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Hepatic and Plasma Endothelin-1 in Dogs with Chronic Hepatitis

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Background: Endothelin (ET)-1 is a 21-amino-acid peptide with potent vasoactive properties, which increases intrahepatic resistance in patients with chronic hepatitis (CH) or cirrhosis. ET-1 concentrations have not been investigated in dogs with CH.

Hypothesis/Objectives: This study compared hepatic and plasma ET-1 levels in healthy dogs and in dogs with CH, and examined the relationship between the plasma ET-1 level and portal vein pressure in dogs with CH.

Animals: Fourteen healthy dogs and twenty dogs with CH were used in this study.

Methods: Prospective case-control study. Hepatic ET-1 mRNA expression was determined by real-time reverse transcription polymerase chain reaction, and hepatic and plasma ET-1 levels were assessed using ELISA. Splenic pulp pressure (SPP), as an indicator of portal vein pressure, was measured laparoscopically.

Results: Hepatic ET-1 mRNA levels were 3.7 times higher in dogs with CH than in healthy dogs ($P = .008$). The median hepatic and plasma ET-1 protein levels were significantly higher in dogs with CH than in healthy dogs (13.20 pg/mg wet liver vs. 3.42 pg/mg wet liver, $P = .004$, and 0.99 pg/mL vs. 0.71 pg/mL, $P = .013$, respectively). Moreover, there was a weak but significant correlation between plasma ET-1 level and SPP in dogs with CH ($P = .036$; $r_s = 0.53$).

Conclusions and clinical importance: The results indicate that ET-1 might play an important role in the pathogenesis of portal hypertension caused by CH.

Key words: Canine; Chronic liver disease; Portal hypertension; Vasoactive peptide.

Chronic hepatitis (CH) is a common liver disease in dogs and is histologically defined by the presence of hepatocellular apoptosis or necrosis, a variable mononuclear or mixed inflammatory cell infiltrate, regenerative nodes of hepatocytes, and fibrosis.[1] Cirrhosis is the final stage of CH, and the progression of CH can lead to portal hypertension (PH) as a result of fibrosis and increased resistance, increased blood flow, or both the conditions in the portal circulation.[2] The clinical consequences of PH include acquired portosystemic collaterals (APSCs), ascites, hepatic encephalopathy, or their combination.[3–5]

Endothelin (ET)-1 is a 21-amino-acid peptide with potent vasoactive properties[6,7] and is markedly overexpressed in the human patients with cirrhotic liver, particularly in sinusoidal endothelial cells and hepatic stellate cells.[7] The physiological functions of ET-1 are mediated by two ET receptors, ETA and ETB. ET-1 is produced and released from sinusoidal endothelial cells and exerts paracrine effects on ETA receptors in vascular smooth muscle and hepatic stellate cells, thereby inducing their contraction. ET-1 also exerts autocrine, intracrine, or both effects on sinusoidal endothelial cells via induction of ETB receptor-mediated release of vascular-relaxing factors, such as nitric oxide and prostacyclin. The major function of these receptors appears to be regulation of vascular tone.[7–10] ET-1 levels in plasma and hepatic tissue are elevated in human patients with CH or cirrhosis, and this increase is proportional to the severity of liver disease.[11–13] Current evidence indicates that elevated plasma and hepatic ET-1 levels might also contribute to PH caused by CH in humans.[11–13]

Plasma ET-1 levels are elevated in dogs with heart failure or respiratory failure and are used as diagnostic biomarkers to assess the severity of heart failure or respiratory failure.[14–18] However, the role of ET-1 in the pathogenesis of liver failure has not been evaluated.

