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RESEARCH ARTICLE

Investigation of Some Cytokines, Acute Phase Proteins and Hepcidin Levels Before and After Treatment in Dogs with Parvoviral Gastroenteritis

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ABSTRACT

The purpose of this study is to demonstrate the value of hepcidin detected in healthy dogs and also to investigate the values of some inflammatory mediators and hepcidin released during the treatment in dogs with parvoviral gastroenteritis. In this study, 20 puppies with parvoviral gastroenteritis, and 15 healthy puppies were used. Dogs in study group were classified as septic after serological confirmation of infection and showing two of sepsis criteria. Serum levels of tumor necrosis factor, interleukin -6, interleukin- 1, serum amyloid A, C reactive protein, haptoglobin, hepcidin and iron were determined in both groups. At the 3rd, 7th, and 14th days of the treatment, blood samples were taken again, and compared with the day 0. Died dogs were examined with cytokines, acute phase proteins and hepcidin by immunohistochemical methods in liver and intestine samples. Hepcidin, TNF, IL-1, IL-6, SAA, Hp and CRP levels were increased significantly compared with in control animals, while their levels were decreased significantly during treatment. For the first time, hepcidin levels in healthy dogs and dogs with sepsis were determined and hepcidin levels were monitored during treatment. It was concluded that hepcidin can be used as prognostic indicator of sepsis.

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INTRODUCTION

Parvoviral enteritis is a viral disease mostly seen in unvaccinated puppies younger than 6 months of age. After the entrance of virus to the body, it proliferates in small intestinal crypt epithelium, lymphopoetic tissue and bone marrow. The intestinal mucosal damage caused by virus results in entrance of bacteria to the circulation which lead to blood and organ infection, and ultimately the systematic inflammatory response syndrome (SIRS) and sepsis (Otto *et al.,* 1997; Prittie, 2004; Nemzek *et al.,* 2007).

Recently discovered hepcidin is peptide in nature and defined as a hormone with multiple functions. Initially, hepcidin was considered as an antimicrobial peptide found in human Blood and urine in human blood and urine (Krause *et al.,* 2000; Park *et al.,* 2001). However, subsequent studies proved that hepcidin is a type II acute phase reactant and has been reported to play a regulator role in iron metabolism (Ganz, 2006). Studies in dogs showed that the structure of hepcidin consists of 85 amino acids and 8 cysteine in C-terminal while in humans 84 aminoacids and similarly 8 cysteine in the C terminal. Studies to date about the structure of hepcidin showed similarity in humans and dog. Due to the similarity of human and canine hepcidin, researchers suggest the use of animal models in evaluation of human diseases (Fry *et al.,* 2004).

Hepatic hepcidin production is under the influence of various stimulants. Low iron levels and erythropoietic activation decreases hepcidin levels and certain cytokines especially interleukin 6 (IL-6) increases hepcidin levels (Lee *et al.,* 2005; Wessling-Resnick, 2010). Inflammation and infection stimulates the increased production of hepcidin (Nemeth *et al.,* 2003). Hepcidin also helps the host defense due to direct antimicrobial properties (Verga and Muckenthaler, 2005; Wessling-Resnick, 2010).

The purpose of this study is to demonstrate the value of hepcidin detected in healthy dogs detected to be never studied before according to our literature researches and also to investigate the values of some inflammatory mediators and hepcidin in dogs naturally infected with parvoviral gastroenteritis during treatment.

MATERIALS AND METHODS

Ethical guidelines: The experiments were performed in accordance with the guidelines for animal research and were approved by the Committee on Animal Research at Mehmet Akif Ersoy University, Burdur.

Animals: Puppies which were brought to our clinic with complaints of diarrhea or bloody diarrhea and vomiting were evaluated. Rapid test kits for the diagnosis of parvoviral enteritis were used in these puppies with the complaints mentioned above and blood was taken from positive ones. Twenty puppies (15 were unvaccinated, 5 were vaccinated) of different breeds and sex (6 female, 14 male), aged 2-6 months with below mentioned criteria selected as study group. Fifteen healthy puppies that were brought to our clinic for routine vaccinations were considered as control group.

