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**Study on the evaluation of hepatic microvascular perfusion and
hepatic fibrosis using ultrasonography in dogs with liver disease**

(犬の肝疾患における超音波検査を用いた肝微小循環および
肝線維化の評価に関する研究)

Masahiro Tamura

GENERAL ABBREVIATIONS

2D-SWE	Two-dimensional shear wave elastography
ARFI	Acoustic radiation force impulses
AUC	Area under the curve
CEUS	Contrast-enhanced ultrasonography
CI	Confidence interval
CV	Coefficient of variation
HA	Hyaluronic acid
PV/Ao	Portal vein-to-aorta ratio
ROC	Receiver operating characteristic
ROI	Region of interest
RR	Rising-rate
RT	Rising-time
SWV	Shear wave velocity
Tchol	Total cholesterol
TIC	Time-intensity curve
TTP	Time-to-peak
Δ HP-PV	Portal vein-to-hepatic parenchyma transit time

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GENERAL INTROCUCTION

Ultrasonography is non-invasive, free of radiation, and easy to use. It is commonly used as the first imaging modality to diagnose dogs with liver disease. However, conventional ultrasonography has some disadvantages including a demand of highly experienced and skilled operator. Additionally, the conventional ultrasonography such as B mode and color Doppler is difficult to assess images quantitatively.

To resolve this issue, two major categories of ultrasonography are recently developed: Contrast-enhanced ultrasonography (CEUS) and elastography. CEUS takes advantages of microbubble technology to improve the echogenicity of blood and evaluate quantitatively tissue perfusion. Second-generation contrast agents (Sonazoid[®], Daiichi-Sankyo, Tokyo, Japan) comprise microbubbles with higher stability and resistance to pressure, providing minimal variability in clinical performance.^{1,2} In liver perfusion studies, CEUS can detect small vessels and evaluate quantitative hemodynamic indices that relate to tissue perfusion. Many studies in humans have evaluated liver perfusion of various hepatic parenchymal disorders, such as viral hepatitis and alcoholic hepatitis.³⁻⁵ The results of these studies indicated the potential of CEUS in enhancing the diagnostic accuracy, understanding pathophysiology, or monitoring and predicting response to treatment of various hepatic disorders.

Congenital portosystemic shunt is a vascular anomaly that connects the portal vein to the systemic circulation and is common diseases in veterinary medicine. A single extrahepatic congenital portosystemic shunt is reported in 75% of affected dogs, whereas dogs with congenital intrahepatic portosystemic shunts accounted for 8%.⁶ The clinical diagnosis of extrahepatic congenital portosystemic shunt is supported by signalment, clinical history, and serum biochemistry (e.g., liver

enzyme activity, hypoalbuminemia, and an increase of serum bile acid or ammonium). Conventional B-mode ultrasound imaging is also commonly used as the first imaging modality to diagnose dogs with extrahepatic congenital portosystemic shunt. However, it is occasionally difficult to detect the abnormal vessels by ultrasonography due to the specific limiting factors, such as gastrointestinal gas, patient conformation, and operator-dependent function.

The liver is a unique organ that receives its blood supply from two sources: the portal vein and the hepatic artery. In a healthy animal, approximately 70%–80% of the blood entering the liver is poorly oxygenated and supplied by the portal vein. This is venous blood flowing from the intestines, pancreas, spleen, and gallbladder. The remaining 20%–30% of the blood supply is well oxygenated and delivered by the hepatic artery.⁷ It is well known that hepatic parenchymal disorder, abnormal vasculature, and various other factors such as the production of vasoactive substances and cytokines, lead to change of hepatic microvascular perfusion.^{8,9} In dogs with extrahepatic congenital portosystemic shunt, vascular anomaly connecting the portal vein to the systemic circulation decreases the portal vein blood flow supply to the liver. Consequently, it is thought that changes in hepatic microvascular perfusion (arterialization) caused by arteriovenous shunting or capillarization of the sinusoid occurs in dogs with extrahepatic congenital portosystemic shunt. Thus, detecting the hepatic microvascular perfusion change using CEUS can help understand the pathophysiology and additional diagnostic tests in dogs with extrahepatic congenital portosystemic shunt.

Ultrasound elastography is a relatively recent imaging technique that can be used to noninvasively evaluate tissue elastic modulus.^{10,11} Similar to conventional B-mode ultrasound imaging, elastography is noninvasive, does not involve radiation, and can be easily learned. Two major categories of elastography techniques are currently widely used in clinical settings for humans.¹² Strain imaging is an elastography technique in which stress is applied by external compression with an ultrasound transducer or internal physiologic pulsation, such as a heartbeat or respiration. Strain

imaging is used to measure the amount of lesion deformation relative to the surrounding normal tissue; it involves use of a color display and provides qualitative or semiquantitative evaluations. In veterinary medicine, strain imaging has been performed on the liver, spleen, and kidneys of clinically normal dogs¹³ and cats.¹⁴ However, strain imaging cannot be used to quantitatively evaluate tissue elastic modulus. A more recently developed and more quantitative elastography technique is shear wave imaging, which overcomes the limitation of strain imaging and consists of ultrasonographic-based transient elastography and shear wave elastography (SWE). Shear wave imaging involves the use of dynamic stress to generate shear waves in parallel or perpendicular dimensions. Measurements of the shear wave propagation speed result in qualitative and quantitative estimates of tissue elasticity. Furthermore, SWE by use of acoustic radiation force impulses (ARFI) has been developed. For SWE, shear wave propagation speed is measured within tissues and allows tissue elastic modulus to be evaluated as shear wave velocity (SWV; reported in meters per second) or the Young modulus (reported in kilopascals). Two types of SWE are currently used in human medicine: point SWE and two-dimensional SWE (2D-SWE). Point SWE is used to measure tissue elastic modulus with the shear wave generated from a fairly limited area of an ROI, which needs to be in a homogeneous area. In contrast, 2D-SWE generates a 2D image of elastic modulus over a larger region of tissue. An arbitrarily sized region of interest (ROI) may be used in 2D-SWE. Furthermore, 2D-SWE provides 2D color velocity maps and also has the advantage of the placement of arbitrary ROIs during real-time imaging.¹⁰⁻¹²

Hepatic fibrosis is histopathologically characterized by the progressive accumulation of extracellular matrix in the liver parenchymal tissue, which subsequently leads to distortion of hepatic architecture. Hepatic fibrosis in dogs usually is caused by chronic diffuse liver diseases, such as chronic hepatitis and cholangiohepatitis.^{15,16} Progression of hepatic fibrosis involves an increase in the liver elastic modulus and resistance of hepatic blood flow, which consequently result in liver cirrhosis

and portal hypertension. In humans with hepatic fibrosis, the stage of hepatic fibrosis and its progression are essential for determination of optimal treatment and prediction of prognosis.¹⁷

Liver biopsy is the gold standard for diagnosing the severity of hepatic fibrosis in dogs and humans. However, the limitations of liver biopsy include the risk of bleeding and anesthesia, invasiveness, and possibility of sampling errors.^{18,19} Hence, non-invasive and easily accessible methods of predicting stages of hepatic fibrosis have been developed in human medicine. Many studies in human patients with hepatic fibrosis have indicated that SWV is correlated with the stage of hepatic fibrosis, and SWE is widely applied in the clinical setting to predict the stage of hepatic fibrosis in humans.²⁰⁻²⁴ However, to the best of our knowledge, no reports have evaluated the use of 2D-SWE for the diagnosis of hepatic fibrosis in dogs with spontaneous hepatic disease.

With the above background, this study was performed in 3 stages to determine the usefulness of quantitative CEUS in dogs with extrahepatic congenital portosystemic shunt to understand the pathophysiology and the feasibility of 2D-SWE in dogs with hepatic fibrosis. In the first stage, I have investigated the perfusion changes of the hepatic artery, portal vein, and hepatic parenchyma in dogs with extrahepatic congenital portosystemic shunt using CEUS. In the second stage, I have established the repeatability and reproducibility of SWV using 2D-SWE in healthy beagle dogs. In the third stage, I have investigated the usefulness of 2D-SWE in detecting hepatic fibrosis in dogs with chronic diffuse liver disease.

CHAPTER 1

QUANTITATIVE EVALUATION OF HEPATIC MICROVASCULAR PERFUSION IN DOGS WITH EXTRAHEPATIC CONGENITAL PORTOSYSTEMIC SHUNT USING CONTRAST-ENHANCED ULTRASONOGRAPHY

1. INTRODUCTION

So far, in dogs, it is often difficult to diagnose with congenital portosystemic shunt by using the conventional B-mode ultrasound imaging due to the specific limiting factors, such as gastrointestinal gas, patient conformation, and operator-dependent function. Assessment of the perfusion changes of the liver in dogs with extrahepatic congenital portosystemic shunt using CEUS would help understand the pathophysiology and additional diagnostic test in dogs with extrahepatic congenital portosystemic shunt.

The present study hypothesized that (1) CEUS can detect the change in hepatic microvascular perfusion in dogs with extrahepatic congenital portosystemic shunt, (2) dogs with extrahepatic congenital portosystemic shunt show an arterialization of the liver and a subsequent compensatory increase in hepatic artery blood flow caused by a decrease in the portal vein blood flow, and (3) the characterization of the simultaneous hepatic microvascular perfusion change, including hepatic artery, portal vein, and hepatic parenchyma, is an additional diagnostic test that can distinguish dogs with extrahepatic congenital portosystemic shunt from healthy dogs.

2. MATERIALS AND METHODS

2.1 Study population

The study was a prospective, exploratory design. Ten client-owned dogs with suspected extrahepatic congenital portosystemic shunt were consecutively enrolled between June 2017 and May 2018 at the Hokkaido University Veterinary Teaching Hospital. Informed owner consent was obtained in all cases. Complete blood count, serum biochemistry, and urinalysis were obtained in all the dogs. In addition, all the dogs had the PV-to-aorta (PV/Ao) ratio measured using abdominal ultrasonography by three trained operators with over 10 years of experience performing liver ultrasound examinations. The inclusion criteria in this study were dogs with morphologic characteristics consistent with an extrahepatic congenital portosystemic shunt that was identified on computer tomography, and dogs receiving surgical treatment. Exclusion criteria were dogs with an acquired shunt or portal hypertension from diagnostic imaging, surgical findings, or measurement of portal vein pressure. Dogs with liver tumor were also excluded.

As a control population, eight healthy beagles were included; three intact males and five intact females. Median (range) of age and body weight were 31 (22–52) months of age and 10.5 (9.4–15) kg, respectively. All the dogs were confirmed to be healthy based on physical examination, complete blood count, serum biochemical panel, echocardiography, and abdominal ultrasonography. All the animal experimental procedures were conducted in accordance with the standard operation protocols of the institutional animal experimental committee reviewed by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The Animal Experimentation Committee, Graduate School of Veterinary Medicine, Hokkaido University approved all the animal experiments (Accession No. 18-0075).

2.2 CEUS

I performed the CEUS preoperatively without sedation in all the dogs. The ultrasound equipment (Aplio 500, Canon Medical Systems, Tochigi, Japan) using a 5–11 MHz broadband linear transducer (PVT-704 AT, Canon Medical Systems, Tochigi, Japan) was optimized for pulse subtracting imaging. The CEUS procedure was performed by the author to ensure consistent imaging conditions throughout the study. All the dogs were positioned in left lateral recumbency for imaging of the liver. The transducer was placed in the intercostal space parallel to the ribs with sufficient gel to minimize rib shadowing. The right division in liver was identified under visual control in B-mode. In a quantitative analysis using a contrast agent, hepatic artery, portal vein, and hepatic parenchyma at the level of porta hepatis were simultaneously scanned in a transverse section via the intercostal approach (Figure 1A). The CEUS examination was performed with a low mechanical index of 0.20, a frame rate of 15 frames/s, a Doppler gain of 80, a dynamic range of 45, and a focus of 3–5 cm in depth. The microbubble contrast agent used was perfluorobutane within a hydrogenated egg phosphatidylserine shell (Sonazoid®). All the dogs received a 22-gauge intravenous catheter in the cephalic vein with a 21-gauge butterfly catheter. Perfusion imaging was evaluated after a contrast agent (0.01 ml/kg) was intravenously injected through catheter, and 3 ml of heparinized normal saline was flushed. This was performed by one person throughout this study. The dose of microbubbles was determined according to methods from our previous reports.²⁵ The data generated for the first 30 s were saved as raw data in the system hardware.

The ROI was placed at hepatic artery, portal vein, and hepatic parenchyma and adjusted manually to maintain the same position when respiratory motion was present. An analysis of time-intensity curve (TIC) was performed using a Contrast Harmonic Imaging Quantification (CHI-Q) RAW Data package (Canon Medical Systems, Tochigi, Japan) installed on ultrasound equipment, and the smoothing function for the TIC was set to 19. The time-to-peak (TTP) in hepatic artery, portal vein,

and hepatic parenchyma was defined as the time interval (in seconds) from the onset of the injection to the peak of the TIC. For the TIC characterizations, the rising-time (RT) was defined as the time interval (in seconds) from the first arrival of contrast agents to the peak of the TIC, and the rising-rate (RR) was defined as an average increase of the signal (in decibels (dB)/s) between the first arrival time of contrast agents and the peak of TIC (Figure 2). In addition, the PV-to-HP transit time (Δ HP-PV) (in seconds) was calculated.

2.3 Assessment of repeatability

Eight healthy beagles were also used for the assessment of repeatability. The CEUS procedure was performed on three different days (minimum one-week interval) in all the dogs without sedation. Repeatability of all the CEUS parameters described above was assessed in this study.

2.4 Statistical analysis

A commercially available software (JMP Pro[®], version 13.1.0, SAS Institute Inc, North Carolina, USA) was used to perform statistical analyses. All the data were expressed as median values with range. Repeatability was assessed by coefficient of variation (CV), and a small CV indicated a highly reliable measurement. In this study, the Shapiro-Wilk test was used to evaluate normal distribution. If the data distribution of measured parameter from healthy and extrahepatic congenital portosystemic shunt dogs was normal, the Student's *t*-test was used. If the data were not normally distributed, the Wilcoxon rank sum test was used. Spearman's rank correlation coefficient was used to assess the correlation between the perfusion parameters and the PV/Ao ratio in dogs with extrahepatic congenital portosystemic shunt. Receiver operating characteristic (ROC) curves with the Mann-Whitney U-test were used to evaluate the accuracy of the parameters in distinguishing extrahepatic congenital

portosystemic shunt dogs from healthy dogs. Additionally, the area under the curve (AUC) was calculated for each parameter. A *P* value less than 0.05 was considered statistically significant.