This study aimed to determine whether hepatic ET-1 mRNA expression and hepatic and plasma ET-1

Abbreviations:

APSCs	acquired portosystemic collaterals
CH	chronic hepatitis
CI	confidence interval
ET	endothelin
HPRT	hypoxanthine phosphoribosyl transferase
PDGF	platelet-derived growth factor
PH	portal hypertension
PHPV	primary hypoplasia of the portal vein
ROC	receiver operating characteristic
RT-PCR	reverse transcription polymerase chain reaction
SPP	splenic pulp pressure
TGF-β	transforming growth factor-β

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protein levels in dogs with CH are different from those observed in healthy beagle dogs. Additionally, it is investigated whether the plasma ET-1 level correlated with portal vein pressure in dogs with CH.

Materials and Methods

Dogs

In this study, 20 dogs were laparoscopically examined for the histopathological evaluation of CH. Liver biopsy specimens were examined histopathologically by an American College of Veterinary Pathologists board-certified pathologist, and CH was diagnosed in the dogs according to the criteria developed by the World Small Animal Veterinary Association Liver Standardization Group.[1] It was confirmed that the dogs had no concurrent diseases, such as congestive heart failure or pulmonary disease, based on clinical signs, auscultation, thoracic radiography, and echocardiography. The presence of APSCs was determined using laparoscopic splenoportography. The study controls comprised 14 beagle dogs. The College of Bioresource Sciences, Nihon University, granted ethical approval for use of the control dogs, and the study proceeded in accordance with the Guide for Animal Experimentation published by the College of Bioresource Sciences, Nihon University. The beagle dogs were confirmed as healthy according to a clinical examination, a complete blood cell count, serum biochemistry, and abdominal radiography and ultrasonography. The median age and body weight of the control group (9 male dogs and 5 female dogs) were 2.6 years (range, 0.9–5.9 years) and 10.8 kg (range, 9.2–11.7 kg), respectively.

Sampling and Treatment of Biopsy Specimens

After giving general anesthesia, laparoscopic liver biopsy was performed in 20 CH dogs and 3 healthy beagle dogs. Additionally, laparotomic liver biopsy was performed in three other healthy beagle dogs. Biopsy of liver tissue was performed in five to six samples from each dog. The samples were extracted using biopsy forceps and subsequently used for mRNA, hepatic ET-1 protein, and histopathological analyses in all dogs. Liver specimens for total RNA extraction were immediately preserved in RNAlater[a] and stored at −20°C until use. Liver specimens for hepatic ET-1 concentration analysis were immediately immersed in liquid nitrogen and stored at −80°C until use. Blood samples collected in ethylenediaminetetraacetic acid tubes were centrifuged at 1,600 × *g* for 15 minutes at 4°C, and the plasma was stored at −80°C until use. Samples for histopathology were immediately placed in 10% neutral buffered formalin, and hematoxylin and eosin-stained sections were prepared.

Hepatic ET-1 mRNA Expression

ET-1 mRNA levels were measured in the liver tissue samples of the 20 dogs with CH and the 6 healthy beagle dogs using real-time reverse transcription polymerase chain reaction (RT-PCR). Stabilized liver tissue (20 mg) was ground thoroughly with a mortar and pestle, and frozen at −80°C prior to addition of 600 μL of lysis buffer containing β-mercaptoethanol. The lysate was transferred onto a QIAshredder spin column[a] in a 2 mL collection tube and centrifuged at 7,000 × *g* for 2 minutes in a microcentrifuge. The supernatant was used for total RNA extraction with the RNeasy Protect Mini Kit.[a] RNA was quantified spectrophotometrically using a Nanodrop ND-2000[b] system and diluted to a concentration of 0.1 μg/μL. Single-stranded cDNA was synthesized from 1 μg of total RNA using a PrimeScript RT-PCR kit[c] according to the manufacturer's instructions. Thermal conditions for reverse transcription were an initial incubation at 42°C for 15 minutes, followed by incubation at 95°C for 5 minutes and at 4°C for 5 minutes. Real-time RT-PCR was performed using Fast SYBR Green Master Mix[d] in an Applied Biosystems 7500 Fast RT-PCR system.[d] The thermal cycles were carried out as follows: 95°C for 30 seconds; 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds; and dissociation at 95°C for 1 second, 65°C for 15 seconds, and 95°C for 1 second. mRNA levels of hypoxanthine phosphoribosyl transferase (HPRT) were determined for each sample as a control and to normalize the data. All reactions were performed in triplicate and included a no-template control. The amplification efficiency for ET-1 (89%) and HPRT (87%) in the samples was determined in triplicate using serial 10-fold sample dilutions. The relative quantity of each transcript was calculated using the $2^{-\Delta\Delta CT}$ method[19] as described by the manufacturer (Applied Biosystems). The primers used are summarized in Table 1.[20]