Laboratory analysis: Blood and stool samples were evaluated for determining antigen, IgM and IgG antibodies with ELISA (Enzyme-Linked Immunosorbent Assay) method. After confirmed serological diagnosis, puppies with at least two of the following sepsis criteria were included in study group: hypo- or hyperthermia (body temperature below 37.8ºC or above 39.4ºC), tachycardia (>140bpm), tachypnea (respiration>30/min), leukopenia (Leukocyte count less than 5.500/mL), and leukocytosis (Leukocyte count greater than 12.500/mL). In the study group blood serum samples were collected at 3rd, 7th and 14th days and stored at -80°C after centrifugation of blood. In control group blood samples were withdrawn once at submission. Iron, TNF-α, IL-6, IL-1, CRP, serum amyloid A, haptoglobin, hepcidin levels were measured after the completion of study. Complete blood counts were carried out immediately after sampling.

Blood serum TNF-α, IL-6, IL-1, CRP, serum amyloid A, haptoglobin, and hepcidin values was measured by ELISA method. Following ELISA kits were used which were produced specially for dogs: TNF-α [Canine (Dog), tumor necrosis factor α (TNF-α) ELISA Kit, Cloud- Clone Crop, USA- Cat. No: 133 of SEA]; IL-6 [Canine (Dog) Interleukin 6 (IL-6) ELISA Kit, Cloud- Clone Crop, USA- Cat. No: SEA 079 CA]; IL-1 [Canine (Dog) Interleukin 1 (IL-1) ELISA Kit, Cloud- Clone Crop, USA- Cat. No. SEA CA 071]; Hepcidin (HAMP) [Dog hepcidin ELISA Kit, CUSABIO, Cat. No: CSB-EL010124DO]; CRP [Canine (Dog) C-Reactive Protein ELISA Kit, CUSABIO, cat.no: CSB-E14262 by; Haptoglobin [Dog (HP) ELISA Kit, CUSABIO, cat.no: CSB-EL010691DO]; Serum Amyloid A [Dog (SAA) ELISA Kit, cat.no: by CSB-E17749]. Serum iron levels were measured with the biochemistry autoanalyser (Gesan Chem 200, Italy).

Treatment protocol: After withdrawal of blood samples at day 0, the treatment was started. All dogs received Ampicillin (22 mg/kg/q8h, IV), Gentamicin (4 mg/kg/q24h, IV), Metaclopromide (0.4 mg/kg/q8, SC), Ranitidine (1-4mg /kg/q12 h, SC) and Lactated ringer+ %5 dextrose (Supplemented with 20mEq/L potassium chloride, IV) for treatment.

Immunohistochemistry: Six puppies were died during the treatment and excluded from the study. A necropsy performed on these puppies and constitute the study group of pathological and immunohistochemical examinations. Liver and intestinal tissues of 5 puppies of similar ages died due to traffic accidents were used as control group. The dogs' organs examined histopathologically before using as control, only healthy animals used as control.

Tissue samples were fixed in 10% buffered formaldehyde solution. The samples then routinely were processed and embedded in paraffin, 5µm sectioned and stained with hematoxylin- eosin (HE) and examined microscopically (Luna, 1967).

Liver and intestine samples were then immunostained with primary antibodies. All antibodies purchased from the Abcam, Cambridge, UK. Tissue sections were immunostained by hepcidin [anti-hepcidin-25 antibody (ab30760)], haptoglobulin [anti-haptoglobin antibody (ab135835)]; serum amyloid A [anti-serum amyloid antibody, [mc1] (ab655)], C-reactive protein [Anti-C Reactive Protein antibody - Aminoterminal end (ab65842)], IL-1 beta [Anti-IL1 beta antibody (ab2105)), IL-6 beta [Anti-IL1 beta antibody (ab2105)] and TNF- [Anti-TNF alpha antibody (ab6671)] antibodies according the manufacturer's instructions. All the slides were analyzed for immunopositivity and a semiquantitative analysis was carried out (Ozmen, 2009).

