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**Application of contrast-enhanced ultrasonography to diagnose and evaluate
chronic intestinal diseases in dogs**

(犬の慢性腸疾患の診断と評価における造影超音波の応用)

Khoirun Nisa

GENERAL ABBREVIATIONS

AT	arrival time
AUC	area under the curve
BI	baseline intensity
BP	blood pressure
CCECAI	canine chronic enteropathy clinical activity index
CE	chronic enteropathy
CEUS	contrast-enhanced ultrasonography
CI	confidence interval
CRP	c-reactive protein
CV	coefficient of variation
DAP	diastolic arterial pressure
GABA	gamma amino butyric acid
GI	gastrointestinal
HR	heart rate
IBD	inflammatory bowel disease
LoA	limit of agreement
MAP	mean arterial pressure
MB	microbubble
MI	mechanical index
MPV	mean pixel value
MRE	magnetic resonance enterography
MRI	magnetic resonance imaging

PFB	perflubutane
PI	peak intensity
ROI	region of interest
SAP	systolic arterial pressure
SD	standard deviation
SMA	superior mesenteric artery
TIC	time-intensity curve
TTP	time-to-peak
WiR	wash-in rate
WoR	wash-out rate
WSAVA	world small animal veterinary association

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GENERAL INTRODUCTION

Chronic intestinal signs (i.e. diarrhea, vomiting, weight loss) in dogs can be very challenging to diagnose and manage as these signs are resulted from intestinal damage in associated with various entities including inflammatory diseases and neoplasia. Among the inflammatory diseases of intestine in dogs, chronic enteropathy (CE) is considered to be the most frequent.¹ The underlying etiology of CE is unknown. CE is diagnosed by exclusion of possible causes of intestinal signs (e.g. infectious, endocrine, or neoplastic diseases, or exocrine pancreatic insufficiency) by blood tests, fecal examinations, and imaging; and detection of intestinal inflammation by histopathology.^{2,3} One important differential diagnosis of CE is intestinal lymphoma which first diagnosed by ultrasound-guided fine needle aspiration of enlarged regional lymph nodes and/or intestinal masses. But the corresponding ultrasound findings are not necessarily presence in dogs with intestinal lymphoma⁴ and negative result from cytological examinations of the lymph nodes and/or masses does not completely rule out intestinal lymphoma.^{5,6} In such cases, histopathology of intestinal biopsies become essential not only to confirm the presence of intestinal inflammation but mainly to exclude intestinal lymphoma.⁷

CE is retrospectively classified into food-responsive, antibiotic-responsive, steroid/immunosuppressant-responsive, or non-responsive/refractory following a sequence of treatment trials using alternative diets, antibiotics, and anti-inflammatory or immunosuppressive agents.⁸ There are no differences in the histopathology among those CE types that limit the advantage of histopathology at the diagnosis.^{2,9,10} Several biomarkers such as clinical scoring, clinicopathological findings, and B-mode ultrasound have been utilized so far in clinical practice to evaluate disease severity and therapeutic

response in dogs with CE. Unfortunately these biomarkers are either subjective, not specific for intestine, or lack of correlation with the therapeutic response.^{11–15} Endoscopy together with histopathology on the other hand best characterize the progression of intestinal inflammation, but are relatively invasive and impractical for repeated evaluation. Due to above reasons, there remains a need for an alternative modality to diagnose and evaluate CE in dogs.

Contrast-enhanced ultrasonography (CEUS) is an imaging modality that combine the use of intravenously injected contrast agent and contrast-specific ultrasound setting. The contrast agent for CEUS is composed by microbubbles (MB) which are low molecular weight gas-filled microspheres encapsulated by an outer shell.¹⁶ Sonazoid®, a second-generation MB contrast agent containing perflubutane (PFB) gas, is highly stable *in vivo*.¹⁷ Using these MBs, organ perfusion can be evaluated in real-time basis with the intensity of contrast enhancement reflecting the concentration of MB within microvessels.^{18,19} Furthermore, a more objective evaluation is provided by an adjunct quantitative analysis of CEUS images which is performed by drawing a region of interest (ROI) on targeted organ and obtaining a number of perfusion parameters.²⁰ In veterinary medicine, qualitative and quantitative CEUS have been utilized for the perfusion analysis of various abdominal organs including the liver, spleen, kidneys, pancreas, and adrenal glands.^{21–26}

The use of CEUS to evaluate intestinal perfusion in veterinary practice is still limited. Few CEUS studies on intestine of healthy dogs have been initiated^{24,25,27} but CEUS of intestine in dogs with intestinal diseases has not yet been reported. In human medicine, CEUS has been utilized to evaluate intestinal perfusion of patients with inflammatory bowel disease (IBD) and revealed changes in post-contrast enhancement

pattern¹⁹ as well as quantitative perfusion parameters compared to healthy controls.²⁸ These findings were related to microvascular reconstruction in the intestine as a direct consequence of chronic inflammation which contribute to the pathogenesis of IBD.^{29,30} CE in dogs has been known to share some common pathogenesis with human IBD.^{3,31} Thus, I hypothesize that changes in intestinal perfusion as assessed by CEUS can be useful in the diagnosis and evaluation of CE dogs.