Measurement of ET-1 Concentration

ET-1 concentrations were determined in blood samples collected from 20 dogs with CH and 14 healthy beagle dogs, and in liver tissue samples collected from the 20 dogs with CH and 6 of the healthy beagle dogs. Briefly, 100 mg of each minced liver tissue was boiled in a 1mL mixture of 1 M acetate and 20 mM hydrochloride for 10 minutes at 100°C, followed by centrifugation at 7,000 × *g* for 10 minutes at 4°C. The supernatant was filtered, lyophilized, and dissolved in 300 μL of buffer solution according to the manufacturer's instructions (Endothelin-1 Assay Kit[e]). The extracted peptide solution was used for ELISA using a human sandwich ELISA kit that had been validated previously for use with canine plasma.[18,21] The kit exhibited a sensitivity of 0.23 pg/mL and was highly specific for ET-1. Prior to measurement, pre-extraction with a Sep-Pac C-18 column[f] was required to obtain a 2-fold concentration of the sample.[18] The ELISA used was specific for ET-1 and did not cross-react with ET-2, ET-3, big ET-1, big ET-2, or big ET-3 (cross-reactivity, <0.1%).

Splenic Pulp Pressure Measurement

Splenic pulp pressure (SPP) was measured in cases of severe liver change or grossly abnormal blood vessels. SPP was not measured in dogs with grossly normal livers. Splenoportography was performed simultaneously. SPP was measured laparoscopically in the 16 dogs with CH by placing the dogs in the right lateral

Table 1. Primer sequences used in real-time RT-PCR.

Gene	Primer	Sequence (5′–3′)	Product size (bp)	Source
ET-1	Forward	GCC CCG CCG ATC CA	61	AC: AB115087
	Reverse	GGT GGC AGAAGT AGACACACTCTTT	61	AC: AB115087
HPRT	Forward	AGC TTG CTG GTG AAA AGG AC	114	Brinkhof et al.[20]
	Reverse	TTA TAG TCA AGG GCA TAT CC	114	Brinkhof et al.[20]

AC, GenBank accession number; HPRT, hypoxanthine phosphoribosyltransferase.

recumbent position and applying CO_2 gas insufflation (Endoflator[g]) of the abdominal cavity. Intra-abdominal pressure was automatically maintained between 8 and 10 mmHg. Two cannulae were placed in the abdomen, and the abdominal cavity was visualized through a laparoscope (Hopkins II, 5 mm, 0°[g]). An 18-gauge over-the-needle intravenous catheter (SRFF 1832; length, 6.4 cm[h]) was percutaneously inserted into the spleen parallel to the long axis. After the pneumoperitoneum was stopped and gas was removed completely from the abdominal cavity, SPP was measured by a laparoscopist (M.S.) via the catheter as previously described.[22] The normal level for SPP in healthy dogs is 6.2 ± 0.8 mmHg, as previously reported.[22]

Statistical Analysis

Data were expressed as the median (range). Differences in hepatic ET-1 mRNA expression and plasma and hepatic ET-1 concentrations between the dogs with CH and healthy dogs were statistically analyzed using the Mann Whitney test. Area under the receiver operating characteristic (ROC) curve and sensitivity and specificity with 95% confidence intervals (CIs) were calculated. The diagnostic validity of CH in dogs with regard to hepatic and plasma ET-1 concentrations was investigated based on the ROC curve. Correlations between the plasma ET-1 concentration and SPP were evaluated using Spearman's correlation coefficient. All data were analyzed using GraphPad PRISM for Mac OS X version 5.0b.[i] Differences were considered significant at $P < .05$.