Statistical analysis: Student's t test was used to determine any significant differences between the groups. The groups were compared by using Chi square test. Time-dependent parameters were analyzed by "repeated variance analysis" or "Cochran Q test". Calculations were made using the SPSS 15.0 program pack. $P<0.05$ was set as the value for significance.

RESULTS

Fifteen (75%) out of 20 animals in the study group were hypothermic (<37.8ºC), 5 puppies were (25%), hyper-thermic (>39.4ºC). Also leukopenia (<5.500 leukocyte/mL) observed 15 (75%) out of 20 dogs was and leukocytosis (>12.500/mL) was observed in 5 (25%) puppies.

ELISA study: At 0 day levels of hepcidin and acute phase proteins were found to be increased in the study group when compared with the control group ($P<0.001$) (Table 1). In the evaluation of the study group, Hepcidin, haptoglobin, TNF-α, CRP, SAA, and IL-6 levels were found to be statistically significant ($P<0.001$) at 3rd, 7th, 14th days when compared with the day 0. IL-1 levels were also statistically significant ($P<0.001$) at 7th and 14th days when compared with the day (Table 2).

Also a negative correlation was observed in control group between Hepcidin and Fe levels between 0, 3rd and 7th day, and a positive correlation at 14th day (Table 3). No statistical difference was observed between males and females in the effect of sex for the hepcidin levels. Within group evaluations showed that hepcidin levels of female dogs (n=6) at day 0, 7th and 14th days was statistically significant ($P<0.05$). When male dogs evaluated there was statistically differences between 0, 3rd, 7th and 14th days were significantly different between ($P<0.001$) (Table 4).

Table 1: Values of some acute phase proteins and hepcidin at Day 0 in control and study group

Parameters	Unit	Control group (n=15)	Study group (n=20) *
Hepcidin	µg/ml	14.35±16.28	52.62±32.09
CRP	µg/ml	6.45±1.55	102.41±29.06
SAA	µg/ml	51.02±8.76	1331.71±742.34
Haptoglobin	g/l	0.89±0.45	2.55±0.56
TNF-α	pg/ml	22.08±9.73	266.09±31.21
IL-1	pg/ml	0.01±0.00	1.48±0.59
IL-6	pg/ml	20.46±2.84	44.27±13.09

*All values (mean±SD) differ significantly (P<0.001) than control group.

Immunohistochemical findings: No hepcidin immunoreaction was seen in the livers of animals in the control group. Hepcidin, immune reaction was increased in the livers of the animals with parvoviral enteritis and some hepatocytes showed mild and medium hepcidin expression (Fig.1A-B).

No hepcidin immune reaction was observed in the intestines of animals in the control group. Moderate to severe hepcidin immune reaction was present in some of the intestinal cells of the puppies died from parvoviral enteritis (Fig.1C-D)

No SAA, CRP, TNF, IL-1β and IL-6 immunoreaction but slight haptoglobulin expressions were observed in the livers of control animals. While haptoglobulin, CRP and TNF markedly expressed, mild to slight expressions were observed in SAA, IL-1β and IL-6 in liver from puppy with parvoviral enteritis (Fig 2). Similarly, negative haptoglobulin, SAA, CRP, TNF, IL-1β and IL-6 immunoreaction were in control intestines (Fig 3). Significant increase was observed in CRP and TNF in guts from puppies with parvoviral enteritis while slight to moderate expressions detected in haptoglobulin, SAA, IL-1β and IL-6. CRP was the highest expressed marker in studied markers both the liver and intestines cells in the study group (Table 5). Significant reaction was found especially in the epithelial cells. Particularly high levels of expressions were found in the regenerated intestinal epithelial cells. After CRP, the highest increased expression was seen in TNF-α. Increased TNF -α activity were observed in intestinal epitheliums as well as liver cells in study group (Fig. 4).