Before clinical application of CEUS to evaluate intestinal perfusion in dogs, the assessment of its repeatability and reproducibility is important to determine the feasibility and the most reliable parameters that allow detection of pathological changes in the intestine. As this modality is aimed for serially repeated assessment (e.g. evaluation of disease progression and therapeutic response), the changes related to disease progression or therapeutic response should be able to be distinguished from physiological changes or measurement errors. Furthermore, it is important to maintain animal cooperation during CEUS acquisition because animal movement interferes with the image analysis. For this purpose, a sedative is sometimes required to restrain uncooperative animals. To my knowledge, repeatability and the effect of sedation on the CEUS of canine intestine are still limited.

Considering the above background, I conducted research on the application of CEUS in the assessment of canine intestinal perfusion focusing on duodenum. Duodenum was selected in this study due to the position which is superficial and rectilinear along the right lateral abdominal wall.³² In dogs, duodenum is relatively easy to identify by ultrasound as it has the thickest wall among the entire segment of small intestine.³² The study was performed within 3 stages. In the first stage, I examined the repeatability and reproducibility of CEUS in assessing duodenal perfusion of healthy dogs. The result of

this stage determined the feasibility of CEUS and the best perfusion parameters for future application in clinical practice. In the second stage, I evaluated the effect of sedation on CEUS-derived parameters which were determined in the first stage. Then proceeded to the third stage, I conducted a cross-sectional study involving client owned dogs to determine the applicability of CEUS in evaluating perfusion changes in dogs with chronic intestinal diseases especially CE and intestinal lymphoma.

CHAPTER 1

REPEATABILITY AND REPRODUCIBILITY OF QUANTITATIVE CONTRAST-ENHANCED ULTRASONOGRAPHY FOR ASSESSING DUODENAL PERFUSION IN HEALTHY DOGS

1. INTRODUCTION

The assessment of canine intestinal perfusion may provide valuable information for the diagnosis and evaluation of dogs with chronic intestinal diseases. In human medicine, intestinal perfusion of Crohn's disease (CD) patients has been evaluated by CEUS. It revealed changes in the enhancement pattern¹⁹ as well as perfusion parameters when compared with healthy controls.²⁸ CEUS has been utilized to estimate the disease activity and predict the treatment response in CD patients.^{33–36} It has also been reported to be correlated with the endoscopic severity³⁷ and shown to be comparable with Magnetic Resonance Enterography (MRE).³⁸ In veterinary medicine, a few studies reported that qualitative and quantitative CEUS enable the characterization of intestinal perfusion in healthy dogs,^{24,25,27} but the application in clinical practice has yet to be established.

Repeatability (intraday variability) and reproducibility (interday variability) assessment of quantitative CEUS in evaluating intestinal perfusion is important as a prerequisite before application in clinical practice. It is necessary to evaluate its feasibility and determine reliable perfusion parameters to detect pathological changes in the intestine. It is even more essential as this modality is aimed at serially repeated assessment (e.g. disease monitoring and treatment evaluation), so that changes related to disease activity or the treatment response can be differentiated from physiological changes and measurement errors. To my knowledge, information on this topic in healthy dogs is still limited. Therefore, chapter 1 aimed to assess the repeatability and reproducibility of quantitative CEUS of the duodenum in healthy dogs.

2. MATERIALS AND METHODS

2.1 Animals

Six beagle dogs (3 males and 3 females, aged 1-4 years, weighing 8.8-12 kg) were enrolled in this study. All dogs were healthy on physical examination and did not present clinical signs or hematologic (complete blood count and serum biochemistry) abnormalities associated to gastrointestinal (GI) diseases. CEUS was conducted on 4 days over a 9-day period (i.e. 3 dogs underwent CEUS on day 1 and 8, while the remaining 3 dogs underwent CEUS on day 2 and 9). On a given day, the 3 dogs were each examined 3 times (i.e. at 9:00, 13:00, and 17:00). The scanning of 3 dogs was performed consecutively according to the same order for each examination (dog 1, 2, 3 for day 1 and 8; and 4, 5, 6 for day 2 and 9). Food was withheld for approximately 12 hrs before experiment. Dogs were sedated with a combination of butorphanol (Vetorphale® 5 mg/ml, Meiji Seika Pharma Co, Ltd, Tokyo, Japan) and midazolam (Dormicum® 5 mg/ml, Astellas Pharma Inc, Tokyo, Japan) at a dosage of 0.2 mg/kg and 0.1 mg/kg for butorphanol and midazolam respectively. Sedation was performed to improve animal cooperation throughout duodenal imaging. During the procedure, the heart rate (HR) and mean arterial pressure (MAP) of all dogs were monitored noninvasively using an oscillometric technique (BSM-5192, Nihon Kohden Co., Tokyo, Japan). All procedures were approved by the Hokkaido University Animal Care and Use Committee.