Results

Dogs

The 20 dogs with CH (n = 20; 3 intact males, 7 castrated males, 3 intact females, and 7 spayed females) included 12 breeds, including American cocker spaniel, Labrador retriever, miniature dachshund (n = 3 each), English cocker spaniel, Welsh corgi (n = 2 each), Irish setter, Jack Russell terrier, Cairn terrier, Maltese, mixed breeds, Shiba, and West Highland white terrier (n = 1 each). The median age and body weight of dogs with CH were 7.1 years (range, 2.2–11.9 years) and 9.4 kg (range, 4.2–38.3 kg), respectively. The median SPP was 10.0 mmHg (range, 7.0–19.0 mmHg) in the 16 CH dogs. The presence of APSCs was confirmed in 12 dogs with CH. In these 12 dogs, the median SPP was 10.5 mmHg (range, 8.0–19.0 mmHg). In contrast, in the 4 dogs without APSCs, the median SPP was 7.0 mmHg (range, 7.0–9.0 mmHg).

Gene Expression of ET-1 and Hepatic and Plasma ET-1 Concentrations

Hepatic ET-1 mRNA expression was significantly higher in dogs with CH than in healthy dogs ($P = .008$; Fig 1). Hepatic ET-1 protein concentrations were significantly higher in dogs with CH (median, 13.20 pg/mg wet liver; range, 3.56–62.60 pg/mg wet liver) than in the healthy dogs (median, 3.42 pg/mg wet liver; range, 1.70–8.68 pg/mg wet liver; $P = .004$; Fig 2). Similarly, plasma ET-1 concentrations in dogs with CH were significantly higher (median, 0.99 pg/mL; range, 0.37–2.98 pg/mL) than those in healthy beagle dogs (median, 0.71 pg/mL; range, 0.25–1.28 pg/mL; $P = .013$; Fig 3).

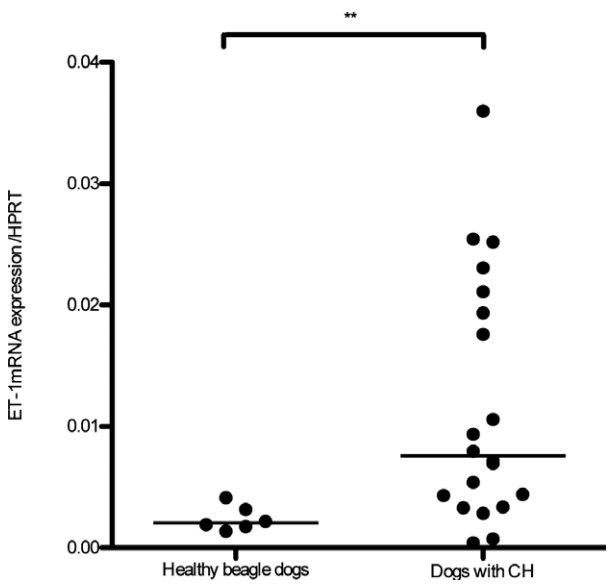

Fig 1. Expression of hepatic ET-1 mRNA in dogs with chronic hepatitis (CH; n = 20) and healthy beagle dogs (n = 6). The expression of hepatic ET-1 mRNA was significantly higher in dogs with CH than in healthy beagle dogs (**$P = .008$). The line represents the median.