DISCUSSION

All animals in the study group diagnosed to have parvo-viral gastroenteritis and were carrying at least two the criteria mentioned for sepsis at the first day they came to our clinic.

Cytokines are key elements of inflammatory response in sepsis and classified as proinflammatory and anti-inflammatory cytokines. The most important of these mediators are TNF-α, IL-1, IL-2 and IL-6. Interleukin-6, TNF-α and proinflammatory cytokines such as IL-1β are the main mediators of acute phase proteins synthesized in the liver (Coskun and Sen, 2012). Endotoxemia, bacteremia and sepsis occurs due to the mucosal barrier damage in the intestine during parvoviral infections, and then TNF-α secretion started as a systemic inflammatory response (Otto *et al.*, 2000; Prittie, 2004). TNF-α and IL-6 levels reported to be increased in dogs with sepsis developed due to parvoviral enteritis (Nemzek *et al.*, 2007). In this study the serum TNF, IL-1 and IL-6 levels in the study group was higher significantly when compared with control group and decreased significantly starting from the third day of the study (P<0.001). Levels of serum TNF, IL-1 and IL-6 are parallel with the studies. Liver and intestinal tissue of died dogs showed significant higher immunohistochemical levels of TNF, IL-1β and IL-6 compared to the control group (P<0.001). In this study, increase in serum and tissue TNF, IL-1β and IL-6 levels was thought to be associated with parvoviral enteritis and sepsis due to secondary bacterial infections.

Haptoglobin (Hp), CRP, SAA, and fibrinogen are commonly used acute phase proteins in diagnosis of inflammation in dogs (Eckersall and Bell, 2010). Kocaturk *et al.* (2010), reported elevated levels of serum CRP, ceruloplasmin and haptoglobin in dogs with parvoviral enteritis in dogs. In this study, serum CRP levels were significantly higher compared to the control group and by the 3rd day of treatment showed a decline (P<0.001). Increase in CRP levels in serum and tissues were thought to be associated with parvoviral enteritis and septicemia occurred due to secondary infections and was parallel with the studies. Also liver and intestinal tissue of died dogs showed significantly immunohistochemical higher CRP levels compared to the control group.

Another increasing acute phase protein in inflammatory reactions in dogs is SAA. During acute phase of inflammation, SAA majorly manufactured in liver and regulated by IL-6 and TNF. Ok *et al.*, (2015) reported significant increases in serum IL-1ß, TNF-α, IFN-γ, CRP and SAA levels in dogs with sepsis. Increased levels of SAA in parvoviral enteritis (Yule *et al.*, 1997) and Leish-maniosis (Martinez-Subiela *et al.*, 2003), SAA and CRP in dogs with piyometra (Jitpean *et al.*, 2014) were demonstrated.

Table 2: Values (mean±SD) of acute phase proteins in study group on various days

Parameters	Unit	Experimental Days				P value
		0	3	7	14	
Hepcidin	(ug/ml)	52.62±32.09	23.9±27.92	16.72±21.10	16.11±20.08	0.001[a,b,c]
CRP	(µg/ml)	102.41±29.06	63.16±11.16	13.87±1.60	13.21±1.46	0.001[a,b,c,d,e,f]
SAA	(µg/ml)	1331.71±742.34	769.61±373.76	128.42±23.15	94.47±20.26	0.001[a,b,c,d,e,f]
Haptoglobin	(g/l)	2.55±0.56	2.15±0.38	1.36±0.35	1.09±0.42	0.001[a,b,c,d,e,f]
TNF-α	(pg/ml)	266.09±31.21	82.81±8.98	81.79±9.57	28.61±3.98	0.001[a,b,c,e,f]
IL-1	(pg/ml)	1.48±0.56	0.12±0.07	0.07±0.05	0.06±0.04	0.001[a,b,c,e]
IL-6	(pg/ml)	44.27±13.09	40.51±11.71	24.28±4.44	23.05±4.03	0.001[a,b,c,d,e,f]

Difference at days: [a]0 vs 3; [b]0 vs 7; [c]0 vs 14; d3 vs 7; [e]3 vs 14; [f]7 vs 14.