2.2 Ultrasonography

B-mode ultrasound was performed prior to CEUS for general imaging of the duodenum, and neither focal nor diffuse abnormalities were found. For CEUS acquisition, dogs were positioned in left lateral recumbency and the ultrasound probe was placed behind the last rib to achieve a longitudinal view of the duodenum. Contrast agent

(Sonazoid[®], Daiichi-Sankyo, Tokyo, Japan) administration and CEUS acquisition (Aplo XG; broadband linear probe, 5-11 MHz, PLT-704AT, Toshiba Medical Systems, Otawara, Japan) were performed based on the bolus method described in a previous study on the pancreas²⁵ with few modifications. In the current study, the mechanical index (MI), imaging depth, and focal zone depth were adjusted to 0.20, 3 cm, and 2 cm, respectively. All CEUS was performed by one sonographer (S.Y.L.) and one contrast agent administrator (S.M.) throughout the study.

2.3 Quantitative analysis

Quantitative analysis was done using image analysis software (ImageJ, US National Institutes of Health, Bethesda, MD, U.S.A.) by one observer (K.N.). One frame per sec for a total 120 sec were analyzed. Four ROIs were manually drawn as large as possible in the duodenal mucosa at approximately the same depth and without including big vessels or adjacent tissue. When a respiratory motion or duodenal movement was present, the ROIs were adjusted manually to maintain the same position within the duodenal mucosa and narrowed depth range. The analysis using 4 ROIs were selected based on a preliminary study that indicated the best intraobserver agreement when compared with other methods (data not shown). If 4 ROIs could not be drawn due to motion artifacts; 1, 2, or 3 ROIs were drawn instead. The software calculated the intensity within each ROI as a gray-scale level ranging from a mean pixel value (MPV) of 0-255. The intensity data were subsequently exported to commercial software (Microsoft Excel 2013, Microsoft, Redmond, WA, U.S.A.), followed by averaging the intensities obtained from 4 ROIs. The averaged intensities were plotted against time to create a time-intensity curve (TIC).

A number of perfusion parameters were acquired from TIC, including the time-

to-peak (TTP), which refers to the time from the arrival time (AT, time point when the intensity is above the baseline, and followed by a further rise) to maximum enhancement; peak intensity (PI) refers to maximum enhancement with subtraction of the baseline intensity (BI, intensity at AT); area under the curve (AUC) refers to the area under the TIC curve above BI and is calculated from AT to 120 sec; wash-in and wash-out rates (WiR and WoR, respectively) refer to the slope of ascending and descending tracts of TIC respectively. WiR and WoR were calculated by performing regression using the same Excel sheet to subsequent points that continued to increase from BI to PI and decrease from PI to the end of the recording, respectively (Figure 1). Quantitative analysis for all scans was performed twice by the same observer to evaluate intraobserver variability.

2.4 Statistical analysis

Statistical analysis was done using statistical analysis programs (JMP pro 12.0.1, SAS Institute Inc., Cary, NC, U.S.A. and IBM SPSS Statistics V22.0, IBM Corp., Armonk, NY, U.S.A.). All perfusion parameters were evaluated for normal distribution using the Shapiro-Wilk test, and the results are expressed as the mean±standard deviation (SD). The following linear fixed effect model was used to analyze intraday, interday, and intraobserver variabilities:

$$Y_{ijkl} = \mu + analysis_i + day_j + dog_k + (day \times dog)_{jk} + \epsilon_{ijkl}$$

where Y_{ijkl} is the l th value measured for dog k on day j in the i th analysis, μ is the general mean, $analysis_i$ is the differential effect of analysis i , day_j is the differential effect of day j , dog_k is the differential effect of dog k , $(day \times dog)_{jk}$ is the interaction term between day and dog, and ϵ_{ijkl} is the model error. The SD of intraday variability was estimated as the residual SD of the model, the SD of interday variability as the SD of the differential effect of day, and the SD of intraobserver variability as the SD of the

differential effect of analysis. The coefficients of variation (CVs) were calculated by dividing each SD by the mean and are written as a percentage (%). Based on previous studies of CEUS in humans and animals, $CV < 25\%$ is considered clinically acceptable.^{39,40} The confidence intervals (CIs) were calculated by multiplying SD by 2.77.⁴¹ The CI represents the difference required between two analyses conducted at the same time or on a single individual for a true change to be detected with a probability of < 0.05 . In addition, the partial correlation coefficient between HR as well as MAP and perfusion parameters were evaluated.

