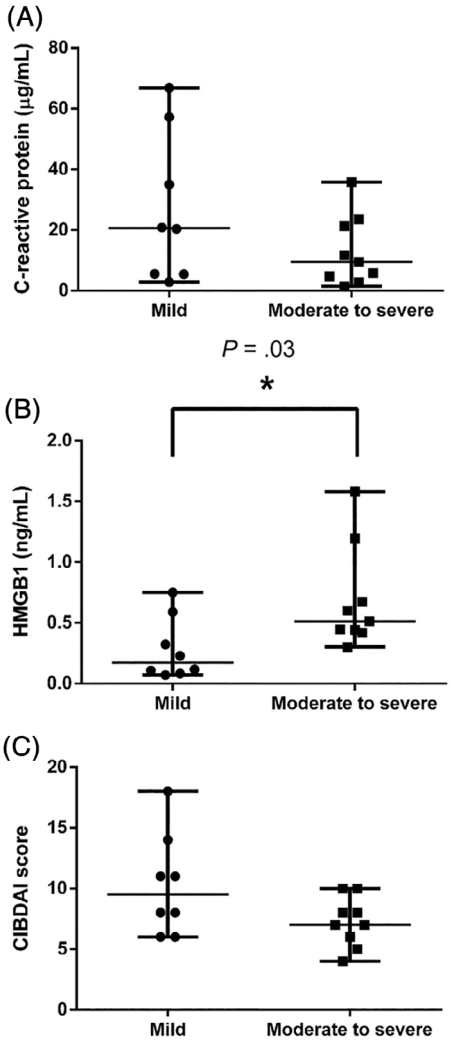

FIGURE 4 Comparison of serum CRP (A) and HMGB1 (B) concentrations and CIBDAI scores (C) between dogs with mild IBD histopathologic severity (n = 8) and dogs with moderate to severe IBD histopathologic severity (n = 9). *$P < .05$ (Mann-Whitney U test). CRP, C-reactive protein; CIBDAI, canine IBD activity index; HMGB1, high-mobility group box 1; IBD, inflammatory bowel disease

3.7 | Correlations of serum CRP and HMGB1 concentrations with CIBDAI score in dogs with IBD

The serum CRP concentration was positively correlated with CIBDAI score ($r_s = .49$, $P = .05$) in IBD dogs (Figure 5). However, significant correlations were not identified between serum CRP and HMGB1 concentrations ($r_s = .18$, $P = .48$) and serum HMGB1 concentration and CIBDAI score ($r_s = .32$, $P = .21$).

4 | DISCUSSION

This study aimed to investigate the role of CRP and HMGB1 in dogs with IBD. The serum HMGB1 concentration was higher in dogs with IBD, and dogs with IBD with moderate to severe duodenal histopathologic severity had higher serum HMGB1 concentrations than did IBD dogs with mild histopathologic severity. However, there was no correlation between serum HMGB1 concentration and CIBDAI score in dogs with IBD. Our results indicate that HMGB1 might be a marker for duodenal histopathologic severity and might be involved in pathogenesis of IBD in dogs.

Diagnosis of IBD as the cause of the chronic signs of gastrointestinal disease requires a stepwise medical treatment approach, including an elimination diet and antibiotics trial, and further evaluation including histologic examination of gastrointestinal biopsies to document inflammation and exclude a diagnosis of alimentary lymphoma. This process can be time-consuming and frustrating for owners.[1] In addition, given that endoscopic biopsy for histologic evaluation is expensive and relatively invasive, biomarkers revealing clinical and histopathological indices would be helpful for the diagnosis of IBD. The receiver operating characteristic curve analysis in the present study supported the assertion that the serum HMGB1 concentration could be a biomarker for IBD in dogs. The cutoff value for the serum HMGB1 concentration was set at 0.18 ng/mL, as this value exhibited the highest sensitivity (96%) and specificity (76%) for predicting IBD in dogs. Thus, the serum HMGB1 concentration could be a useful screening tool for the diagnosis of IBD in dogs. In the present study, the diagnosis of IBD was also established after the exclusion of gastrointestinal diseases, such as pancreatitis, as causes. Clinically, the