3. RESULTS

3.1 Study population

Eighteen dogs were initially included in the study, and no side effects were noticed in any of the dogs. One dog was excluded because it was impossible to perform quantitative analysis due to poor image quality. Finally, 17 dogs, including eight healthy dogs and nine dogs with extrahepatic congenital portosystemic shunt were enrolled in the present study. Dogs in the extrahepatic congenital portosystemic shunt group were three intact males, one castrated male, one intact female, and four spayed females. The median age was 38 (range, 6–111) months, and weight was 2.4 (1.3–7.0) kg. The extrahepatic congenital portosystemic shunt dog breeds included two each of mixed-breed dogs and Yorkshire terriers, and one each of Miniature Schnauzer, Shiba, Toy Poodle, Maltese, and Chihuahua. Shunt morphology was classified based on a previous report²⁶ and included three each of spleno-caval and spleno-azygos, and one each of spleno-phrenic, right gastric-caval, and right gastric-azygos with caudal loop. The median PV/Ao ratio in the extrahepatic congenital portosystemic shunt group was 0.55 (range, 0.41–0.91).

3.2 Assessment of repeatability

I initially assessed the repeatability of CEUS parameters in all healthy dogs without sedation. Table 1 presents the CV and 95% confidence interval (CI) in the present study. The CVs of all the parameters in hepatic microvascular perfusion were 4.8–15.0% with moderate to high (relatively low CV) repeatability.

3.3 CEUS analysis

In the healthy dogs, after the contrast agent administration, the microbubbles first reached the hepatic artery (Figure 1B) and, then, immediately reached peak enhancement. After a delay of several seconds, the microbubbles reached portal vein and hepatic parenchyma. They reached peak enhancement in portal vein (Figure 1C). Then, the microbubbles gradually reached peak enhancement in hepatic parenchyma (Figure 1D). Conversely, in the dogs with extrahepatic congenital portosystemic shunt, immediately after the microbubbles reached the hepatic artery (Figure 3A), they reached the hepatic parenchyma and rapidly reached peak enhancement (Figure 3B). Then, the microbubbles finally reached peak enhancement in portal vein (Figure 3C).

The TIC was made for the healthy and extrahepatic congenital portosystemic shunt dogs. In the healthy dogs, the TTP of hepatic parenchyma was long, and the TIC of hepatic parenchyma showed a gradually rising curve (Figure 4A). On the other hand, the TTP of hepatic parenchyma in dogs with extrahepatic congenital portosystemic shunt was earlier than in healthy dogs, and the TIC of hepatic parenchyma in extrahepatic congenital portosystemic shunt dogs showed a rapidly rising curve (Figure 4B).

The TTP and Δ HP-PV measured from TIC were compared between healthy and extrahepatic congenital portosystemic shunt dogs and are shown in Table 2. The TTP of hepatic artery in extrahepatic congenital portosystemic shunt dogs was significantly different from healthy dogs ($P = 0.0023$). Furthermore, the TTP of hepatic parenchyma in extrahepatic congenital portosystemic shunt dogs was significantly earlier than in healthy dogs ($P = 0.0018$). Conversely, the TTP of the portal vein was not different between extrahepatic congenital portosystemic shunt and healthy dogs. The Δ HP-PV in extrahepatic congenital portosystemic shunt dogs was shorter compared with that in healthy dogs ($P = 0.0018$).

Table 3 shows the characteristics of TIC in hepatic artery, portal vein, and hepatic parenchyma. In the extrahepatic congenital portosystemic shunt dogs, a rapidly rising form of TIC of

hepatic parenchyma was like that of portal vein. The RT of the hepatic artery in the extrahepatic congenital portosystemic shunt dogs was significantly earlier than in the healthy dogs ($P = 0.0153$). The RR of hepatic artery in extrahepatic congenital portosystemic shunt dogs was significantly higher compared to that in healthy dogs ($P = 0.0033$). In the hepatic parenchyma, the RT in extrahepatic congenital portosystemic shunt dogs was significantly earlier than that in healthy dogs ($P = 0.0024$). Additionally, the RR of hepatic parenchyma in extrahepatic congenital portosystemic shunt dogs was significantly higher compared to that in healthy dogs ($P = 0.0007$). On the other hand, the RT and RR in portal vein were not significantly different between dogs with extrahepatic congenital portosystemic shunt and healthy dogs. In addition, the RT in hepatic parenchyma ($r = 0.8703$, $P = 0.0023$) and $\Delta\text{HP-PV}$ ($r = 0.8167$, $P = 0.0072$) showed good correlation with PV/Ao ratio in dogs with extrahepatic congenital portosystemic shunt. However, the TTP and RR in hepatic parenchyma did not significantly correlate with PV/Ao ratio.

3.4 ROC analysis

Sensitivity and specificity were determined by selecting the cut-off point along each ROC curve that maximized sensitivity and maintained maximal or near maximal accuracy to avoid excessively reducing specificity. According to the ROC analysis, AUC was 0.94 on RT of hepatic parenchyma, 0.99 on RR of hepatic parenchyma, and 0.95 on $\Delta\text{HP-PV}$, and there was a significant difference (P value of all parameters < 0.0005). The cut-off value was < 14.8 s on RT of hepatic parenchyma, providing 100% sensitivity and 75.0% specificity for distinguishing extrahepatic congenital portosystemic shunt dogs from healthy dogs. The cut-off value was > 0.16 dB/s on RR of hepatic parenchyma, providing 100% sensitivity and 87.5% specificity for distinguishing extrahepatic congenital portosystemic shunt dogs from healthy dogs. The cut-off value was < 7.3 s on $\Delta\text{HP-PV}$,

providing 100% sensitivity and 75.0% specificity for distinguishing extrahepatic congenital portosystemic shunt dogs from healthy dogs.

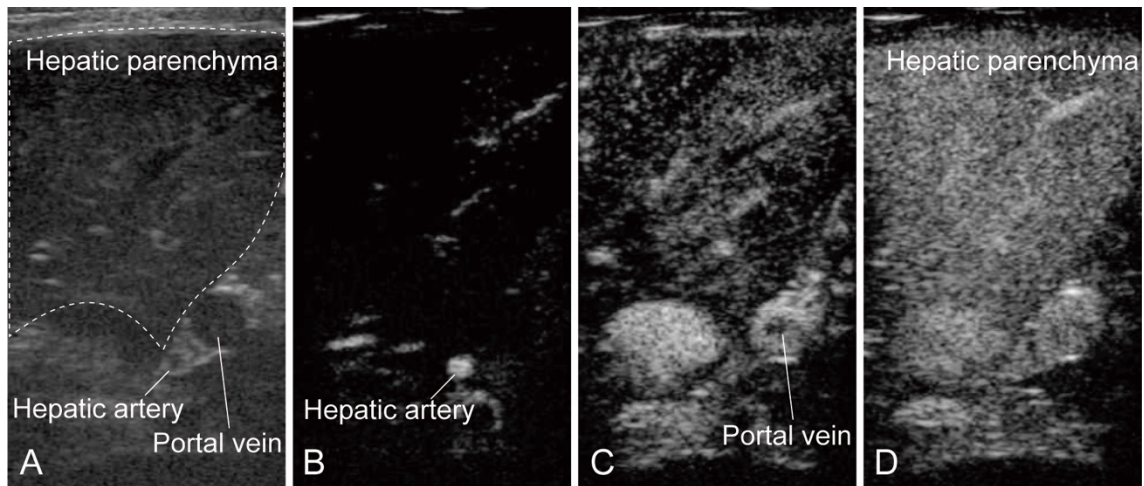


Figure 1. Images of hepatic artery, portal vein, and hepatic parenchyma at the level of porta hepatis in healthy dogs.

A, B-mode image before a bolus injection of Sonazoid. The hepatic artery, portal vein, and hepatic parenchyma were scanned. B, Three seconds after a bolus injection of Sonazoid. First, the microbubbles reached the hepatic artery. C, 11 seconds after a bolus injection of Sonazoid. The microbubbles reached peak enhancement in portal vein. D, 24 seconds after a bolus injection of Sonazoid. Finally, the microbubbles reached the peak enhancement in hepatic parenchyma.

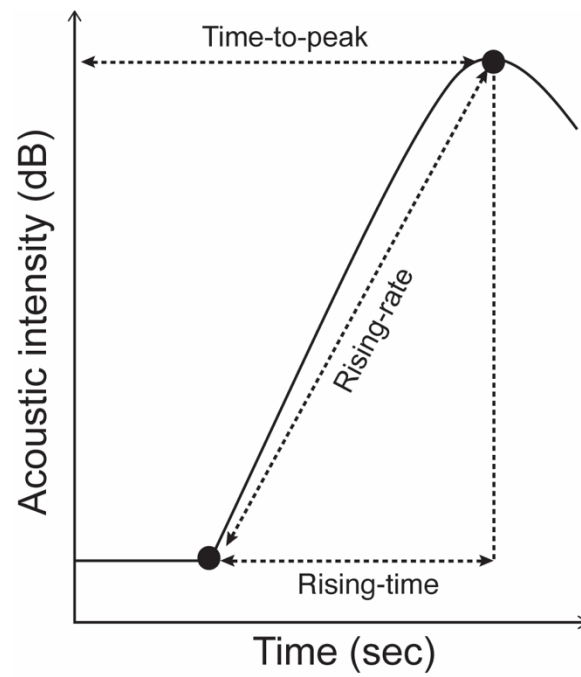


Figure 2. Schematic illustration of contrast-enhanced ultrasonography parameters measured from time-intensity curve.

Parameters	Mean $\pm$ SD	95% CI	CV (%)
HA TTP (s)	3.74 $\pm$ 1.18	3.24–4.24	14.1
PV TTP (s)	9.18 $\pm$ 1.33	8.62–9.73	7.3
HP TTP (s)	19.88 $\pm$ 3.76	18.29–21.47	4.8
Δ HP-PV (s)	10.71 $\pm$ 4.11	8.97–12.44	12.9
HA RT (s)	2.77 $\pm$ 0.64	2.50–3.04	10.9
HA RR (dB/s)	0.71 $\pm$ 0.18	0.63–0.78	15.0
PV RT (s)	5.70 $\pm$ 0.95	5.29–6.10	8.2
PV RR (dB/s)	0.29 $\pm$ 0.07	0.26–0.32	14.8
HP RT (s)	17.69 $\pm$ 4.02	15.99–19.39	6.1
HP RR (dB/s)	0.12 $\pm$ 0.03	0.11–0.14	7.9

Table 1. Repeatability of contrast-enhanced ultrasonography parameters in healthy dogs.

s, second; dB, decibel; HA, hepatic artery; PV, portal vein; HP, hepatic parenchyma; TTP, time-to-peak; Δ HP-PV, portal vein to hepatic parenchyma transit time; RT, rising-time; RR, rising-rate; SD, standard deviation; CI, confidence interval; CV, coefficient of variation.

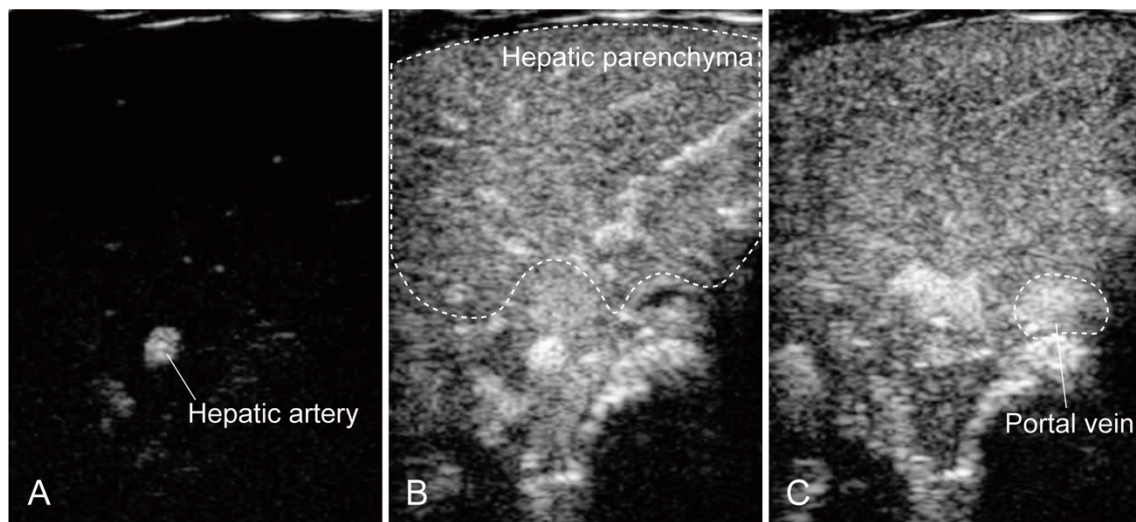


Figure 3. Images of the hepatic artery, portal vein, and hepatic parenchyma at the level of porta hepatis in extrahepatic congenital portosystemic shunt dogs.

A, One second after a bolus injection of Sonazoid. First, the microbubbles reached the hepatic artery. B, Four seconds after a bolus injection of Sonazoid. The microbubbles reached peak enhancement in the hepatic parenchyma before that in the portal vein, which appeared different compared to healthy dogs. C, 10 seconds after a bolus injection of Sonazoid. Finally, the microbubbles reached the peak enhancement in the portal vein.

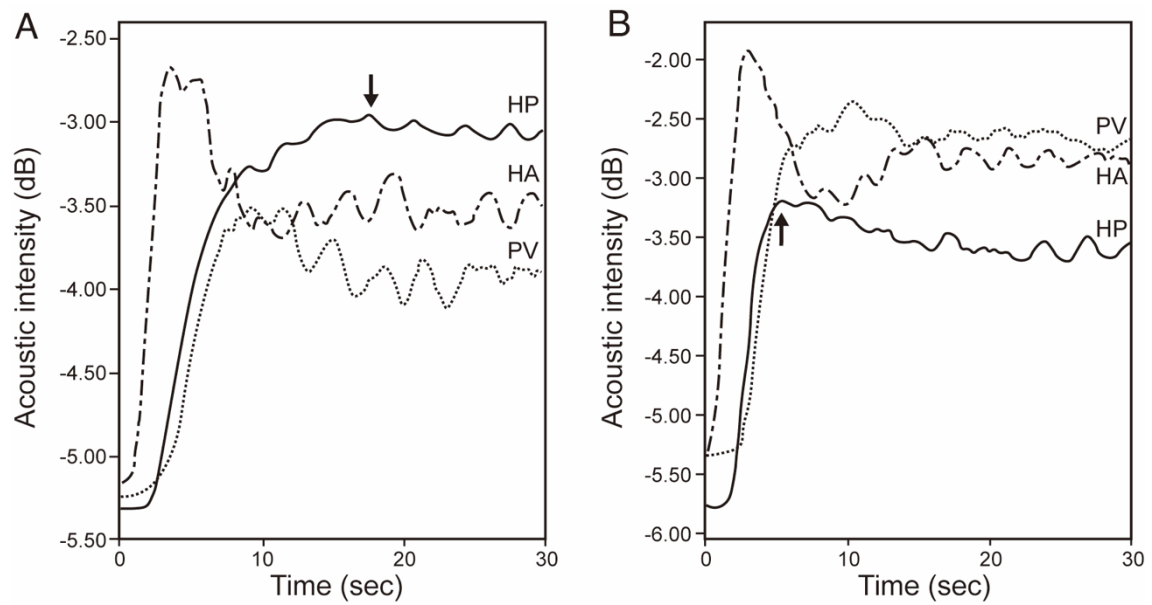


Figure 4. Time-intensity curve (TIC) of the hepatic artery (HA; chain line), portal vein (PV; dotted line), and hepatic parenchyma (HP; solid line) in a healthy (A) and an extrahepatic congenital portosystemic shunt dog (B).