The median plasma ET-1 concentrations in CH dogs with APSCs (1.33 pg/mL; range, 0.64–2.98 pg/mL) were slightly but not significantly higher than those measured in CH dogs without APSCs (0.86 pg/mL; range, 0.37–1.53 pg/mL; $P = .114$). The sensitivity and specificity of hepatic ET-1 concentration (cut-off, 6.66 pg/mg wet liver) were 80.0% (95% CI, 56.3–94.3%) and 83.3% (95% CI, 35.9–99.6%), respectively. The sensitivity and specificity of plasma ET-1 concentrations (cut-off, 0.82 pg/mL) were 70% (95% CI, 45.7–88.1%) and 64.3% (95% CI, 35.1–87.2%), respectively. The areas under the ROC curves were 0.90 (95% CI, 0.76–1) and 0.75 (95% CI, 0.59–0.92) for hepatic and plasma ET-1 concentrations, respectively.

Relationship between Plasma ET-1 Concentration and SPP

There was a weak but significant correlation between plasma ET-1 concentration and SPP in dogs with CH ($r_s = 0.53$; $P = .036$, n = 16; Fig 4).

Discussion

Dogs with PH caused by CH and cirrhosis exhibited PH symptoms. Portal vein pressure is rarely measured, and the presence of APSCs indicates PH in dogs. The main treatment for PH is infusion and diuretics. The ET family consists of three members, ET-1, ET-2, and ET-3, among which ET-1 is a potent vasoconstrictor, that are widely expressed in a variety of tissues such as the blood vessels, heart, lungs, and liver.[7] Elevated hepatic ET-1 mRNA expression and circulating ET-1 protein in human patients with PH caused by liver diseases, such as CH and cirrhosis, have been

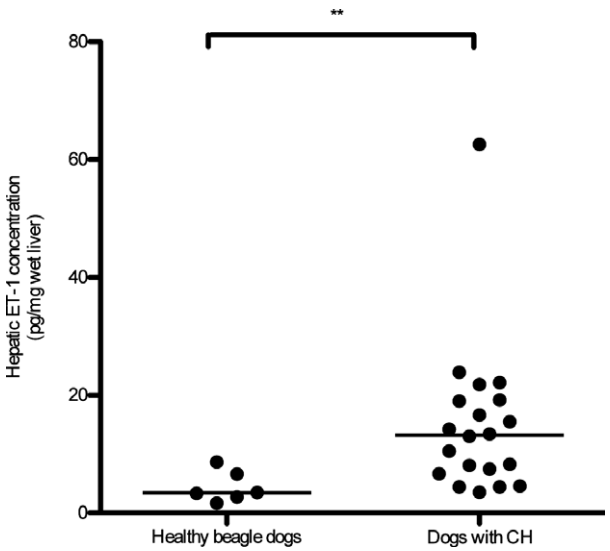

Fig 2. Hepatic ET-1 concentration in dogs with chronic hepatitis (CH; n = 20) and healthy beagle dogs (n = 6). The hepatic ET-1 concentration was significantly higher in dogs with CH than in healthy beagle dogs (**P = .004). The line represents the median.

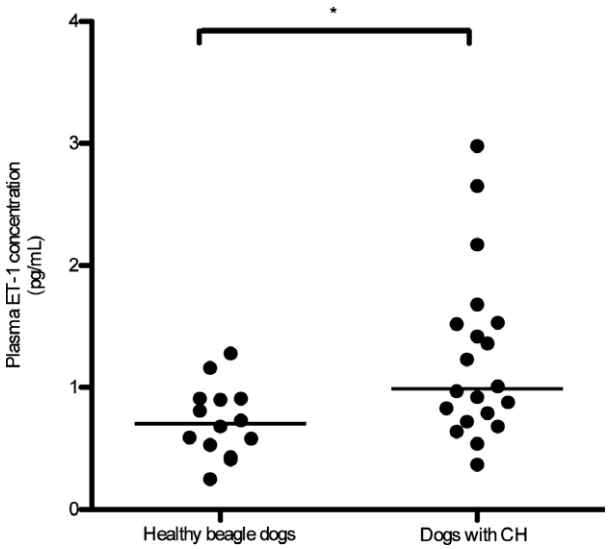

Fig 3. Plasma ET-1 concentration in dogs with chronic hepatitis (CH; n = 20) and healthy beagle dogs (n = 14). The plasma ET-1 concentration was significantly higher in dogs with CH than in healthy beagle dogs (*P = .013). The line represents the median.