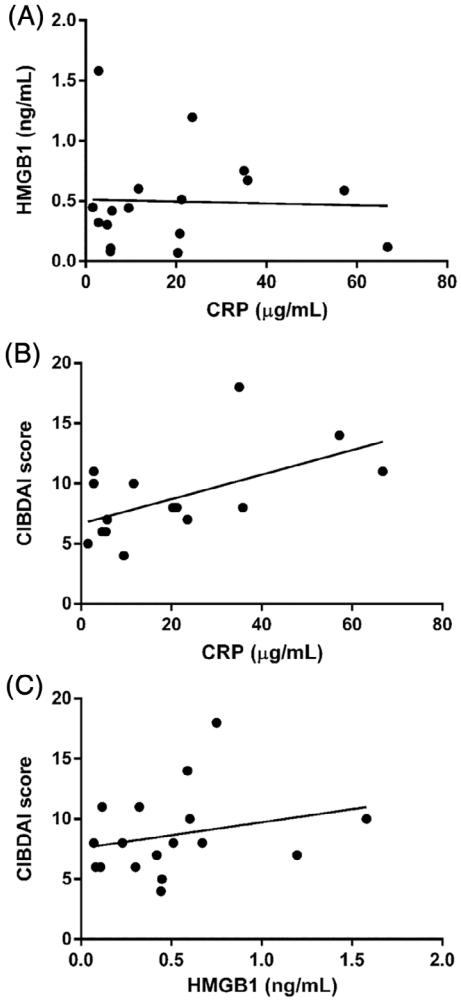

FIGURE 5 Spearman's correlations between (A) serum CRP and HMGB1 concentrations, (B) serum CRP concentration and CIBDAI score, and (C) serum HMGB1 concentration and CIBDAI score in IBD dogs. The serum CRP concentration was positively correlated with CIBDAI score. CIBDAI, canine IBD assessment index; CRP, C-reactive protein; HMGB1, high-mobility group box 1

diagnosis of pancreatitis in dogs is established based on clinical signs compatible with pancreatitis, abnormalities consistent with pancreatitis on physical examinations, ultrasonography, and canine pancreatic lipase immunoreactivity (≥400 μg/L).[17,18] We excluded dogs with abnormal canine pancreatic lipase immunoreactivity (≥200 μg/L) or ultrasonographic findings compatible with pancreatitis. Therefore, none of the dogs in the present study were suspected of having pancreatitis at the time IBD was diagnosed. However, we did not serially measure canine pancreatic lipase immunoreactivity during IBD management and did not perform histopathologic examination of the pancreas, which is the gold standard for the diagnosis of pancreatitis. A canine pancreatic lipase immunoreactivity ≥200 μg/L had low sensitivity (30%) in dogs with mild pancreatitis confirmed with histopathology of the pancreas,[19] implying that the effect of mild pancreatic injury on increased serum HMGB1 concentrations cannot be excluded in our dogs with IBD. In rats with experimentally induced acute necrotizing pancreatitis, the intracellular expression of HMGB1 is initially high in the pancreas and was then released into the bloodstream, leading to higher serum HMGB1 concentrations.[20] High serum HMGB1 concentration was also identified in dogs with acute pancreatitis diagnosed based on canine pancreatic lipase immunoreactivity ≥400 μg/L.[21] Most importantly, the possible effect of concurrent pancreatitis cannot be completely excluded, although only a small portion of IBD dogs might have mild pancreatitis. Consequently, the use of the serum HMGB1 concentration as a single marker might require caution during the process of diagnosing IBD, especially in dogs with abnormal canine pancreatic lipase immunoreactivity or ultrasonographic findings compatible with pancreatitis.