In the healthy dog, TIC showed the gradually rising curve, and the microbubbles reached the peak intensity of hepatic parenchyma (arrow) after that of portal vein. On the other hand, in an extrahepatic congenital portosystemic shunt dog, TIC showed the rapidly rising curve and the microbubbles reached the peak intensity of hepatic parenchyma (arrow) before that of portal vein. The time-to-peak of hepatic parenchyma in an extrahepatic congenital portosystemic shunt dog was markedly earlier than that in a healthy dog.

Parameters	Healthy Dogs (n = 8)	EH-CPSS (n = 9)	P Value
HA TTP (s) ^a	3.9 (3.1–4.6)	2.4 (1.3–3.3)	0.0023
PV TTP (s) ^a	9.3 (7.7–10.9)	8.6 (5.0–10.3)	0.1346
HP TTP (s) ^a	19.9 (13.5–24.4)	8.5 (5.5–16.5)	0.0018
Δ HP-PV (s) ^a	10.6 (3.8–15.1)	0.1 (–4.8–7.3)	0.0018

Table 2. Time-to-peak differences among the hepatic artery, portal vein, and hepatic parenchyma in healthy and extrahepatic congenital portosystemic shunt dogs.

Letters in superscript within a row are expressed as median (range). s, second; HA, hepatic artery; PV, portal vein; HP, hepatic parenchyma; TTP, time-to-peak; Δ HP-PV, portal vein to hepatic parenchyma transit time; EH-CPSS, extrahepatic-congenital portosystemic shunt.

Parameters	Healthy Dogs (n = 8)	EH-CPSS (n = 9)	P Value
HA RT (s) ^a	2.6 (2.4–3.5)	2.2 (1.2–2.7)	0.0153
HA RR (dB/s) ^a	0.84 (0.47–0.93)	1.20 (0.74–1.70)	0.0033
PV RT (s) ^a	5.9 (4.0–6.8)	6.0 (4.7–8.6)	0.4998
PV RR (dB/s) ^a	0.33 (0.24–0.43)	0.38 (0.30–0.66)	0.0825
HP RT (s) ^a	16.7 (11.8–22.0)	7.7 (4.1–14.8)	0.0024
HP RR (dB/s) ^a	0.14 (0.08–0.16)	0.33 (0.16–0.62)	0.0007

Table 3. Time-intensity curve characteristics in the hepatic artery, portal vein, and hepatic parenchyma.

Letters in superscript within a row are expressed as median (range). s, second; dB, decibel; HA, hepatic artery; PV, portal vein; HP, hepatic parenchyma; RT, rising-time; RR, rising-rate; EH-CPSS, extrahepatic-congenital portosystemic shunt.

4. DISCUSSION

Portal venous hypoperfusion in dogs with congenital portosystemic shunt causes stereotypical histological responses of the liver, which include a reduction or absence of portal vein profile in portal tracts and an increased number of arteriolar profiles. Particularly, an increased number of arterioles would reflect a compensatory increase in hepatic artery blood flow due to a decrease in the portal vein blood flow.^{27,28} However, detecting these mechanical alterations is difficult because assessing liver perfusion requires real-time and high-resolution diagnostic imaging techniques. Therefore, changes in hepatic microvascular perfusion in dogs with extrahepatic congenital portosystemic shunt have not been demonstrated using diagnostic imaging techniques. A previous pilot study was the first to report changes in hepatic parenchymal perfusion in three dogs with extrahepatic congenital portosystemic shunt using CEUS.²⁹ However, this study did not examine the simultaneous hepatic microvascular perfusion change including hepatic artery, portal vein, and hepatic parenchyma. Therefore, a compensatory increase in hepatic artery blood flow has not yet been demonstrated. In the present study, I assessed the perfusion of hepatic artery and portal vein, as well as that of hepatic parenchyma. Additionally, I also determined whether assessing microvascular perfusion using CEUS can contribute as an additional diagnostic test to diagnose dogs with extrahepatic congenital portosystemic shunt.

In the present study, I initially evaluated the repeatability of CEUS parameters because one of the disadvantages of CEUS is its relatively high degree of variability, which may cause diagnostic uncertainty in humans. A previous study has reported that patient variables such as body temperature, heart rate, cardiac output, blood pressure, and respiratory rate, and physiologic factors such as phagocytosis ability will affect the number of circulating microbubbles and their characteristics.³⁰ Meanwhile, little is known about the factors affecting CEUS parameters in dogs and cats. To the best of our knowledge, there are only two studies investigating the TTP in hepatic artery and portal vein in

anesthetized healthy dogs. One of these studies described that TTP in the aorta (instead of hepatic artery) and portal vein were 13.5 ± 3.37 s and 27.5 ± 4.09 s, respectively, using Levovist® (Schering AG, Berlin, Germany) as a contrast agent.³¹ The other study showed that TTP in hepatic artery and portal vein were respectively 10 s and 30 s, using the same contrast agent as that used in the current study.³² In my study, TTP of hepatic artery and portal vein in healthy dogs is 3.74 ± 1.18 s and 9.18 ± 1.33 s, respectively, which are much earlier than that of the previous two reports. For hepatic parenchyma, two reports described that TTP of hepatic parenchyma was 45 ± 26.63 s, which is also slower than my data (19.88 ± 3.76 s).^{32,33} These different results in TTP may be caused by many factors, such as the injected speed of the contrast agent or saline, type of contrast agent, setting of ultrasound machine, difference of dog's body size, or use of anesthesia. Therefore, to reduce this variability, I do not use anesthesia, and the contrast agent is injected by the same person throughout the study in addition to standardizing image acquisition and data processing. However, I cannot control the variability caused by the physiological or physical factors of the dog and the contrast agent. For example, differences in a dog's body size may influence the TTP in terms of distance to the liver from the cephalic vein where the contrast agent is administered. Hence, I propose RT, RR, and Δ HP-PV to reduce the influence of these other factors. The RT and RR directly reflect perfusion within the liver, because they show from first arrival of the echogenic bubbles of contrast agent to peak enhancement. In the Δ HP-PV, the portal vein serves as an in vivo reference. Additionally, I initially investigate the repeatability for the hepatic microvascular perfusion with the same dog on different days. A CV < 25% has been considered clinically acceptable based on previous study of CEUS in human and animals.^{34,35} Consequently, parameters of hepatic parenchyma show highly repeatability (CVs; $\leq 7.9\%$). On the other hand, parameters of the hepatic artery and portal vein are moderately repeatable, because parameters within the vessel may be more directly affected by a dog's condition. However, the interday

CVs for all the parameters are $\leq 15\%$, and all the parameters are feasible for assessing hepatic microvascular perfusion in dogs.

CEUS with a single contrast agent (Sonazoid®) was conducted to characterize the simultaneous hepatic microvascular perfusion in the hepatic artery, portal vein, and hepatic parenchyma in healthy dogs. The TIC of hepatic parenchyma in healthy dogs shows a gradually rising curve characterized by a long TTP and RT and low RR findings. First, contrast injected via the cephalic vein reaches the liver through the hepatic artery blood flow. The hepatic artery forms the peribiliary capillary plexus network around bile ducts to nourish the biliary system, and finally, some of the capillaries enter directly into sinusoids or occasionally the terminal portal venules. Furthermore, portal vein supplied from the intestines, pancreas, spleen, and gallbladder enters the sinusoids. The mixture of both hepatic artery and portal vein blood penetrates through the sinusoids and collects in a central vein that drains into the hepatic veins. The gradually rising curve of hepatic parenchyma likely is reflecting the extensive vascular bed characterized by slow, continuous flow.

The TIC of hepatic parenchyma in extrahepatic congenital portosystemic shunt dogs showed a difference from that of healthy dogs. The TIC of hepatic parenchyma in extrahepatic congenital portosystemic shunt dogs shows rapidly rising curve characterized by a short TTP and RT and high RR. Furthermore, parameters of the hepatic artery in extrahepatic congenital portosystemic shunt dogs also showed a difference from that of healthy dogs. $\Delta\text{HP-PV}$ in extrahepatic congenital portosystemic shunt dogs is fairly early compared with healthy dogs, which supports a previous pilot study in which the perfusion of hepatic parenchyma was evaluated in three dogs with extrahepatic congenital portosystemic shunt using CEUS.²⁹ In a normal liver, it is known that the portal vein provides approximately 70%–80 % of the blood supply, carrying nutrients and various other substances to the liver.⁷ However, in extrahepatic congenital portosystemic shunt dogs, portal vein blood flow is reduced, leading to an increase in arterial blood flow to the liver, in other words ‘arterialization’.^{36,37} The rapid

enhancement of hepatic parenchyma and parameters of hepatic artery reflect a compensatory increase in the hepatic arterial blood flow (arterialization) in the livers of dogs with extrahepatic congenital portosystemic shunt. Interestingly, PV/Ao ratio in extrahepatic congenital portosystemic shunt is significantly correlated with the RT of hepatic parenchyma and $\Delta\text{HP-PV}$. From these findings, PV/Ao ratio not only indicates the decreased portal vein blood flow, but also may indirectly reflect the degree of arterialization.

One of the most difficult ultrasonographic challenges in veterinary medicine is the diagnosis of extrahepatic congenital portosystemic shunt. Sensitivity and specificity of conventional ultrasonography for the diagnosis of extrahepatic congenital portosystemic shunt has been reported to be 74% to 98% and 67% to 100%, respectively.³⁸⁻⁴⁰ However, due to the specific limiting factors such as gastrointestinal gas, patient conformation, and operator-dependent nature, it is occasionally difficult to detect abnormal vessels by ultrasonography. Therefore, several reports have described the usefulness of additional diagnostic tests, such as PV/Ao ratio or portal vein flow velocity for the diagnosis of extrahepatic congenital portosystemic shunt.⁴⁰⁻⁴³ In this study, I evaluate whether the assessment of hepatic microvascular perfusion using CEUS can reliably distinguish dogs with extrahepatic congenital portosystemic shunt from healthy dogs. As a screening test, it is important that the test has high sensitivity. Therefore, I selected a cut-off point along each ROC curve that maximizes sensitivity and maintains maximal or near maximal accuracy to avoid excessively reducing specificity in the present study. However, a compensatory increase in the hepatic arterial blood flow (arterialization) may be caused by other liver disease. Several reports have described that human patients with cirrhosis or portal hypertension have reduced portal vein blood flow to the liver and increased hepatic artery blood flow.⁴⁴⁻⁴⁷ In dogs, our previous study described that dogs with portal hypertension experimentally induced by intraportal injection of microspheres showed the hepatic hemodynamic change by using CEUS with a single contrast agent (Sonazoid®).⁴⁸ However, portal

hypertension may be distinguishable with a thorough sonographic examination. Cirrhosis, which is the end stage of hepatic fibrosis, is caused by chronic liver disease such as chronic hepatitis or cholangiohepatitis. Cirrhosis can also be easily distinguished, because the liver may be small and irregular in shape with regenerative nodules. In addition, a dog with cirrhosis will have an abnormal liver function and a large amount of free peritoneal fluid.¹⁵ For this reason, the assessment of hepatic microvascular perfusion using CEUS in addition to conventional B-mode ultrasound imaging may be useful in distinguishing dogs with extrahepatic congenital portosystemic shunt from healthy dogs.

This study has several limitations. First, this study does not include dogs with various parenchymal liver diseases leading to changes in hepatic microvascular perfusion. Further study is needed to establish CEUS characteristics in dogs with various parenchymal liver diseases. Second, a histopathological examination of the liver in healthy dogs was not performed. Third, this study enrolled extrahepatic congenital portosystemic shunt dogs that were mainly small- to medium-sized dogs. In Japan, small- to medium-sized dogs, including Miniature Schnauzer, Toy Poodle, or Yorkshire terrier were reported to have a high risk for extrahepatic congenital portosystemic shunt,⁴² which is similar to reports in North America and the Netherlands.^{26,49} Although the difference in the dog's body size may influence CEUS parameters (especially TTP) in terms of distance to the liver from cephalic vein, this study showed that the TTP of portal vein was similar for healthy dogs and extrahepatic congenital portosystemic shunt dogs. Furthermore, blood pressure may affect CEUS parameter and was not measured in both the groups of this study. Therefore, the RT, RR, and Δ HP-PV were assessed to reduce the influence of these limitations. Thus, it is likely the changes in hepatic microvascular perfusion in this study are caused by the existence of shunt vessels. Last, this study is not undertaken by a blinded operator. Further blinded study is needed to confirm the accuracy of this study.

5. SUMMARY

In this chapter, hepatic microvascular perfusion was characterized by simultaneously evaluating the perfusion of the hepatic artery, portal vein, and hepatic parenchyma in dogs with extrahepatic congenital portosystemic shunt using CEUS. The enhancement of hepatic artery and hepatic parenchyma in dogs with extrahepatic congenital portosystemic shunt was significantly more rapid than that in healthy dogs. These results reflect a compensatory increase in the hepatic artery blood flow (arterialization) caused by a decrease in portal vein blood flow. The results of the present study will help enhance the understanding of pathophysiology of dogs with extrahepatic congenital portosystemic shunt. Moreover, the RT and RR of hepatic parenchyma and $\Delta\text{HP-PV}$ for dog in this study showed the highest discretionary accuracy. The assessment of hepatic microvascular perfusion using CEUS may help distinguish dogs with extrahepatic congenital portosystemic shunt from healthy dogs.

CHAPTER 2

EVALUATION OF LIVER AND SPLEEN ELASTIC MODULUS OF HEALTHY DOGS BY USE OF TWO-DIMENSIONAL SHEAR WAVE ELASTOGRAPHY

1. INTRODUCTION

Chronic liver damage results in an increase in the extracellular matrix produced by fibroblast-like cells, and the progression of liver fibrosis leads to stiffer liver parenchyma.⁵⁰ Additionally, portal hypertension, which is caused by fibrotic hepatopathies, often results in splenomegaly and a stiffer spleen in humans.⁵¹ Severity of liver fibrosis and portal hypertension may be evaluated by the quantification of liver and spleen elastic modulus by use of 2D-SWE in human patients with chronic liver disease.⁵²⁻⁵⁵ In contrast to the literature for humans, no reports have evaluated the feasibility of using 2D-SWE for the evaluation of severity of liver fibrosis in dogs with chronic liver disease.