Table 3: Results of serum hepcidin and Fe in study group on various days

Parameters	Unit	Study Group (Days)				P value
		0	3	7	14	
Hepcidin	(ug/ml)	52.62±32.09	23.90±27.92	16.72±21.10	16.11±20.08	0.001[a,b,c]
Fe	(mcg/dl)	38.25±19.44	46.20±17.00	86.95±42.23	88.60±32.14	0.001[a,b,c,d,e]
Correlation coefficient		-0.109	-0.064	-0.205	0.064	
Statistical evaluation		P<0.646	P<0.787	P<0.385	P<0.790	

Difference at days: [a]0 vs 3; [b]0 vs 7; [c]0 vs 14; d3 vs 7; [e]3 vs 14.

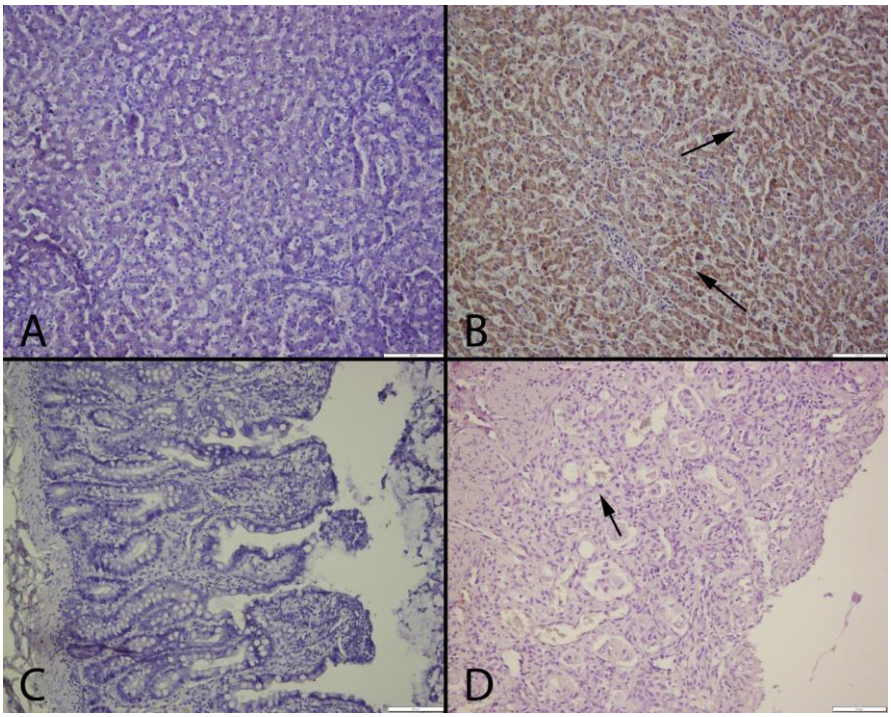

Fig. 1: Hepcidin immunorections. (A) Negative hepcidin immunoreaction in control liver; (B) Marked hepcidin expression in hepatocytes (arrows) in a puppy died from parvoviral enteritis; (C) No expression in control gut; (D) Slight immune reaction in regenerated crypt cells (arrow), Streptavidin biotin peroxidase method, Bars=100µm.