In humans, HMGB1 is considered to have an important role in pathogenesis of IBD.[22] Increased HMGB1 concentrations in the enteric cavity mainly because of passive release by necrotic cells and, active secretion by interstitial inflammatory cells and intestinal epithelial cells after inflammatory stimulation. The secreted HMGB1 binds to receptors such as toll-like receptors 2 and 4 on epithelial and inflammatory cells, indicating that the autocrine activity of HMGB1 is amplified in the intestine of humans with IBD.[23] In addition, inflammatory cells in the intestinal mucosa might actively secrete HMGB1, which can further promote the expression of downstream inflammatory cytokines and amplify intestinal inflammatory reactions.[24] These processes result in increased extracellular HMGB1 concentrations. Thus, as in humans, dogs with IBD might have high extracellular HMGB1 concentrations because of overexpression of toll-like receptors 2 and 4, receptors for HMGB1.[25,26] In this study, dogs with IBD with a moderate to severe duodenal histopathologic grade had significantly higher serum HMGB1 concentrations than those with mild histopathologic grade, indicating that the serum HMGB1 concentration might be related to histopathologic severity. Severely damaged intestinal cells or infiltrated immune cells release HMGB1. Therefore, increased serum HMGB1 concentrations might have occurred in the dogs with IBD after passive release by necrotic cells and, active secretion by interstitial inflammatory cells and intestinal epithelial cells.

In contrast, there was no difference in serum CRP concentration between dogs with IBD with mild duodenal histopathologic severity and those with moderate to severe histopathologic severity. CRP is a positive type-2 acute-phase protein produced by hepatocytes in response to inflammation, infection, or cancer, thus it can be a nonspecific marker of inflammation.[27-29] Serum CRP has been considered as a useful biomarker for assessing the response to treatment and changes in CIBDAI score in dogs with IBD in that serum CRP concentrations decline after administration of prednisone,[30] which is consistent with our findings of reduced serum CRP concentrations and CIBDAI scores after treatment. However, there was no correlation between CRP concentration and histopathological disease severity in dogs with IBD.[31] Therefore, CRP might be not useful for assessing histopathologic severity.

We observed a correlation between the serum CRP concentration and CIBDAI score in dogs with IBD. However, this is not consistent with the result of a previous study, which found no direct correlation between CRP concentration and CIBDAI.[31] In that study, dogs with

IBD had abnormal CRP concentrations although the CRP concentrations (range, <0.001 to 27.65 μg/mL) were lower than those (range, 1.53-67.69 μg/mL) observed in our study. This discrepancy might be because of differences in methodology, population, or both. We used ELISA to measure serum CRP concentrations, while immunoturbidimetric assay was used in the previous study. Furthermore, the median age of the dogs with IBD in the previous study was 3.7 years and the minimum CIBDAI score was 1, whereas the dogs included in our study were older (median age, 8 years) and had more clinical signs (CIBDAI scores >4) although it cannot be also directly compared. These differences might have led to the difference in the results regarding correlation between the serum CRP concentration and CIBDAI score.

Other markers, such as fecal/serum calprotectin and S100A12, reflect the clinical severity and classification of chronic enteropathies (food-responsive and antibiotic-responsive enteropathies, and IBD), and they might be used as predictors of partial or no response to treatment in dogs with IBD.[32-35] Therefore, such tests are useful in dogs with chronic signs of gastrointestinal disease. However, the test results might be altered by other gastrointestinal inflammation,[36] and they are currently not readily available. Serum CRP has been widely used as a marker of IBD progression and response to treatment in dogs, although it is a nonspecific biomarker of inflammation.[15,28,30] Furthermore, serum CRP concentrations are significantly increased in dogs with IBD compared to dogs with food-responsive and antibiotic-responsive enteropathies, and the concentrations are correlated with clinical severity in all dogs with chronic enteropathy.[34] There are conflicting results regarding CRP in dogs with chronic enteropathy,[37] but the clinical severity correlates with serum CRP concentrations in dogs with IBD.[15,30] Therefore, the serum CRP concentration was analyzed because only dogs with IBD were included in the present study.