Assessments of repeatability (intraday variability) and reproducibility (interday variability) of 2D-SWE in the evaluation of liver and spleen elastic modulus are important prerequisites before 2D-SWE can be used in clinical settings. Therefore, the objective of chapter 2 was to evaluate the applicability, repeatability, and reproducibility of 2D-SWE of the liver and spleen in healthy dogs. Furthermore, results of the study could provide reference values for tissue elastic modulus that might be used in the evaluation of dogs with liver and splenic diseases.

2. MATERIAL AND METHODS

2.1 Animals

Eight healthy beagles (5 sexually intact females and 3 sexually intact males) were included in the study. Dogs were between 1 and 4 years of age, and body weight was between 9.7 and 15 kg. All dogs were confirmed to be healthy on the basis of results of a physical examination, CBC, serum biochemical analysis, echocardiography, and abdominal ultrasonography. All animal experimental procedures were conducted in accordance with standard protocols of the institutional animal experimental committee reviewed by the Association for Assessment and Accreditation of Laboratory Animal Care International. All animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Veterinary Medicine at Hokkaido University (Accession No. 18-0075).

2.2 Measurement of 2D-SWE

Food was withheld from dogs for 12 hours, and conventional B-mode ultrasonography and 2D-SWE then were performed with an ultrasound scanner (Canon Medical Systems) as described in chapter 1. The author performed examinations to ensure consistent imaging conditions throughout the study. Conventional B-mode ultrasonography with a 7.0-MHz convex transducer (PVT-712 BT, Canon Medical Systems, Tochigi, Japan) was performed before 2D-SWE; it was used for general imaging of the liver and spleen. The ultrasonographer performed 2D-SWE with a 3.5-MHz convex transducer (PVT-375 BT, Canon Medical Systems, Tochigi, Japan) 3 times in 1 day (4-hour intervals) and on 3 separate days (1-week interval). Dogs were not sedated for ultrasonography or 2D-SWE.

In accordance with recommended guidelines for the clinical use of elastography in humans¹¹, all dogs were initially positioned in left lateral recumbency for imaging of the right lobe of the liver. The probe was placed parallel to and within the intercostal space with a sufficient amount of gel to

minimize rib shadowing. The probe was positioned to acquire images of the parenchyma of the right lobe of the liver during visual assessment in 2D B-mode ultrasonography; measurements were obtained at depths of up to 45 mm. All dogs were positioned in dorsal recumbency for acquisition of spleen images. The probe was maintained perpendicular to the curve of the body surface, and measurements were obtained in the parenchyma of the main portion of the spleen at depths of up to 35 mm. The 2D-SWE of the liver and spleen was performed during the end-expiratory phase or respiration to minimize effects of respiratory motion.

The display mode could be changed among 3 options after data acquisition: speed mode, elasticity mode, and propagation mode (Figure 5). The speed mode displayed SWV with a map (range, 0.5 to 6.5 m/s). In contrast, the elasticity mode displayed the Young modulus with another map (range, 0 to 120 kPa). Both maps were obtained so that I could display distributions of shear wave propagation speed in semitransparent 2D color images, which was overlaid on the B-mode image. SWV and the Young modulus were displayed as a gradation of colors on the 2 maps, with increasing elastic modulus in an ascending order of blue, green, yellow, and red. Regions that were not color coded on elasticity images indicated absence of a shear wave.

The propagation mode could be used to provide guidance to evaluate whether ROI placement was accurate in the speed or elasticity mode. A tissue ROI generally needs to be placed in an area with parallel contour lines. The ROI was set at 8 to 10 mm in diameter and excluded regions that were not color coded. The ROI was positioned in the parenchyma of the right lobe approximately 10 mm deep to the liver capsule and in the parenchyma of the spleen approximately 5 mm deep to the spleen capsule (Figure 6). At least 10 valid measurements were obtained for each dog. Mean values of SWV and the Young modulus were considered representative of elasticity values in the liver and spleen.

To compare liver elastic modulus between the right and left lobes of the liver, the left lobe

of the liver of each dog was evaluated by use of 2D-SWE. All dogs were positioned in right lateral recumbency for imaging of the left lobe of the liver, and liver elastic modulus was measured by use of the same protocol used for the right lobe of the liver. Assessment with 2D-SWE was performed 3 times in 1 day (4-hour intervals).

2.3 Statistical analysis

All data of continuous variables were expressed as mean $\pm$ SD. Statistical analyses were performed with commercially available software (as described in chapter 1). A fixed-effect linear model was used to analyze intraday and interday variabilities as follows:

$$Y_{ijk} = \mu + \text{dog}_i + \text{day}_j + (\text{dog X day})_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the first value measured for dog i on day j , μ is the overall mean, dog_i is the differential effect of dog i , day_j is the differential effect of day j , $(\text{dog X day})_{ij}$ is the interaction between dog i and day j , and ε_{ijk} is the model error. The SD of intraday variability was estimated as the residual SD of the model, and the SD of interday variability was estimated as the SD of the differential effect of day. The CV was obtained by dividing each SD by the mean. A small CV indicates a highly reliable measurement, and a CV $< 30\%$ was considered clinically acceptable on the basis of a study⁵⁶ on SWE in humans. The 95% CI was calculated by multiplying SD by 2.069.⁵⁷ The CIs provided an estimated range of values that were likely to include an unknown population parameter from the data obtained, and they represented a probability of < 0.05 that a true change for an individual dog would be detected. Comparison between the right lobe of the liver and spleen elastic modulus was conducted by use of a linear mixed model, with the measurement number (6 measurements) for each dog, organ (right lobe of the liver and spleen), and interaction term as categorical fixed effects and dog as a fixed effect. An F test was performed to assess effects of measurement number and organ. A t test was performed to assess significant differences between organs. Bland-Altman analysis with modifications for repeated

measures was performed to assess the agreement of liver elastic modulus between the right and left lobes of the liver.⁵⁸ Mean of the difference (bias) and the 95% CI for bias were calculated. The 95% CI for bias was compared with 0 and was considered significant when it did not contain 0. The 95% limits of agreement (mean of the difference $\pm$ [1.96 X SD]) were also calculated. For all statistical comparisons, values of $P < 0.05$ were considered significant.

3. RESULTS

3.1 Assessment of repeatability and reproducibility

The 2D-SWE was successfully performed on the liver and spleen of all 8 dogs. All variables were summarized (Table 4). There was high repeatability and reproducibility (ie, low CVs) for 2D-SWE of the right lobe of the liver. Intraday and interday CVs for SWV in the right lobe of the liver were 3.9% and 4.6%, respectively, whereas intraday and interday CVs for the Young modulus in the right lobe of the liver were 8.7% and 10.0%, respectively. There was also high repeatability and reproducibility for 2D-SWE of the spleen. Intraday and interday CVs for SWV in the spleen were 8.0% and 6.1%, respectively, whereas intraday and interday CVs for the Young modulus in the spleen were 20.7% and 12.2%, respectively.

3.2 2D-SWE analysis between the right lobe of the liver and spleen

Mean $\pm$ SD values for 2D-SWE of the right lobe of the liver were 1.51 ± 0.08 m/s and 6.93 ± 0.79 kPa. Mean values for 2D-SWE of the spleen were 2.18 ± 0.27 m/s and 14.66 ± 3.79 kPa. Elastic modulus was compared between the right lobe of the liver and spleen by use of a linear mixed model and reported as the least squares mean and 95% CI (Figure 7). Results of 2D-SWE for both SWV and the Young modulus were significantly ($P < 0.001$) higher in the spleen than in the right lobe of the liver.

3.3 Comparison between the right and left lobes of the liver

Elastic modulus was compared between the right and left lobes of the liver. Mean $\pm$ SD values for 2D-SWE of the left lobe of the liver were 1.42 ± 0.07 m/s and 6.02 ± 0.61 kPa. Results of Bland-Altman analysis with modifications for repeated measures were graphically illustrated (Figure 8). Mean bias for 2D-SWE between the right and left lobes of the liver by use of SWV was 0.09 m/s (95%

CI, 0.02 to 0.17 m/s). Mean bias for 2D-SWE between the right and left lobes of the liver by use of the Young modulus was 0.83 kPa (95% CI, 0.15 to 1.51 kPa). Elasticity values for both the SWV and Young modulus were significantly higher in the right lobe of the liver than in the left lobe of the liver.

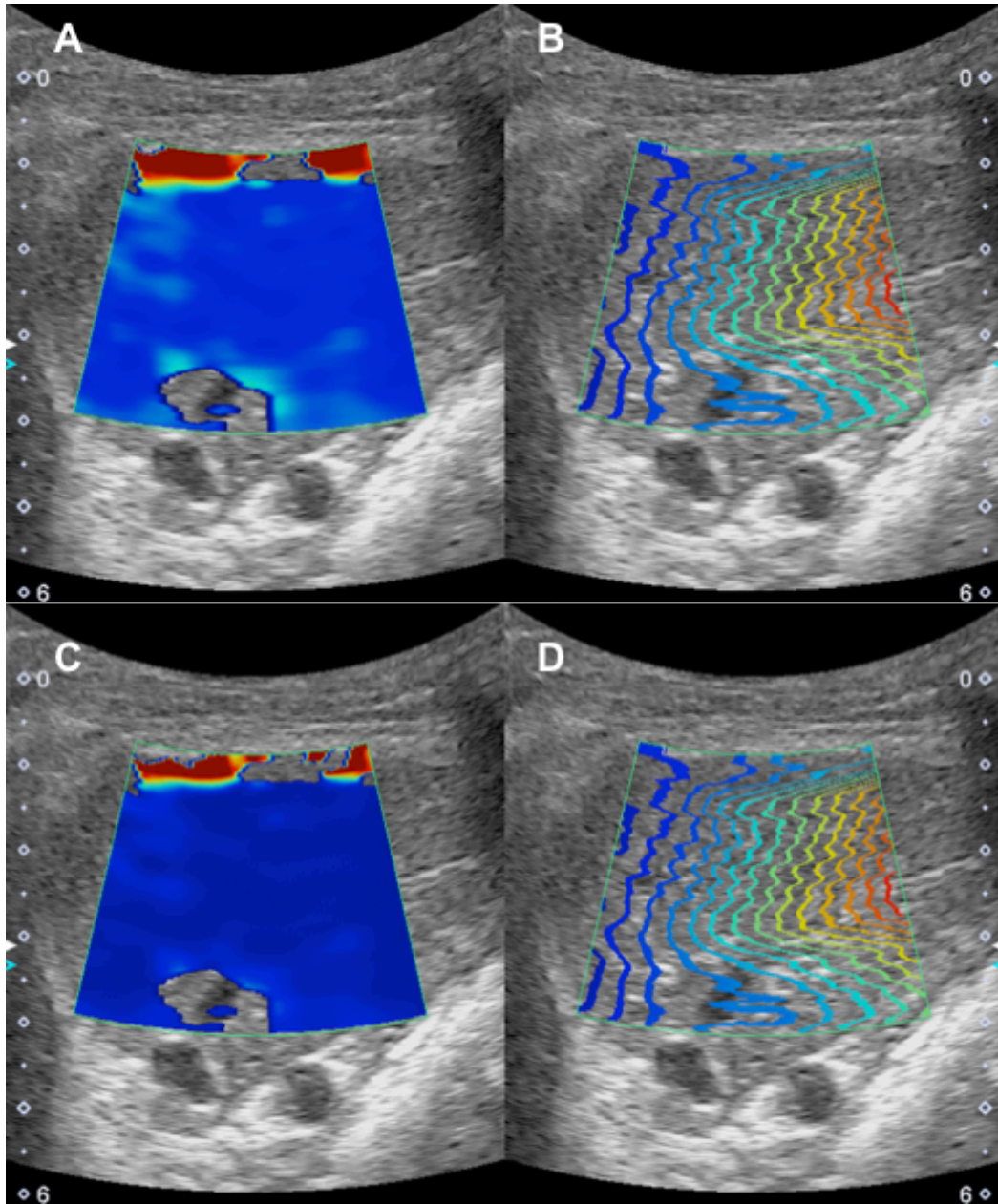


Figure 5. Images of the right lobe of the liver of a dog for the speed mode (A and B) and elasticity mode (C and D) of 2D-SWE.

Images for the speed mode are the shear wave velocity map (A) and map for the propagation mode (B); images for the elasticity mode are the shear wave elasticity map (C) and map for the propagation mode (D). Notice the consistent parallel contour lines for the speed and elasticity modes.

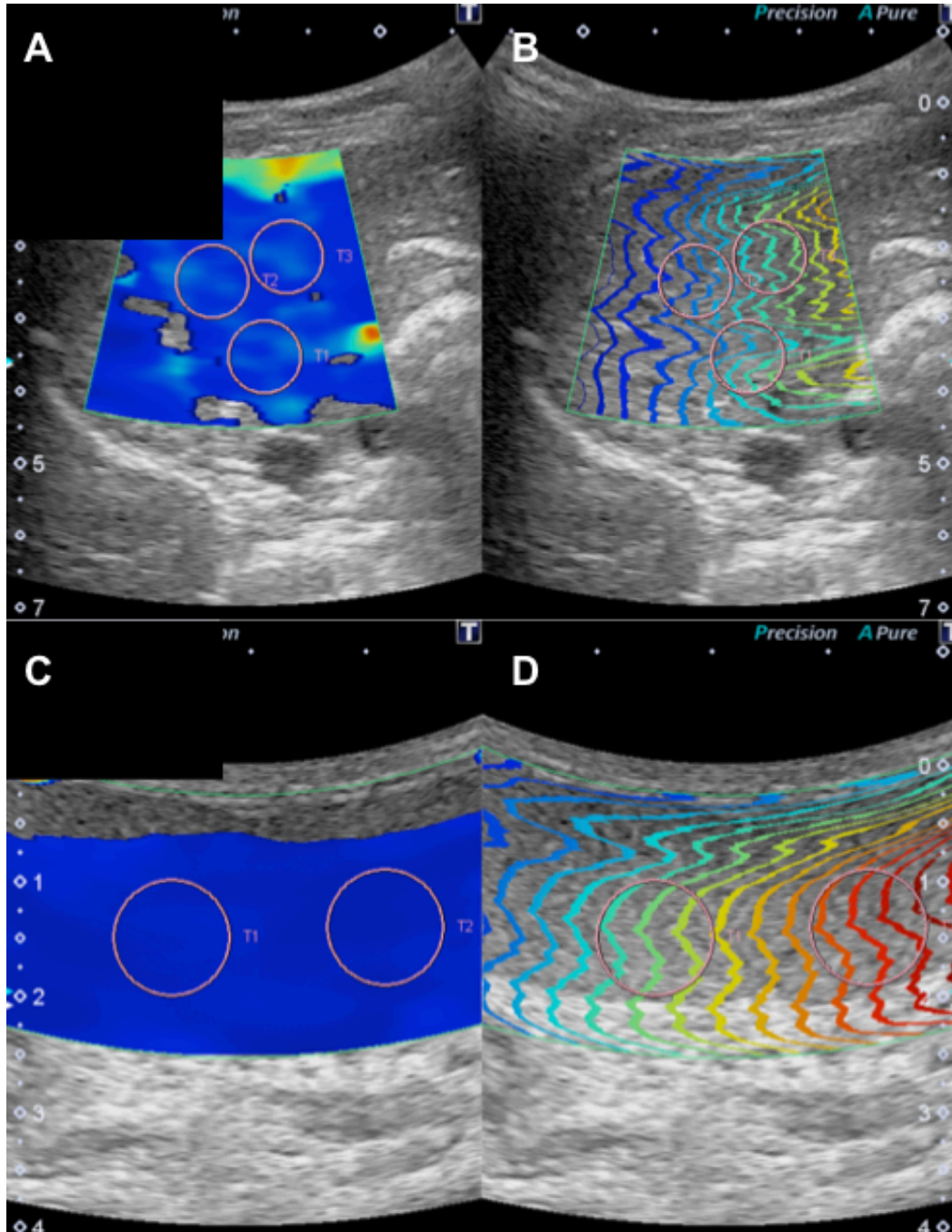


Figure 6. Images of the right lobe of the liver (A and B) and spleen (C and D) of a dog used to measure elastic modulus for the speed mode of 2D-SWE.