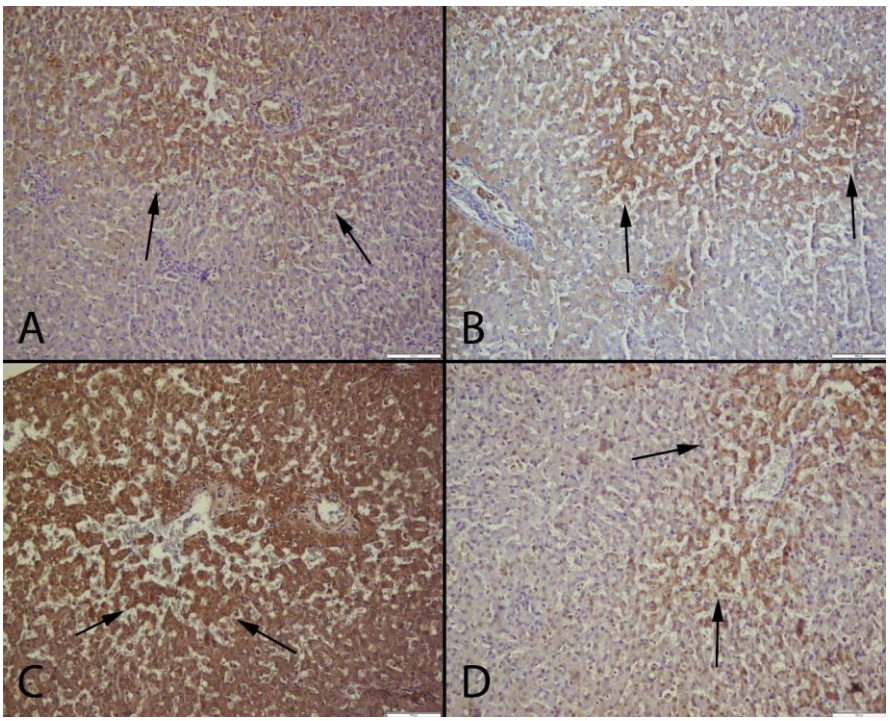

Fig. 2: Expressions of different markers in livers in study groups. (A) Haptoglobulin immunoreaction of liver (arrows) from a puppy with parvoviral enteritis, increase expression in hepatocytes (arrows); (B) Increased SAA expression in liver cells (arrows) in study group; (C) Marked increase in CRP immunoreaction in hepatocytes (arrows) in parvovirirus group; (D) Increase in TNF expression in liver cells (arrows), Streptavidin biotin peroxidase method, Bars=100µm.

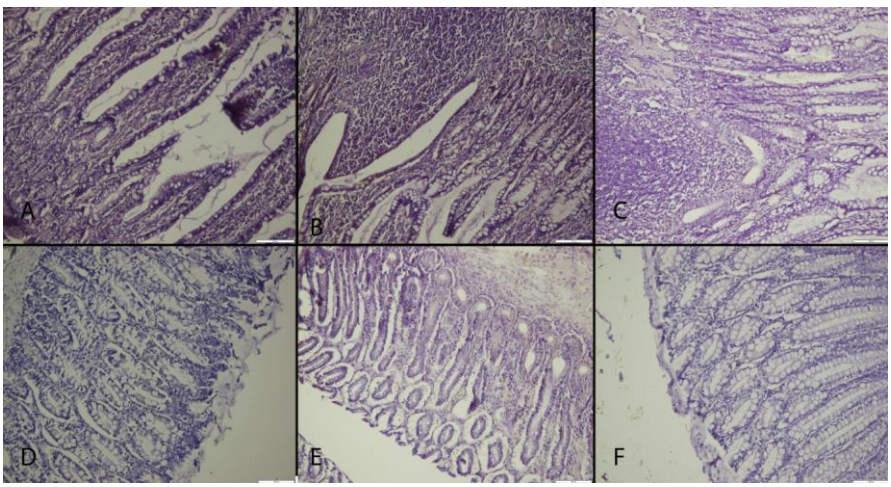

Fig. 3: Negative haptoglobulin (A), CRP (B), SAA (C), IL6 (D), IL1 (E) and TNF-α (F) immunoreaction in control guts, Streptavidin biotin peroxidase method, Bars=100µm.