We observed significant differences in serum total cholesterol concentrations between dogs with IBD with mild duodenal histopathologic severity and those with moderate to severe histopathologic severity, indicating a correlation between the serum total cholesterol concentration and histopathologic severity. Thus, dogs with IBD with a more severe histopathologic grade because of severely damaged duodenal lacteals might experience large amounts of cholesterol loss[38] based on the negative correlation between the total cholesterol concentration and lacteal dilatation grade in the present study. Serum ALP activity was significantly higher in IBD dogs with moderate to severe duodenal histopathologic severity than those with mild histopathologic severity. In contrast, a previous study found that duodenal mucosal expression and activity as well as fecal concentrations of intestinal ALP were lower in dogs with IBD, especially in those with moderate or severe disease.[39] Serum ALP activity is considered to be unrelated to intestinal ALP activity. Furthermore, we did not detect relevant hepatobiliary disease in any of the dogs with IBD in this study. Considering the high HMGB1 concentrations observed in IBD dogs with advanced histopathologic severity, the elevated serum ALP activity might have been because of severe intestinal inflammation, but the exact mechanism could not be identified.

This study was performed with duodenal biopsy samples. Severe mucosal lesions in the duodenum are associated with a negative outcome in dogs with chronic enteropathies.[37] In addition, inflammatory cells in the intestinal mucosa actively secrete HMGB1 during intestinal inflammation in humans.[24] Moreover, standardized scoring of histology findings specifically for the jejunum and ileum is not included in the WSAVA guidelines, although the scoring system used for duodenal biopsies has been validated for ileal lesions,[2,5,40] IBD could be diagnosed based on mucosal inflammation on the histopathologic review of duodenal biopsy samples in many studies.[3,14,41] Therefore, we focused on the association between duodenal histopathologic scores and serum HMGB1 concentration in IBD dogs. However, the results might differ if other areas of the small or large intestines are involved. In addition, the number of duodenal histopathologic samples could affect the results. At least 10 endoscopic duodenal biopsy samples were obtained in the present study,[2] which is a higher number than that suggested for endoscopic duodenal biopsy samples (at least 8 individual tissue pieces).[42] Furthermore, only tissue sections of adequate diagnostic quality were used in the present study, and the dogs with IBD were divided semiquantitatively according to the duodenal histopathologic grade (mild and moderate to severe); thus, the histopathologic grades would be minimally affected.

This study has several limitations. First, we did not examine the fecal HMGB1 concentration, which has been the focus of studies on humans and rodents.[8,43] Quantifying and comparing the serum and fecal HMGB1 concentrations of dogs with IBD might provide insight into the pathogenesis of IBD in dogs. Second, the sample size of this study was small, especially for comparing the serum CRP and HMGB1 concentrations and CIBDAI scores between dogs with mild duodenal histopathologic severity ($n = 8$) and those with moderate to severe IBD histopathologic severity ($n = 9$). Therefore, because of the small sample size, some results might have prone to type II error and should be cautiously interpreted. Our objective was to provide insight into the role of the serum HMGB1 concentration in dogs with IBD, and collect data that could be used to calculate the sample size of a larger, more definitive study; thus, no a priori sample size calculation was performed. Finally, duodenal endoscopic biopsy samples were individually evaluated by several pathologists rather than by a single pathologist, implying a limitation of the present study because there are known interobserver differences among pathologists evaluating endoscopic intestinal biopsy samples.[2,3,44]

5 | CONCLUSIONS

We found that IBD dogs with moderate to severe duodenal histopathologic grades had significantly higher serum HMGB1 concentrations than those with a mild histopathologic grade, indicating the possible role of HMGB1 in the pathogenesis of IBD in dogs. Furthermore, the serum CRP concentration was positively correlated with clinical score, and the serum CRP concentration reduced after treatment. Therefore, serum CRP could be used as a surrogate marker for assessing response to treatment in IBD dogs.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the IACUC at Chungbuk National University (CBNUA-1046-17-01).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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