Elastic modulus was measured as shear wave velocity (meters per second) in the parenchyma of the right lobe of the liver and as the Young modulus (kilopascals) in the parenchyma of the spleen. Notice the ROIs (T1, T2, and T3; 10 mm) used for the measurements (circles).

variable	Mean ± SD	Intraday			Interday		
		CI*	SD†	CV (%)	CI*	SD‡	CV (%)
Liver							
Shear wave velocity (m/s)	1.51 ± 0.08	0.17	0.08	3.9	0.17	0.08	4.6
Young modulus (kPa)	6.93 ± 0.79	1.61	0.78	8.7	1.66	0.80	10.0
Spleen							
Shear wave velocity (m/s)	2.18 ± 0.27	0.62	0.30	8.0	0.52	0.25	6.1
Young modulus (kPa)	14.66 ± 3.79	8.83	4.27	20.7	7.12	3.44	12.2

Table 4. Results of 2D-SWE for the right lobe of the liver and spleen of 8 healthy dogs.

For all 8 dogs, 2D-SWE was performed 3 times in 1 day (4-hour intervals) to determine intraday CV and on 3 separate days (1-week interval) to determine interday CV. *Calculated as $SD \times 2.069$. †Estimated as the residual SD of the model. ‡Estimated as the SD of the differential effect of day.

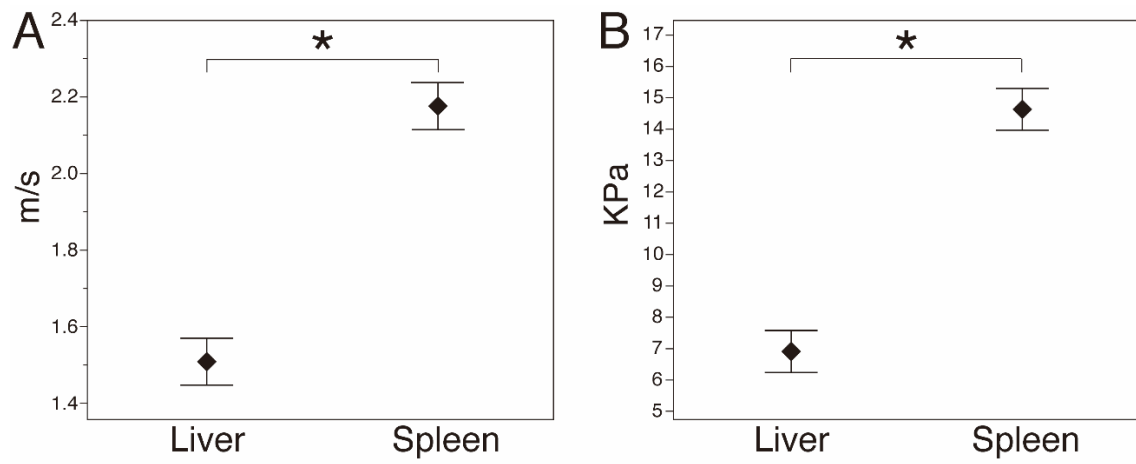


Figure 7. Comparison between the elastic modulus of the right lobe of the liver and spleen of 8 dogs measured as the shear wave velocity (A) and Young modulus (B) of 2-D SWE.

Data represent least squares mean and 95% CI. *Value differs significantly ($P < 0.001$) from the value for the right lobe of the liver.

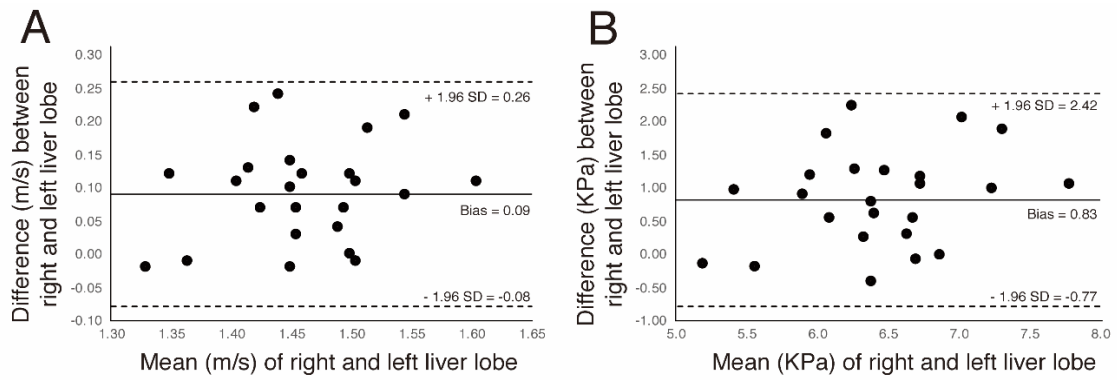


Figure 8. Bland-Altman plots with modifications for repeated measures of differences in elastic modulus between the right and left lobes of the liver of 8 dogs measured as the shear wave velocity (A) and Young modulus (B) of 2-D SWE.

The mean of the difference (bias; solid horizontal line) and 95% CI for the bias ($\text{mean} \pm [1.96 \times \text{SD}]$; dashed horizontal lines) are indicated.

4. DISCUSSION

Histologic examination of liver biopsy specimens is the criterion-referenced method for diagnosis and monitoring of chronic hepatitis and cirrhosis, but it is sometimes difficult to obtain biopsy specimens because of a patient's poor condition and complications associated with the procedure.^{18,19} For humans, investigators of many studies⁵³⁻⁵⁵ have reported that shear wave imaging is an alternative noninvasive diagnostic modality to liver biopsy and is useful in noninvasively assessing the severity of liver fibrosis through evaluation of liver elastic modulus. Although the cause of chronic hepatitis in dogs currently remains unknown, some histopathologic features of chronic hepatitis in dogs are similar to those of humans with chronic hepatitis, such as mixed inflammatory cell infiltration and fibrosis.⁵⁹ The usefulness of shear wave imaging for the diagnosis of liver fibrosis has been investigated in veterinary medicine. Investigators of 1 study⁶⁰ reported that transient elastography is applicable for dogs with experimentally induced hepatic disease. Those authors concluded that the best measurements of liver elastic modulus are obtained in the right lobe of the liver with a dog positioned in left lateral recumbency.⁶⁰ However, transient elastography, which is a 1-D modality, cannot display an anatomic B-mode image, and its performance is inferior to techniques that involve the use of ARFI. Investigators reported that transient elastography is less accurate than 2D-SWE for the assessment of severe fibrosis in humans with chronic hepatitis C.⁶¹ In another study that involved the use of 2D-SWE, liver elastic modulus obtained in the right lobe of the liver was positively correlated with the stage of liver fibrosis in dogs with CCl₄-induced liver fibrosis.⁶² However, 2D-SWE was performed on anesthetized dogs in that study, which did not provide reference values for liver elastic modulus in healthy dogs. In accordance with the recommended guidelines for the clinical use of elastography in humans¹¹ and results for studies of dogs,^{60,62} I initially evaluated the feasibility of 2D-SWE for use on the abdomen of conscious dogs, including assessing repeatability and reproducibility of measurements,

and also obtained reference values for tissue elastic modulus for both the SWV and Young modulus of the right lobe of the liver and spleen in healthy dogs.

Mean SWV for the right lobe of the liver of the healthy dogs of the present study was 1.51 m/s, which is similar to findings obtained by use of 2D-SWE for the right lobe of the liver of healthy humans (1.4 m/s).⁵⁵ The liver is a highly vascular organ with large-diameter capillaries lined by endothelial cells between rows of plates or cords of hepatocytes. The sinusoids also contain Kupffer cells of the reticuloendothelial system. This arrangement imparts relative softness to the liver.

Results obtained in the study reported here indicated that SWV of 2D-SWE was highly repeatable and reproducible in conscious dogs; the CV for all variables was < 10%. I speculated that the clinically acceptable high repeatability and reproducibility of SWV of 2D-SWE in the present study relied on the proper propagation mode, which is a component of the novel technology for 2D-SWE. The proper propagation mode was displayed as arrival time contours with the shape of contour lines. This mode allowed operators to preliminarily confirm whether shear waves propagated as expected and verify the reliability of the data obtained. When the contour lines were nearly straight and consistently parallel to each other, reliability of the data was high. In contrast, when the contour lines were irregularly distorted and chaotic, the reliability of data was low. When the latter was encountered, elastography had to be repeated to yield reliable results. Because shear wave propagation speed becomes faster as tissue elastic modulus increases, the distance between contour lines is wider (ie, blue to green) in regions with greater tissue elastic modulus and narrower (ie, blue to red) in regions with lower tissue elastic modulus. The reliability of data may be verified by examining the propagation map. In chronic liver disease, the shear wave propagation speed does not change, and the intervals between the contour lines are constant. Thus, use of this novel technology may allow objective assessment of the reliability of data simply by the examination of contour lines and may enable investigators to select suitable areas for measurements of shear wave propagation speed.

On the other hand, intraday and interday CVs were higher for the Young modulus than for the SWV of 2D-SWE and indicated moderate repeatability and reproducibility. The elastic modulus of tissue may be estimated and expressed in kilopascals by a physical quantity called the modulus of elasticity (ie, the Young modulus), which is defined as the ratio of the applied stress to the introduced strain from the Hooke law.¹² However, accurate data on the modulus of elasticity have not yet been obtained because it cannot directly measure applied stress. On the other hand, shear wave propagation speed may be calculated as the displacement of localized tissue under short-duration ARFI; therefore, SWE may be used to measure the elasticity of tissue by use of the following formula: $E = 3\rho c_s^2$, where E is tissue elastic modulus measured in kilopascals, ρ is density of the tissue, and c_s is SWV measured in meters per second. By use of this principle, SWV of 2D-SWE is proportionally related to E , and it also offers quantitative elastic information. The value for E may be calculated by use of the aforementioned formula when the density of tissue is the same as that of water (ie, 1 g/mL); however, soft tissues are inherently nonlinear, viscoelastic, and heterogeneous.¹¹ In addition, because E is calculated by use of the value for SWV, I speculated that all CVs were higher for the Young modulus of 2D-SWE than for the SWV of 2D-SWE. Authors of a recent report¹⁰ advocate the reporting of findings for humans as the SWV as part of a standardized approach to enable comparison among modalities and machines. Results of the present study also supported the use of SWV of 2D-SWE from the aspect of repeatability and reproducibility.

Elastic modulus of the spleen also was evaluated in the present study. The spleen is a good target for the assessment of elastic modulus by ultrasonographic elastography. There is a change in spleen elastic modulus with portal hypertension because human patients commonly have splenomegaly secondary to portal hypertension.^{52,63} Spleen elastic modulus measured by use of transient elastography is increased in patients with hepatitis C virus–induced cirrhosis and portal hypertension, and transient elastography values may be used to predict the onset or severity of portal

hypertension.⁶⁴ On the other hand, splenomegaly is a rare finding in dogs with portal hypertension.⁶⁵ However, splenic congestion may occur secondary to portal hypertension and lead to an increase in spleen elastic modulus. Thus, similar to the situation for humans, evaluations of spleen elastic modulus may predict the onset or severity of portal hypertension in dogs with chronic hepatitis and cirrhosis. The value of E for the spleen of healthy dogs in the present study was similar to that in the spleen of healthy humans.⁶⁶ Anatomically, the spleen comprises the parenchyma, including red and white pulp, and also fibroelastic supporting tissue that forms the capsule, trabeculae, and a fine reticulum. This arrangement causes the spleen to be a relatively stiff organ. Elasticity values for both the SWV and Young modulus were significantly higher in the spleen than in the right lobe of the liver of the dogs of the present study.

In the present study, elastic modulus in the left lobe of the liver was measured to enable us to evaluate the feasibility of this technique for assessment of the left lobe of the liver and to compare liver elastic modulus between the right and left lobes of the liver. Values for both the SWV and Young modulus were significantly higher in the right lobe of the liver than in the left lobe of the liver. Studies of humans have revealed differences in liver elastic modulus between sides, and those investigators suggested that imaging of the left lobe in humans is often influenced by the movement of body organs, such as the heart, lungs, diaphragm, and stomach.^{20,67} Furthermore, findings of that study⁶⁷ indicate that ARFI elastography of the right lobe of the liver is more accurate for diagnosing liver fibrosis in humans than is ARFI elastography of the left lobe of the liver. However, that type of study has not been performed in veterinary medicine. Although 2D-SWE can be used to assess liver elastic modulus in the right and left lobes of the liver of dogs, differences in tissue elastic modulus between the right and left lobes of the liver should be expected, and the same lobe of the liver should be evaluated throughout follow-up assessments in dogs.

The present study had several limitations. Cytologic or histologic examinations of the liver

of the dogs were not performed. Thus, a possibility of the existence of liver disease cannot be completely excluded. However, the laboratory beagles used in the present study had no clinical signs of illness or abnormalities at the time physical examination, clinicopathologic examinations (including measurement of fasting and postprandial total bile acid concentrations), and abdominal ultrasonography were performed for the study. Additionally, occult disease was ruled out on the basis of follow-up examinations conducted for 6 months. Furthermore, all dogs used in the study were beagles. Additional studies with smaller and larger breeds are needed to characterize liver and spleen elastic modulus in dogs of various body sizes.