reported,[11–13,23] and hepatic and plasma ET-1 levels are closely related to the severity of liver fibrosis and PH.[11–13] Elevated circulating ET-1 levels in dogs with pulmonary hypertension caused by idiopathic pulmonary fibrosis and heartworm disease have been reported.[18,24] Plasma ET-1 levels are significantly higher in dogs with congestive heart failure than in dogs with heart disease without congestive heart failure.[17] Therefore, elevated ET-1 levels are an exacerbating factor in dogs with pulmonary hypertension and heart disease. However, hepatic ET-1 mRNA and plasma and hepatic

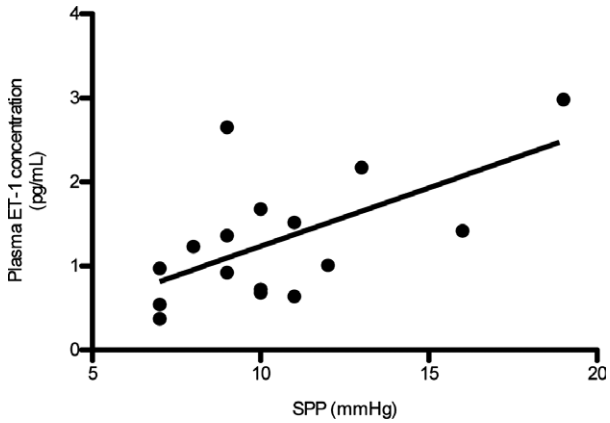

Fig 4. Relationship between plasma ET-1 concentration and splenic pulp pressure (SPP) in dogs with chronic hepatitis (CH). There was a weak but significant correlation between plasma ET-1 levels and SPP in dogs with CH (r_s = 0.53; P = .036, n = 16).

ET-1 protein levels in dogs with PH caused by liver disease have not been reported.

This study demonstrated that hepatic ET-1 mRNA expression was significantly higher in dogs with CH than in healthy beagle dogs. Similar observations were previously reported by Leivas et al.[23] and Tièche et al.[25] for expression of the ET-1 gene in humans and rats. Additionally, ET-1 mRNA is upregulated by cytokines, including transforming growth factor-β (TGF-β), and growth factors, such as platelet-derived growth factor (PDGF), in a variety of cells.[7] Although we did not investigate the levels of TGF-β and PDGF, previous studies showed that increased expression of the PDGF and TGF-β genes and elevated plasma TGF-β contribute to fibrosis in dogs with CH.[26,27] Additionally, several studies reported a 2- to 5-fold increase in plasma ET-1 levels in human patients with cirrhosis.[28–30]

Plasma ET-1 concentrations are significantly higher in patients exhibiting higher scores associated with liver disease, and hepatic ET-1 concentrations are correlated with the extent and severity of hepatic fibrosis in human patients.[12,13] In accordance with the increased mRNA expression, hepatic and plasma ET-1 protein concentrations in dogs with CH were significantly higher than those in healthy beagle dogs. Elevated hepatic ET-1 concentrations indicated that ET-1 might contribute to increased intrahepatic resistance by constriction of the sinusoid, and may exacerbate PH caused by CH and cirrhosis. The factors involved in the elevation of plasma ET-1 concentrations include increased hepatic, splanchnic, and renal ET-1 production as well as decreased hepatic clearance.[28] Moreover, downregulation of the ETB receptor was previously reported.[7,31] Hepatic ET-1 concentrations may be more suitable than plasma ET-1 concentrations for diagnosis of CH. Plasma ET-1 concentrations may change relative to hepatic ET-1 levels through changes in the portal hemodynamics depending on the presence or absence of APSCs[32] and the effect of clearance in the lungs and liver. However, the cut-off value for the hepatic ET-1 concentration in the present study was 6.66 pg/mg wet

liver, whereas the concentrations measured in healthy beagle dogs and CH dogs were within a range of 3.56–8.68 pg/mg wet liver. Therefore, we believe that cases presenting values such as these require attention for potential diagnosis of CH.