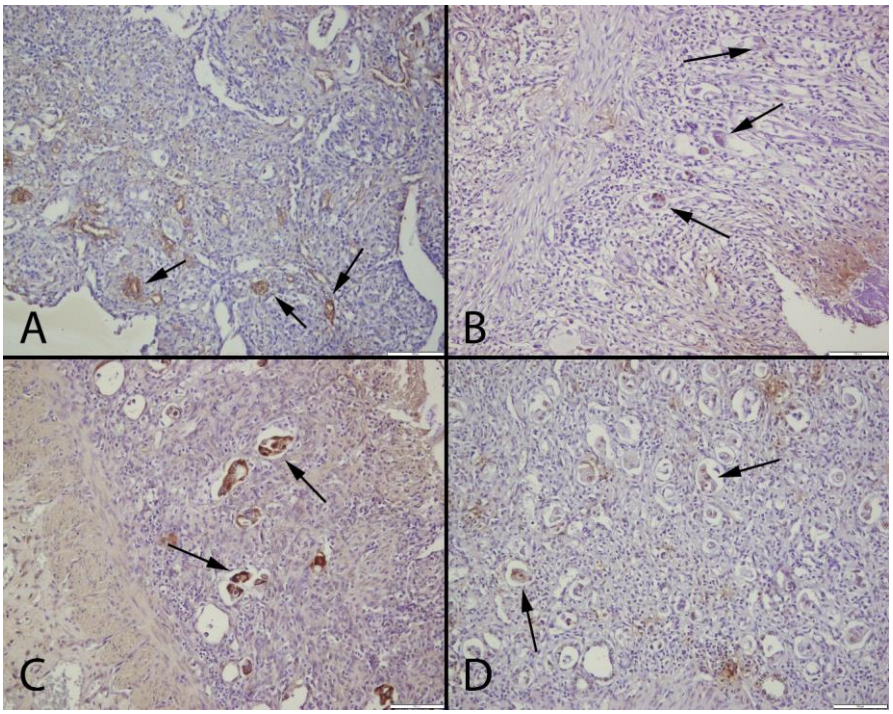

Fig. 4: Expressions of different markers in guts in study groups. (A) Increased haptoglobulin positive immunoreaction of crypt epithelial cells (arrows) from a puppy; (B) Slight Increased SAA expression in regenerated crypt epithelial cells (arrows); (C) Marked increase in CRP immunoreaction in regenerated cells (arrows); (D) Increase in TNF expression in regenerated crypt cells (arrows), Streptavidin biotin peroxidase method, Bars=100µm.

Table 4: Effect of sex for the hepcidin levels (mean±SD; µg/ml)

Day	Female (n=6)	Male (n=14)
0	50.2±34.4	53.7±32.4
3	23.1±30.1	24.2±28.1
7	15.3±18.3	17.3±22.8
14	14.7±17.5	16.7±21.7
Within group P value	$P<0.05^{b,c}$	$P<0.001^{a,b,c}$

Table 5: Values (mean±SD) of immunohistochemical scores in parvoviral enteritis and control group

	Organ	Control (n=5)	Parvoviral enteritis (n=6)	P value
Hepcidin	liver	0.00±0.00	1.83±0.40	<0.05
Hepcidin	gut	0.00±0.00	0.33±0.51	<0.001
Haptoglobulin	liver	0.00±0.00	0.66±0.81	<0.01
Haptoglobulin	gut	0.00±0.00	0.16±0.40	<0.05
SAA	liver	0.00±0.00	0.66±1.21	<0.05
SAA	gut	0.00±0.00	0.83±0.98	<0.01
CRP	liver	0.66±0.81	1.83±0.98	<0.001
CRP	gut	0.00±0.00	1.66±0.81	<0.01
TNF	liver	0.00±0.00	1.16±0.98	<0.001
TNF	gut	0.00±0.00	1.33±0.81	<0.01
IL-1β	liver	0.00±0.00	0.66±0.81	<0.01
IL-1β	gut	0.00±0.00	0.83±0.98	<0.001
IL-6	liver	0.00±0.00	0.16±0.40	<0.05
IL-6	gut	0.00±0.00	0.16±0.40	<0.05