5. SUMMARY

In this chapter, the feasibility, repeatability, and reproducibility of variables obtained by 2D-SWE in healthy dogs were established. Results of this chapter indicated that intraday and interday CVs for elasticity values obtained for healthy conscious dogs by use of a novel 2D-SWE technique were clinically acceptable. Elasticity values obtained for healthy dogs in the present study may serve as reference values for the assessment of liver fibrosis and for predicting the onset or severity of portal hypertension in dogs with hepatic diseases.

CHAPTER 3

USEFULNESS OF NONINVASIVE SHEAR WAVE ELASTOGRAPHY FOR THE ASSESSMENT OF HEPATIC FIBROSIS IN DOGS WITH HEPATIC DISEASE

1. INTRODUCTION

Therapeutic management and prognosis of chronic diffuse liver disease in humans generally depend on the extent and progression of hepatic fibrosis. Although the etiology of chronic liver disease in dogs is different from that in humans, both species have the similar histological appearance and progression of hepatic fibrosis.^{15, 59} Therefore, the evaluation of hepatic fibrosis is highly likely of importance to small animal veterinarians.

Instead of invasive liver biopsy, non-invasive and easily accessible methods of predicting stages of hepatic fibrosis have been developed in human medicine. Hyaluronic acid (HA), which is an essential component of the extracellular matrix, is widely used as a direct marker for the assessment of hepatic fibrosis in human medicine.⁶⁸⁻⁷⁰ Similarly, several studies in veterinary medicine have reported that the blood HA concentration is increased in dogs with hepatic disease.^{71,72} However, another report did not find a positive correlation between serum HA concentration and the stage of hepatic fibrosis in dogs with hepatic disease.⁷³

2D-SWE is widely used for the evaluation of stage of hepatic fibrosis in human patients, because many studies in human patients with hepatic fibrosis have indicated that SWV is correlated with the stage of hepatic fibrosis.²⁰⁻²⁴ In chapter 2, I described that 2D-SWE is a feasible technique for assessing SWV in healthy dogs. However, to the best of our knowledge, no reports have evaluated the use of 2D-SWE for the diagnosis of hepatic fibrosis in dogs with spontaneous hepatic disease.

The aim of this chapter was to evaluate the usefulness of 2D-SWE for the detection of clinically relevant hepatic fibrosis ($\geq$ F2) in dogs with hepatic disease. I hypothesized that SWV measured by 2D-SWE would be different between dogs without clinically relevant hepatic fibrosis (F0-1) and dogs with clinically relevant hepatic fibrosis ($\geq$ F2).

2. MATERIALS AND METHODS

2.1 Animals

This study was a prospective, cross-sectional observational study. From June 2017 to January 2019, 32 client-owned dogs with histopathologically diagnosed acute or chronic hepatobiliary disease examined at the Hokkaido University Veterinary Teaching Hospital were included. Signalment which consisted of age, breed, sex, and body weight were recorded at the time of recruitment. Laboratory findings, including CBC and serum biochemistry, were extracted from the medical records of all the included dogs. Dogs with congenital portosystemic shunt, rupture of the gallbladder, obstructive jaundice, or hepatic tumors were excluded based on abdominal ultrasound and computed tomography findings. Informed owner's consent was obtained in all cases.

Eight healthy beagles were included in the study: 3 were intact males and 5 were intact females. Their age range was 1–4 years and body weight was 9.7–15 kg. All dogs were confirmed healthy based on physical examination, CBC, serum biochemistry, echocardiography, and abdominal ultrasonography. All animal experimental procedures were conducted in accordance with the standard operation protocols of the institutional animal experimental committee reviewed by the Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC International). All animal experiments were approved by the Hokkaido University Veterinary Teaching Hospital.

2.2 Measurement of SWV

The ultrasound equipment used in this chapter was the same as described in chapter 2. According to the chapter 2 and the recommended guidelines for the clinical use of elastography in humans,¹¹ all dogs were positioned in left lateral recumbency for imaging of the right liver lobe. The probe was placed parallel to and within the intercostal space, and sufficient gel was applied to minimize rib

shadowing. Liver image acquisition was positioned within the right lobe parenchyma using the intercostal approach under visual control in the two-dimensional B mode. If gas in the duodenum, colon, or lung hindered imaging of right liver lobe, the probe was positioned more dorsally. Dogs with ascites also were included in the study. Subsequently, 2D-SWE of the liver was performed during the normal end-expiratory phase to minimize the effects of respiratory motion. The reliability of data obtained by 2D-SWE was confirmed by use of the proper propagation mode as described in the chapter 2 (Figure 9A, C). Data reliability is high when the contour lines are nearly straight and regularly parallel to each other, but when the contour lines are irregularly distorted and chaotic, data reliability is low. Additionally, the map of the SWV (range, 0.5–6.5 m/s) displayed as the speed mode was displayed by gradual colors, with increasing elastic modulus indicated in an ascending order of blue, green, yellow, and red (Figure 9B, D). Regions that were not color-coded on the elasticity images reflected the absence of a shear wave. The diameter of the ROI was set as 10 mm and placed on the areas with parallel contour lines in the hepatic parenchyma. At least 10 validated measurements were made for each dog. The median values of the SWV were representative of the liver modulus values.

The SWV of all dogs with hepatic disease was measured using 2D-SWE before liver biopsy or euthanasia and necropsy. The measurements of SWV were performed by a single sonographer to ensure consistent imaging conditions throughout the study. The SWV of dogs with hepatic disease was compared to those of 8 clinically healthy beagle dogs; the latter were obtained from the chapter 2 study evaluating the feasibility of 2D-SWE.

2.3 Histopathological examination and scoring of fibrosis or necroinflammation of liver

Liver biopsy samples in all dogs were collected by surgical laparotomy, laparoscopy, or necropsy. Liver biopsy specimens were fixed in 10% formalin and embedded in paraffin, and the paraffin section was stained with hematoxylin and eosin. All histological examinations were performed by an

American College of Veterinary Pathologists board-certified veterinary pathologist according to the criteria developed by the World Small Animal Veterinary Association Liver Standardization Group.⁷⁴ After routine histopathological examination, hepatic fibrosis and necroinflammatory activity were evaluated semiquantitatively according to a histological scoring scheme that was adapted from the Ishak scheme used in human medicine; this procedure was performed by a single pathologist, who was blinded to the SWV variable.^{74,75} In this scoring scheme, hepatic fibrosis is staged as follows: 0: absent, 1: mild, 2: moderate, 3: marked, or 4: very marked. Furthermore, all dogs were divided into the following groups: dogs without clinically relevant hepatic fibrosis (F0–1) and dogs with clinically relevant hepatic fibrosis ($\geq$ F2). Necroinflammatory activity was graded as follows: 0: absent, 1: slight, 2: mild, 3: moderate, 4: marked, or 5: very marked. Additionally, all dogs were divided into those without necroinflammatory activity (A0) and those with necroinflammatory activity ($\geq$ A1).

2.4 Measurement of serum HA concentration

The serum HA concentrations were compared between dogs with and without clinically relevant hepatic fibrosis. Serum samples were obtained from dogs with hepatic disease before surgical laparotomy, laparoscopy, or necropsy and stored at -80 °C until HA measurement. Serum HA concentrations were measured using a commercially available enzyme-linked immunosorbent assay kit (Hyaluronan ELISA Kit, Echelon Biosciences, Utah, USA), which was used for the measurement of serum HA concentration in dogs in a previous study.⁷³ Absorbance was read at 405 nm using a microplate reader (Benchmark Microplate Reader, BIO-RAD, California, USA). All samples were examined in duplicate, and the mean optical density was calculated.

2.5 Statistical analysis

Statistical analyses were performed using a commercially available statistical program (described in

chapter 1). All continuous variables are presented as median and ranges. The Mann-Whitney U-test was used to compare laboratory findings and HA concentrations between dogs with and without clinically relevant hepatic fibrosis. The Kruskal-Wallis test was used to analyze the overall difference among the 3 group dogs, and post-hoc multiple comparisons were determined by the Steel-Dwass test. The difference was considered statistically significant when P values were < 0.05 .

3. RESULTS

3.1 Study population

Thirty-two dogs with hepatic disease initially were enrolled in the study, but 4 dogs were excluded because of poor ultrasound image quality (i.e. unable to obtain proper contour lines in the propagation mode) for the measurement of SWV; imaging of the right liver lobe was interfered with because of shadowing of gas in the duodenum, colon, or lung in 2 dogs and because of uncontrollable panting in 2 dogs. Finally, 28 dogs with hepatobiliary disease were included in the study (Table 5). The following breeds were presented: 4 miniature schnauzers, 3 miniature dachshunds, 3 beagles, 3 toy poodles, 3 Shih Tzus, 2 American cocker spaniels, 2 Chihuahuas, 2 mixed dogs, and 1 each of Cairn terrier, Scottish terrier, pug dog, Border collie, Pomeranian, and Yorkshire terrier. Histopathological diagnosis of 20 of the 28 dogs with hepatic disease was as follows: 11 cases of cholangiohepatitis; 3, vacuolar hepatopathies; 3, chronic hepatitis; and 3, primary hypoplasia of the portal vein. The last 8 of the 28 dogs were diagnosed with minimal non-specific changes, including mildly scattered lipogranulomas or mononuclear infiltrates with hydropic or histologically normal liver. All hepatic samples were considered adequate and the stages of hepatic fibrosis and necroinflammatory activity grades were evaluated (Table 5). Three dogs that had clinically relevant hepatic fibrosis also had mild ascites.

3.2 Laboratory findings and serum hyaluronic acid concentration

The results of laboratory findings and serum HA concentrations in all dogs with hepatic disease are presented in Table 6. The laboratory findings were measured in all dogs, whereas serum HA concentration was measured in 20 of the 22 dogs without clinically relevant hepatic fibrosis and 5 of the 6 dogs with clinically relevant hepatic fibrosis. Only serum albumin and total cholesterol concentrations were significantly lower in dogs with clinically relevant hepatic fibrosis than in those

without clinically relevant hepatic fibrosis ($P = 0.0010$ and $P = 0.023$, respectively). Serum HA concentration was not significantly different between dogs with (median, 192 ng/mL; range, 151–305 ng/mL) and without clinically relevant hepatic fibrosis (median, 162 ng/mL; range, 117–211 ng/mL, $P = 0.11$).

3.3 Comparison of SWV among hepatic fibrosis stages

SWV results were compared among healthy dogs ($n = 8$), dogs without clinically relevant hepatic fibrosis ($n = 22$), and dogs with clinically relevant hepatic fibrosis ($n = 6$). The speed map of SWV of dogs without clinically relevant hepatic fibrosis is displayed in blue (Figure 9B), and that of dogs with clinically relevant hepatic fibrosis is displayed in green in the major parts and blue in minimal parts (Figure 9C). Figure 10 shows the box plots of SWV measured by 2D-SWE in healthy dogs, dogs without clinically relevant hepatic fibrosis, and dogs with clinically relevant hepatic fibrosis. The median SWV in healthy dogs, dogs without clinically relevant hepatic fibrosis, and dogs with clinically relevant hepatic fibrosis was 1.51 (range, 1.44–1.66) m/s, 1.56 (range, 1.37–1.67) m/s, and 2.04 (range, 1.81–2.26) m/s, respectively (Kruskal-Wallis test, $P < .001$). The SWV measured by 2D-SWE in dogs with clinically relevant hepatic fibrosis was significantly higher than those in healthy dogs ($P = 0.0067$) and dogs without clinically relevant hepatic fibrosis ($P < 0.001$). Conversely, SWV in dogs without clinically relevant hepatic fibrosis was similar to those of healthy dogs ($P = 0.95$).

3.4 Comparison of SWV among different amounts of necroinflammatory activity

Figure 11 shows the SWV measured by 2D-SWE for the 3 groups: healthy dogs ($n = 8$), dogs without inflammatory activity ($n = 17$), and dogs with inflammatory activity ($n = 11$). The data obtained for healthy dogs are the same as shown in Figure 10. The median SWV was 1.57 (range, 1.37–2.26) m/s for dogs without inflammatory activity and 1.61 (range, 1.45–2.24) m/s for dogs with inflammatory

activity. No significant difference was found in the values among healthy dogs, dogs without inflammatory activity, and dogs with inflammatory activity (Kruskal-Wallis test, $P = 0.11$).

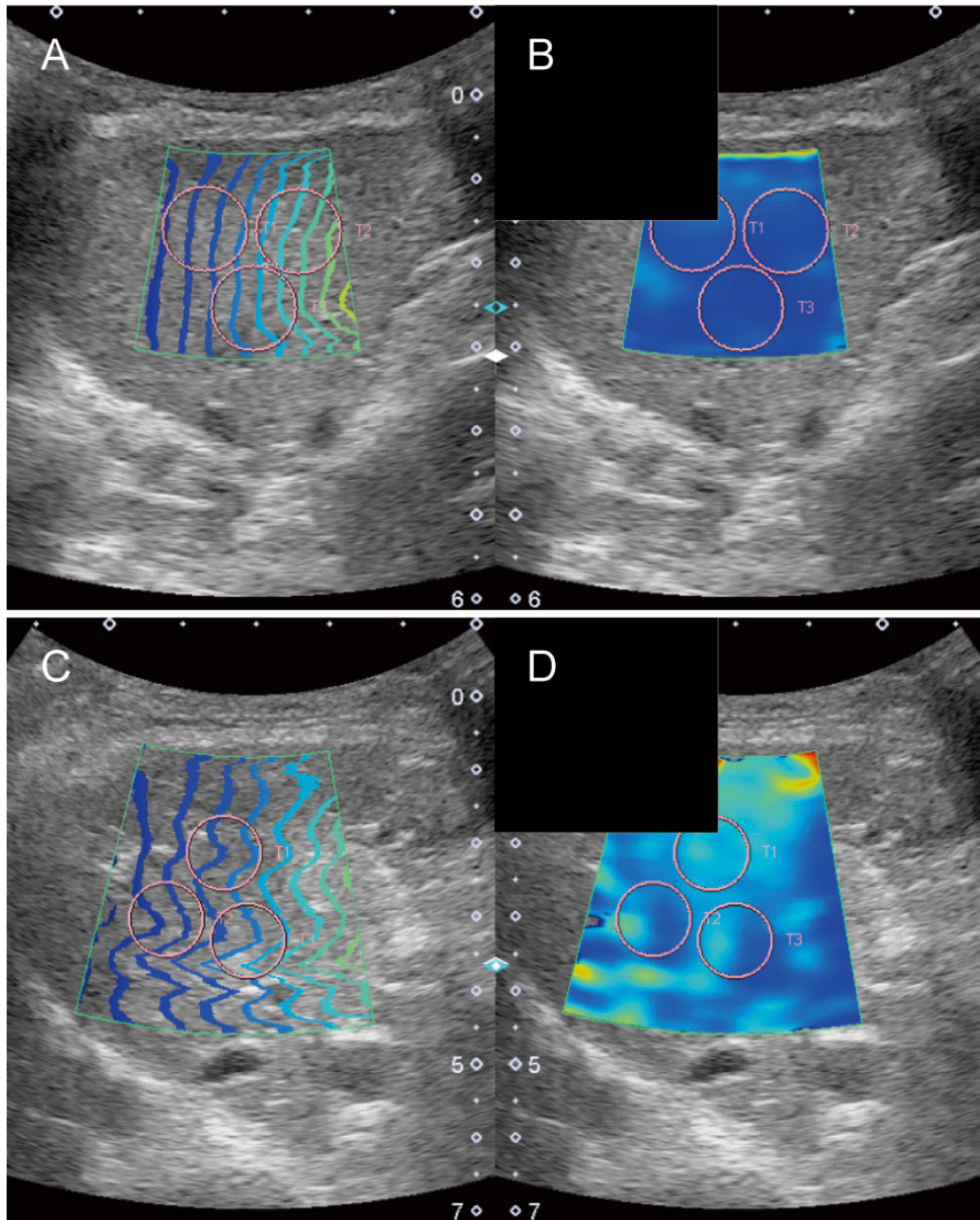


Figure 9. (A) Representative images of the right lobe of the liver via the intercostal approach for the speed mode in a dog without clinically relevant hepatic fibrosis (F0, A0; A and B) and a dog with clinically relevant hepatic fibrosis (F3, A0; C and D).