Plasma ET-1 levels in the hepatic veins have been found to be significantly correlated with the hepatic venous pressure gradient in human patients with cirrhosis.[33,34] Notably, we found a weak correlation between plasma ET-1 levels and SPP in the 16 dogs with CH, in which SPP could be measured. Therefore, these results suggest that plasma ET-1 might be involved in increased portal vein pressure in dogs with CH.

It has been reported that administration of ET-1-receptor antagonists reduces portal vein pressure in carbon tetrachloride-treated mice.[35] Additionally, nonselective ET-1 antagonists exhibit antifibrotic effects.[35] The ETB receptor mediates nitric oxide production, which is presumably important in maintaining sinusoidal relaxation in normal liver tissue, whereas in the injured liver, the ETB receptor mediates fibrogenic and contractile responses in hepatic stellate cells. In this state, blocking this effect leads to specific antifibrogenic and portal-hypotensive responses.[35] The efficacy of ET receptor antagonists has been evaluated in dogs with experimental congestive heart failure.[36,37] Therefore, ET-1 receptor antagonists may be applied to treat dogs with PH caused by CH.

Liver diseases that cause PH in dogs include primary hypoplasia of the portal vein (PHPV), CH, and cirrhosis.[1] In humans, idiopathic PH is a disease similar to PHPV in dogs. Although plasma ET-1 concentrations are high in humans with idiopathic PH, hepatic ET-1 concentrations and immunoreactivity are low in these patients.[38] Nitric oxide levels and expression of vascular cell adhesion molecule-1 are reportedly elevated in the spleen and blood of patients with idiopathic PH as compared to healthy subjects according to immunochemical staining results. Therefore, to clarify the role of ET-1 and other vasoactive agents, such as nitric oxide and vascular cell adhesion molecule-1, in disease pathogenesis, it may be useful to reveal the pathogenic mechanisms in dogs with PHPV to promote the discovery of therapeutic targets.

This study had the following six limitations: (1) a small number of dogs were included, and the controls were of a single breed; (2) all dogs included were diagnosed with CH, although various subtypes of this disease may exist in dogs; (3) we do not know the specific site of ET-1 production in the liver; (4) although reports have indicated that ET-1 levels do not increase with age in dogs, in contrast to humans,[14,18] the control beagle dogs used in this study were very young relative to the dogs with CH; (5) we did not apply a grading classification to the stained liver tissues, despite correlations between the severity of liver disease and hepatic and plasma ET-1 concentrations being reported in humans;[11,13] and (6) despite the MIQE guidelines for real-time RT-PCR published in 2009,[39] we used only one reference gene in this study.

In conclusion, we found significantly higher plasma ET-1 levels, hepatic ET-1 mRNA levels, and hepatic ET-1 levels in dogs with CH than in control beagle dogs. Based on these results, ET-1 may be involved in the pathogenesis of PH in dogs with CH. These findings suggest an avenue for the treatment of PH caused by CH in dogs.

Footnotes

[a] Qiagen, Valencia, CA
[b] Thermo Scientific, Yokohama, Japan
[c] TaKaRa, Shiga, Japan
[d] Applied Biosystems, Branchburg, NJ
[e] IBL, Gunma, Japan
[f] Waters Corporation, Milford, MA
[g] Karl Storz GmbH & Co. KG, Tuttlingen, Germany
[h] SR-FF1832; length, 6.4 cm; Terumo, Tokyo, Japan
[i] GraphPad Prism; GraphPad Software Inc., San Diego, CA

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Conflict of Interest Declaration: The authors declared that there is no conflict of interest.

Off-label Antimicrobial Declaration: The authors declared that there is no off-label use of antimicrobials.

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