Haptoglobin synthesized by hepatic parenchymal cells and one of the acute phase proteins present freely in blood. Cytokines, interleukin- I, interleukin- 6, and tumor necrosis factors are important mediators for the production of Haptoglobins. Kogika *et al.* (2003) reported a significant increase in the level of serum haptoglobin in dogs with hemorrhagic gastroenteritis. There are numerous haptoglobin functions but primary task is creating a stable complex with free hemoglobin to prevent the loss of iron. Thus it is identified that the Hp shows bacteriostatic effect by limiting the availability of iron necessary for bacterial growth (Sevgisunar and Şahinduran, 2014). This function is similar to hepcidin. In this study, serum Haptoglobulin levels in the study group were significantly higher when compared with the control group and showed a significant decline (P<0.001) after day 3. In the present study the level of serum haptoglobin is parallel to the findings of Kocaturk *et al.* (2010) and Kogika *et al.* (2003). Also, the SAA and haptoglobulin levels determined immuhistochemically in the liver and intestinal tissues of died dogs were significantly higher than the control group. The increase in the serum and tissue SAA and haptoglobulin levels is believed to be associated with sepsis caused by parvoviral enteritis and secondary infection.

Kali *et al.* (2015) reported that hepcidin does not only play an important role in iron metabolism but it is also vital in a wide range of hematological and non-hematological disorders. Kali *et al.* (2015) determined that hepcidin is induced by IL-6 and can be used as an important marker of inflammatory reactions during inflammation and sepsis. Parallel to Kali *et al.* (2015) in this study it was demonstrated that production of hepcidin is induced by IL-6. Hepcidin is also reported to be superior to CRP and WBC in the follow up and prognosis of pancreatitis (Arabul *et al.*, 2013). Wu *et al.* (2013) reported that in low birth weight children, sepsis resulted in 4 times higher hepcidin values compared to low birth weight children without sepsis and decreased after treatment. They concluded that hepcidin along with blood culture is more useful than CRP in the diagnosis sepsis. In this study nearly 4-fold increase in hepcidin levels was seen in the study group compared to control group and after 14th day of study decreased to the average level of control group. This result is similar to the study of Wu *et al.* (2013).

Iron levels in the study group on day 0 was determined to be lower than the value in the control group (P<0.001). We suggested it as a result of anemia and infection in animals. However, after treatment on days 3rd, 7th and 14th it was seen that iron values approach normal levels. Iron levels in the body are balanced by hepcidin (Ganz, 2006). Badial *et al.* (2011) reported an increase in hepcidin production and decrease in iron levels in experimental inflammatory reaction in sheep. In a study performed by Kossiva *et al.* (2012) on children with

infectious disease, serum hepcidin levels of the patients were higher than the control group, and serum iron (Fe) level in patients was lower than the control group (73.71±34.91 ng/ml) and stated the degree of significance as (P<0.001). In our study hepcidin levels in study group was higher than the control group, and serum iron (Fe) level in study group was lower than the control group, and the significance level was found (P<0.001) respectively. This result is in accordance with the results of Kossiva *et al.* (2012). In this study, changes in the levels of hepcidin was followed up throughout the study and showed compatibility with the result of (Kossiva *et al.*, 2013) which stated that hepcidin increased during acute infection in children and gradually decreased in the follow-up. Results of the present study concluded that hepcidin is a diagnostic marker not only in diagnosis also in prognosis of the disease.

Conclusions: By this study important information was obtained about the proinflammatory response and acute phase response of sepsis in dogs with parvoviral enteritis. Hepcidin, TNF, IL-1, IL-6, SAA, Hp, CRP levels in the study group was significantly (P<0.001) higher than the control group and significant decrease (P<0.001) in TNF, IL-1, IL-6, SAA, Hp, CRP levels were seen during the study. Also for the first time hepcidin levels in healthy and dogs with sepsis were determined and course of hepcidin were observed during the follow up of treatment. Thus, the significant increase in hepcidin levels in presence of sepsis in dogs and decrease by the duration of treatment was demonstrated for the first time. It was concluded that hepcidin can be used as an indicator for the diagnosis of sepsis.

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