Images for the speed mode are the map for the propagation mode (A, C) and the shear wave velocity map (B, D). Notice the consistent parallel contour lines for the speed modes, and the region of interest (T1, T2, and T3; 10 mm) used for the measurements (circles).

Characteristics	Dogs with hepatic disease (n = 28)
Age (years)	11 (1–15)
Sex (number of dogs)	3M, 4MC, 4F, 17FS
Body weight (kg)	6.1 (2.2–22.2)
Fibrosis staging	
F0 (absent)	17
F1 (mild)	5
F2 (moderate)	2
F3 (marked)	1
F4 (very marked)	3
Necroinflammatory activity grading	
A0 (absent)	17
A1 (slight)	6
A2 (mild)	4
A3 (moderate)	1
A4 (marked)	0
A5 (very marked)	0

Table 5. Age and body weight are reported as medians with the ranges in parentheses.

Numbers in the fibrosis staging and necroinflammatory activity grading indicate number of dogs.

	F0–1 dogs (n = 22)	≥ F2 dogs (n = 6)	P value
Platelet (14.8–48.4×10 ⁴ /μL)	41.9 (23.6–132)	38.9 (27.5–57)	0.54
ALT (17–44 IU/L)	261 (46→1000)	708 (164–952)	0.093
Albumin (2.6–4.0 g/dL)	3.4 (2.7–4.3)	2.6 (2.2–3.1)	0.0010
Glucose (75–128 mg/dL)	103 (84–119)	108 (95–117)	0.38
T-Bil (0.1–0.5 mg/dL)	0.1 (0.1–1.0)	0.2 (0.1–2.6)	0.58
Tchol (111–312 mg/dL)	265 (129–451)	179 (63–258)	0.023
HA (ng/mL)	162 (117–211)	192(151–305)	0.11

Table 6. Routine laboratory findings and hyaluronic acid concentration are reported as medians with the ranges in parentheses.

Reference intervals are listed after each laboratory finding in the first column. Abbreviations: ALT, alanine aminotransferase; T-Bil, total bilirubin; Tchol, total cholesterol; HA, hyaluronic acid.

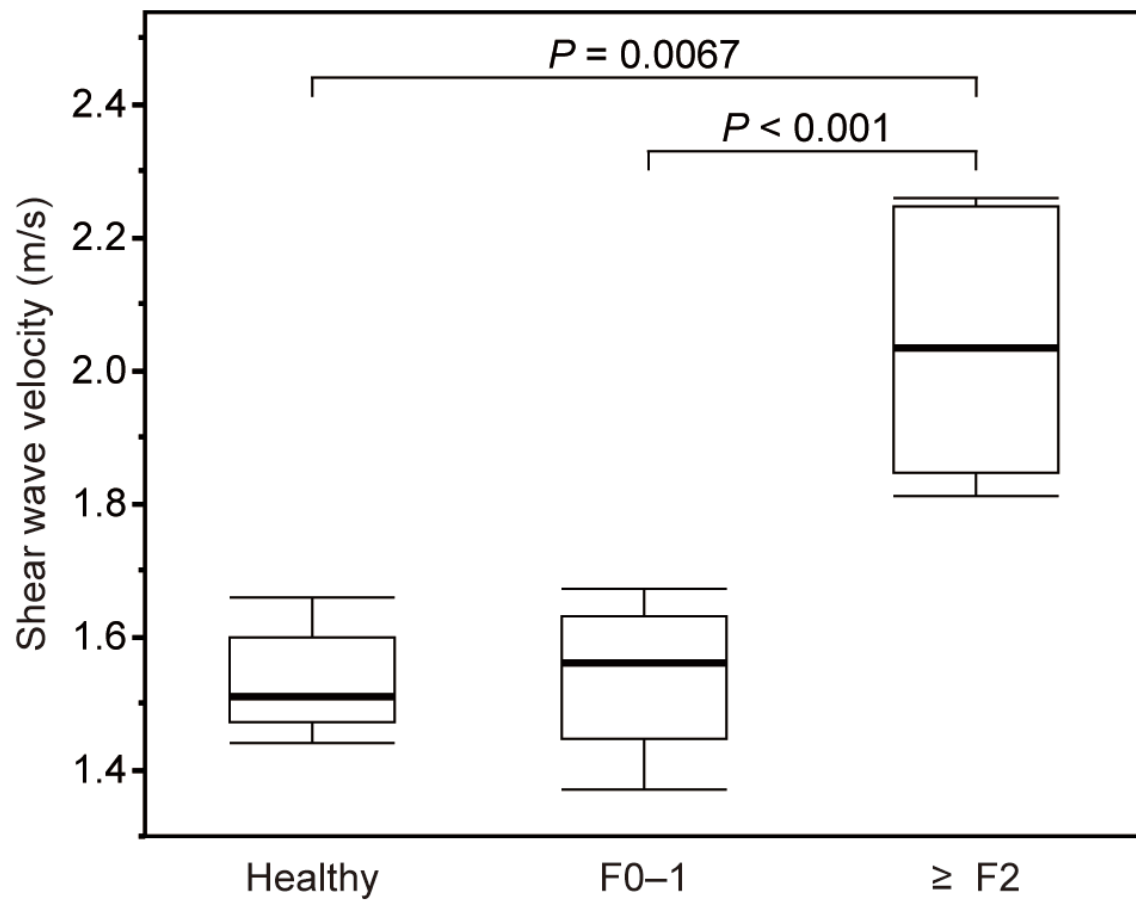


Figure 10. Shear wave velocity in healthy dogs ($n = 8$), dogs without clinically relevant fibrosis (F0–1, $n = 22$), and dogs with clinically relevant hepatic fibrosis ($\geq F2$, $n = 6$).

The box extends from 25% to 75% percentile with the median and the whiskers extend to limits of the data.

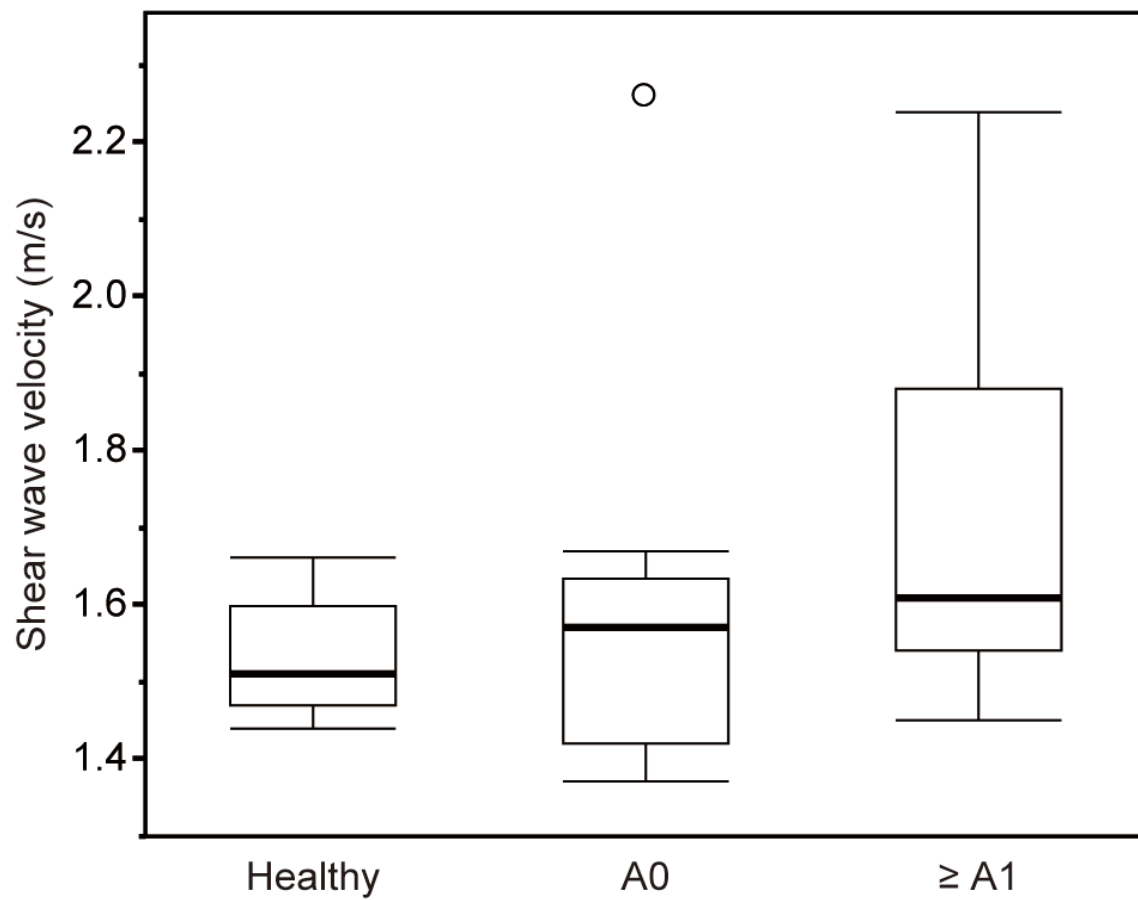


Figure 11. Shear wave velocity in healthy dogs (n = 8), dogs without significant necroinflammatory activity (A0, n = 17), and dogs with necroinflammatory activity ($\geq$ A1, n = 11).

The box extends from 25% to 75% percentile with the median and the whiskers extend to limits of the data.

4. DISCUSSION

Liver biopsy is a criterion-referenced method for the definitive diagnosis and monitoring of hepatic disease and hepatic fibrosis. Repeated liver biopsies are required to evaluate the progression of disease and monitor treatment efficacy, but it is sometimes difficult to perform liver biopsy because of the patient's clinical condition and the risk of life-threatening complications. Conventional B-mode ultrasound imaging would be useful for diagnosing hepatic parenchymal disorders such as chronic hepatitis, especially in the advanced stage, because of its ability to detect surface irregularity and regenerative nodules in the liver. However, it is considered a non-specific and insensitive method for the diagnosis of hepatic fibrosis. Thus, more specific and sensitive methods are needed.

I investigated whether 2D-SWE could differentiate between dogs with clinically relevant hepatic fibrosis ($\geq F2$) and dogs without clinically relevant hepatic fibrosis (F0–1). Consequently, SWV in dogs with clinically relevant hepatic fibrosis was significantly higher than that in dogs without clinically relevant hepatic fibrosis. Similar to findings in human patients, this result indicated that the liver elastic modulus assessed by 2D-SWE in dogs successfully reflected the severity of hepatic fibrosis secondary to hepatic parenchymal disorders such as chronic hepatitis or cholangiohepatitis. However, no significant difference was found between healthy dogs and dogs without clinically relevant hepatic fibrosis, even though all dogs without clinically relevant hepatic fibrosis were clinically diagnosed with hepatic disease and had increased hepatic enzyme activity. In humans, a report indicated that SWV measured using 2D-SWE overlapped among F0, F1, and the healthy control group.⁶⁶ Thus, my results indicated that it is difficult to predict the absence of or mild hepatic fibrosis (F0 or F1 stage) using 2D-SWE in dogs.

On the other hand, no significant difference was found in the SWV among healthy dogs, dogs with necroinflammatory activity, and dogs without necroinflammatory activity. Accordingly, I

presume that the necroinflammatory activity has a much lesser effect on SWV than does hepatic fibrosis. However, several previous studies in humans have reported that the SWV would be affected by conditions of severe liver inflammation such as acute hepatitis, and this finding is attributed to the presence of inflammatory cells infiltrates, tissue edema, and hepatocyte swelling in the liver with severe necroinflammatory activity.^{76,77} In my study, the majority of dogs with hepatic disease were graded as having only mild necroinflammatory activity (A0 to A2), and only 1 dog was graded as A3. Thus, it was difficult to determine the effect of necroinflammatory activity on SWV because of the small number of cases. Further studies with a larger sample of dogs with severe necroinflammatory activity are warranted.

Serum albumin and total cholesterol concentrations were significantly lower in dogs with clinically relevant hepatic fibrosis than those in dogs without clinically relevant hepatic fibrosis, although the concentrations in most dogs with hepatic disease were within reference ranges. In dogs with hepatic disorders, hypoalbuminemia tends to occur when approximately 70% of liver function has been lost. Hypcholesterolemia also could be detected in end-stage hepatic disease in dogs.⁷⁸ In humans, several indirect markers of hepatic failure have been reported for predicting the presence of hepatic fibrosis in clinical settings. In patients with chronic hepatitis B, serum albumin concentrations have been reported to be significantly lower in patients with clinically relevant hepatic fibrosis ($\geq$ F2) than in patients without clinically relevant hepatic fibrosis (F0–1).⁷⁹ Although the decreases in albumin and total cholesterol concentrations could help predict the presence of severe hepatic fibrosis in dogs as an indirect indicator, these changes are not specific for hepatic disorders.

Conversely, HA was not significantly different between dogs with and without clinically relevant hepatic fibrosis in this study. Hyaluronic acid is a high molecular weight glycosaminoglycan primarily synthesized by hepatic stellate cells and degraded by sinusoidal endothelial cells. It had been reported to possess high diagnostic accuracy for the non-invasive evaluation of hepatic fibrosis and

cirrhosis in human patients.⁶⁸⁻⁷⁰ However, in veterinary medicine, only 3 reports have investigated the usefulness of HA in predicting hepatic fibrosis. One report indicated that plasma HA concentration was significantly increased in dogs with cirrhosis,⁷¹ whereas another report indicated that the serum HA concentration correlated with hepatic fibrosis stage.⁷² One study however found no positive correlation between serum HA concentration and hepatic fibrosis stage,⁷³ which is similar to the results of this study. Discrepancy in results may be caused by differences in assay types, sample size, or histological sample population.

This study had several limitations. First, sample size was small, especially for dogs with clinically relevant hepatic fibrosis. However, despite small sample size, these results indicated the usefulness of 2D-SWE as a non-invasive diagnostic tool for the assessment of hepatic fibrosis. Further studies with larger sample size are needed to determine the sensitivity, specificity, and the cut-off value of 2D-SWE for distinguishing among fibrosis groups. Second, sampling errors might have occurred in some dogs with hepatic diseases, possibly because the region measured by 2D-SWE may not have completely coincided with that of liver biopsy. Additionally, it was impossible to collect biopsy specimens from multiple liver lobes because of the high risk of the bleeding. In the previous study of 70 dogs undergoing necropsy, some histological changes such as hepatic fibrosis were nonuniformly distributed among lobes.⁸⁰ Conversely, several previous reports in humans indicated that minimal or no significant differences in hepatic lesion were detected among hepatic lobes.⁸¹⁻⁸³ Further studies are needed to elucidate the effect of histopathological variations among liver lobes and the result of SWE measurement. Third, although this study included 3 dogs with mild ascites secondary to clinically relevant hepatic fibrosis, mild ascites did not separate the right liver lobe from the abdominal wall in these 3 dogs. Transient elastography is limited to patients that do not have perihepatic ascites or fluid in the abdomen surrounding the liver, and cannot be performed in patients with ascites. On the other hand, the recommended guidelines for the clinical use of elastography in humans indicated that SWE

can measure the SWV of patients with ascites because the ultrasound push beam, which generates the shear waves, propagates through fluids and appears not to be influenced by the presence of ascites.¹¹ Because the influence of ascites on the measurement of SWV using 2D-SWE is not well understood in veterinary medicine, further studies also are needed to evaluate the influence of ascites on SWV in dogs.

5. SUMMARY

In this chapter, the usefulness of 2D-SWE in the detection of the clinically relevant hepatic fibrosis ($\geq$ F2) in dogs with hepatic disease was investigated. It was shown that SWV measured by 2D-SWE is increased in dogs with clinically relevant hepatic fibrosis compared to dogs without clinically relevant hepatic fibrosis and healthy dogs. These results indicated that 2D-SWE is a useful method for non-invasively predicting clinically relevant hepatic fibrosis in dogs.

GENERAL CONCLUSION

The goal of this study was to establish the clinical usefulness in the assessment of hepatic microvascular perfusion and hepatic fibrosis using a novel ultrasonography in dogs with liver disease. The findings of the present study indicate that CEUS is useful methods to evaluate the hepatic microvascular perfusion in dogs with extrahepatic congenital portosystemic shunt and 2D-SWE is a feasible method for non-invasively evaluating the hepatic fibrosis in dogs with chronic diffuse liver diseases.

In chapter 1, I have characterized perfusion images and parameters of hepatic parenchyma, hepatic artery, and portal vein in healthy and extrahepatic congenital portosystemic shunt dogs. Briefly, all dogs underwent CEUS without sedation, and a TIC was generated. Sonazoid was used as the contrast agent. The TTP, RT, and RR in the hepatic parenchyma, hepatic artery, and portal vein as well as the Δ HP-PV were calculated and compared between healthy and extrahepatic congenital portosystemic shunt dogs. The relationship between the PV/Ao ratio and each perfusion parameter was also examined. The TTP, RT, and RR of hepatic parenchyma were significantly shorter in extrahepatic congenital portosystemic shunt dogs than in healthy dogs. In contrast, the TTP, RT, and RR of portal vein did not significantly differ between the two groups. Δ HP-PV was fairly earlier in extrahepatic congenital portosystemic shunt dogs than in healthy dogs. Furthermore, the RT of hepatic parenchyma and Δ HP-PV correlated well with the PV/Ao ratio in dogs with extrahepatic congenital portosystemic shunt. CEUS effectively revealed changes in hepatic microvascular perfusion in extrahepatic congenital portosystemic shunt dogs. In extrahepatic congenital portosystemic shunt dogs, the rapid enhancement of hepatic artery and parenchyma appears to reflect a compensatory increase in hepatic arterial blood flow (arterialization) secondary to a decrease in portal vein blood flow. Furthermore, the PV/Ao ratio reflects not only decreased PV blood flow, but also indirectly reflects

the degree of arterialization. The results of the present study suggested the applicability of CEUS as an additional diagnostic test to distinguish extrahepatic congenital portosystemic shunt dogs from healthy dogs.

In chapter 2, I have determined the feasibility, repeatability, and reproducibility of SWV using 2D-SWE in healthy beagle dogs. The interday and intraday CVs for 2D-SWE in liver and spleen were clinically acceptable. On the other hand, SWV measured by 2D-SWE was significantly different between the right and left lobe of the liver. These findings indicated that 2D-SWE can be used to assess the elastic modulus in the right and left lobes of the liver and in the spleen of dogs, but differences in elastic modulus between the right and left lobes of the liver should be considered, and the same lobe of the liver should be assessed throughout follow-up evaluations in dogs.

In chapter 3, I have investigated the usefulness of 2D-SWE for the detection of clinically relevant hepatic fibrosis in dogs with hepatic disease. Briefly, SWV measurement was performed in all dogs using 2D-SWE before liver biopsy or euthanasia and necropsy. Hepatic fibrosis stages and necroinflammatory activity grades were histopathologically evaluated. All dogs were divided into dogs without clinically relevant hepatic fibrosis (F0–1) and those with clinically relevant hepatic fibrosis ($\geq$ F2). Additionally, all dogs were divided into those without necroinflammatory activity (A0) and those with necroinflammatory activity ($\geq$ A1). Consequently, the median SWV was significantly higher in dogs with clinically relevant hepatic fibrosis than those without clinically relevant hepatic fibrosis, and in healthy dogs. However, there was no significant difference in the SWV between dogs without clinically relevant hepatic fibrosis and healthy dogs. On the other hand, the median SWV was not significantly different among groups, indicating that the necroinflammatory activity has a much lesser effect on SWV than hepatic fibrosis. These findings suggested that 2D-SWE would be a useful method for non-invasively predicting significant hepatic fibrosis in dogs.

Future study plans should include a bigger population, with multiple institution involvement

for validation of these CEUS and 2D-SWE data. Further research of hepatic fibrosis is also needed to classify among each fibrosis stage. Moreover, additional research should include comparison between quantitative histological fibrosis area and SWV measured by 2D-SWE.

In conclusion, through this study I was able to detect the changes in the hepatic microvascular perfusion of extrahepatic congenital portosystemic shunt dogs by CEUS. Moreover, I was able to establish a suitable 2D-SWE protocol for measuring SWV and have clarified that 2D-SWE would be useful for noninvasively predicting the presence of hepatic fibrosis in dogs with chronic diffuse liver disease.

JAPANESE SUMMARY (要旨)

Study on the evaluation of hepatic microvascular perfusion and hepatic fibrosis

using ultrasonography in dogs with liver disease

(犬の肝疾患における超音波検査を用いた肝微小循環および

肝線維化の評価に関する研究)

高い空間・コントラスト分解能を有し非侵襲的に生体内部を描画できる超音波検査は、犬の肝疾患において最も重要な検査法の1つとして広く普及している。しかし、これまでの超音波画像による診断は、臓器の断面画像から得られる形態的特徴および画像輝度パターンの定性的評価が中心となり、定量性の面では不十分であった。そこで近年、より客観性を持った定量診断法として造影超音波検査 (CEUS) と Shear Wave Elastography (SWE) が開発され、新たな診断のブレイクスルーとして人医療において様々な研究が行われている。

CEUS は、造影剤の血管内投与により非侵襲的に血流動態や組織灌流動態をリアルタイムに観察できる画像診断検査法である。CEUS による造影増強効果は組織灌流と良好に相関するため、組織内の微小血管の灌流状態をリアルタイムに評価することができる。

肝外性先天性門脈体循環シャント (EH-CPSS) は、門脈系と体循環系の間に生じた異常な側副血管により門脈血が直接体循環に流入する疾患である。肝機能低下に関連した臨床症状を呈し、無症状から肝不全、肝性脳症とその症状は多岐にわたる。EH-CPSS は症例数の多さと早期診断・早期治療の必要性から獣医学の肝疾患分野において非常に重要な疾患である。EH-CPSS は、先天性の血管奇形の疾患であるため、肝臓の血流動態 (肝微小循環) の評価は病態生理のみならず診断にも有用である。しかしながら、肝微小循環の評価には非常に優れた時間分解能が必須となり、古くから造影剤が使用されてきた CT や MRI 検査をもってしても時間分解能に乏しい欠点を理由に、評価することは困難であった。第 1 章で

は、この問題を解決するために CEUS に着目し、CEUS による定量的評価が犬の EH-CPSS に伴う肝微小循環の変化を捉えるのに有用か検討した。

各種画像検査で確定診断された EH-CPSS の犬 9 頭を前向きに本研究に組み込み、健常犬と比較した。右肋間アプローチにて右肝実質、門脈本幹、肝動脈を描出し CEUS を実施し、画像所見を比較した。また時間輝度曲線を作成し、最高輝度到達時間 (TTP)、流入時間 (RT)、上昇率 (RR) の 3 つの値を算出した。まず画像所見において、健常犬では造影剤投与直後すぐに肝動脈が造影増強され、次に門脈、その後、肝実質が造影増強された。一方、EH-CPSS 罹患犬では、肝動脈が造影増強された後すぐに、肝実質が造影増強された。その後、数秒遅れて門脈が造影増強された。次に各種パラメーターの比較では、EH-CPSS 罹患犬では健常犬に比べ、肝動脈および肝実質の TTP および RT の有意な短縮と、RR の有意な上昇を認めた。CEUS では、EH-CPSS 罹患犬において短絡血管の存在により肝臓に流入する門脈血流が減少し、代償性に肝動脈血流が増加する“肝臓の動脈化”を捉えることができた。

第 2 章および第 3 章では、犬の肝疾患における SWE の有用性について検討した。SWE とは、組織に剪断波を発生させてその伝播速度から組織の弾性を定量的に測定できる画像検査法である。肝線維化は、慢性肝炎をはじめとするびまん性肝疾患などによって引き起こされる持続的な炎症により生じる病理組織学的な変化である。持続的な肝線維化は、肝実質の再生性結節を伴い終末像である肝硬変へと進行し、門脈高血圧症や肝性脳症などの重篤な合併症を引き起こす。既に医学領域では肝線維化の進行が予後と強く相関する事が多くの研究で示され、肝線維化評価は治療適応の決定および予後予測に極めて重要とされている。そして近年、犬においても肝線維化の重症度と予後との関連性が報告され、獣医療においても肝線維化評価の重要性が指摘されている。

肝線維化評価のゴールドスタンダードは肝生検による病理組織学的な評価とされてきたが、疼痛や出血などの合併症のリスクが問題となる。人医療において、SWE による肝硬度測定が病理組織学的な肝線維化ステージと強く相関することが示され、SWE は非侵襲的な

肝線維化診断法として広く臨床応用されている。さらに近年登場した最新鋭の 2D-SWE は、これまでの SWE と比較し高い再現性および汎用性が明らかになっている。一方で、犬における SWE を用いた肝硬度測定の情報乏しく、臨床応用の可能性は明らかにされていない。第 2 章および第 3 章では、2D-SWE を用いて犬の肝線維化に対する非侵襲的診断法の可能性について検討した。

第 2 章では、8 頭の健常犬を用いて測定値の再現性、および測定部位間における肝硬度の差について検討した。肋間アプローチにて、それぞれ肝右葉および肝左葉の肝硬度を測定し、左右の肝硬度を比較した。さらに日内および日間変動に関しても、変動係数を算出して評価した。その結果、左右ともに測定の再現性は高く、臨床応用可能であった。一方、左右の肝硬度は Bland-Altman 解析で固定誤差が認められたため、臨床現場で 2D-SWE を使用して経時的に症例の肝硬度を評価する場合には同じ領域で測定することが推奨された。

第 3 章では臨床例を用いて、2D-SWE による非侵襲的肝線維化予測法の可能性について検討した。本研究では、最終的に肝生検により肝疾患と診断された 28 頭の犬を前向きに組み入れ、肝生検実施の前に全ての犬の肝硬度を測定した。Ishak 分類を使用し、病理組織学的に肝線維化 (Fibrosis: F) ステージを 5 段階、炎症 (Necroinflammatory activity: A) グレードを 6 段階に分類した。対照群として健常なビーグル犬 8 頭 (健常群) を使用した。臨床上有意な肝線維化である F2 を基準に有意な肝線維化なし (F0-1 群) とあり ($\geq$ F2 群) の 2 群に分類し、さらに健常群を加えて 3 群で比較検討した。炎症についても、炎症なし (A0 群) と炎症あり ($\geq$ A1 群) に分類して 3 群で比較した。その結果、 $\geq$ F2 群の肝硬度は、F0-1 群および健常群と比較して有意に高値を示した。一方、F0-1 群と健常群では統計学的に有意差は認められなかった。炎症に関しては、3 群間で統計学的な有意差は認められなかった。以上の結果より、人の報告と同様に、犬においても 2D-SWE による肝線維化予測が可能であることが明らかになった。SWE による肝硬度測定は、組織の弾性と粘性の両者 (粘弾性) を計測し定量化している。組織学的な肝線維化の進行が組織の弾性を増加させ、肝硬度が上

昇したと推察される。一方、肝硬度に対する炎症の影響は低いことが明らかになった。

今後の研究課題として、EH-CPSS に対する CEUS の診断精度を検証するため、さらなる症例の蓄積および多施設での検討が必要である。また 2D-SWE による非侵襲的な肝線維化診断に関しても、症例を蓄積して肝線維化ステージ毎の診断精度（感度および特異度）を明らかにする必要がある。さらに、本研究における病理組織学的な肝線維化ステージは定性的な手法を用いたが、今後線維化の特殊染色により定量化した線維化面積率と肝硬度との関連についても検討したい。

最後に、本研究により CEUS を用いることで EH-CPSS の肝微小循環の変化を捉え、診断へ応用できる可能性が示された。また 2D-SWE は、犬においても非侵襲的に肝線維化を検出できることが明らかになった。今後、犬の肝疾患における早期診断や適切な病態把握および治療効果判定への寄与が期待される。

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