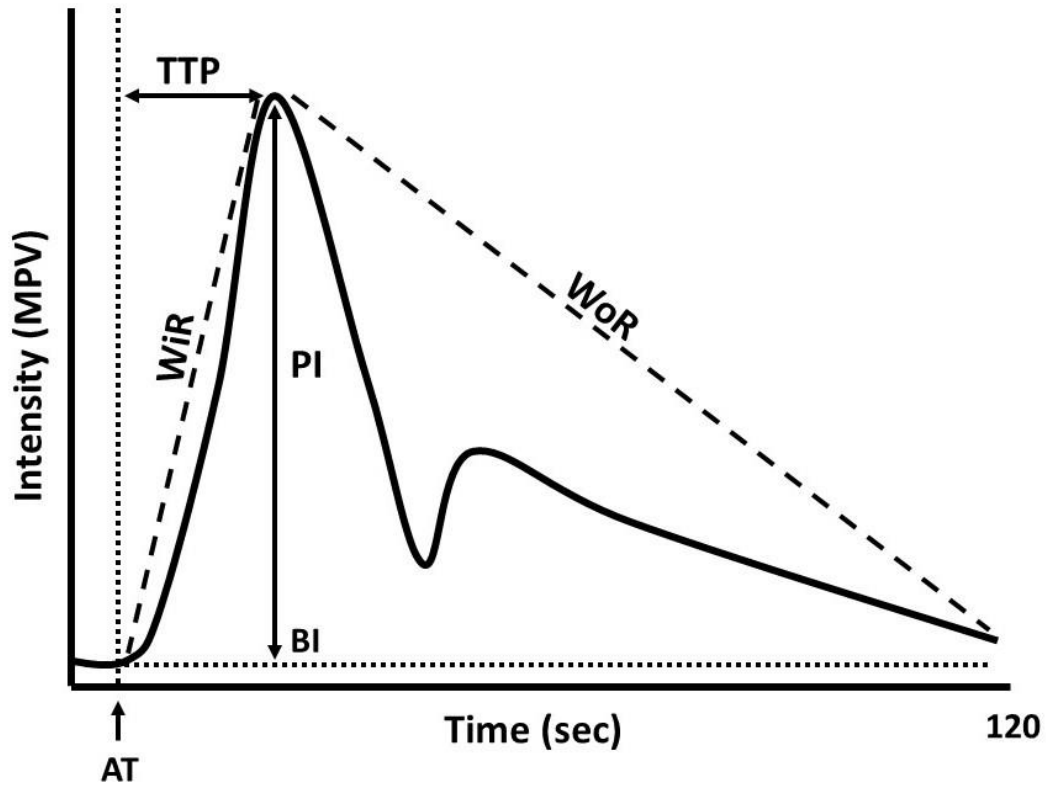


Figure 1. Schematic TIC describing wash-in and wash-out after bolus injection. The second peak indicated recirculation. The arrival time (AT) refers to the time point when the intensity rose above the baseline, followed by a continuous increase. The baseline intensity (BI) refers to the intensity at AT. Five parameters were analyzed: time-to-peak (TTP) refers to the time from AT to PI; peak intensity (PI) refers to the maximum enhancement subtracted by BI; area under the curve (AUC) refers to area under the TIC curve above BI, calculated from AT to 120 sec; wash-in and wash-out rates (WiR and WoR, respectively) refer to slope of ascending and descending tracts of TIC. WiR and WoR were calculated by performing regression to subsequent points that continued to increase from BI till PI and decrease from PI to the end of the recording.

3. RESULTS

No dogs showed immediate or delayed adverse reaction (e.g. vomiting, syncope) after the bolus injection of Sonazoid®. Selected frames from a total of 36 CEUS examinations of the duodenum (6 dogs; 3 times within one day, and on 2 different days) were satisfactory for quantitative analysis. Four ROIs were drawn in selected frames of 34 CEUS examinations, while only 1 and 2 ROIs could be drawn in those of another 2 examinations due to motion artifacts.

Subjectively, the duodenum was enhanced within several seconds after contrast injection (Figure 2A). The contrast enhancement began from the perivisceral vessels, moved towards the duodenal lumen centripetally, and involved all layers of the duodenal wall. Contrast enhancement was homogeneous along the imaged duodenal segment at PI (Figure 2B). This was followed by contrast elimination during wash-out (Figure 2C). The generated TIC showed biphasic decreases (Figure 2D). From the AT, initial rapid wash-in and wash-out stopped approximately 20-30 sec after injection, followed by recirculation. The enhancement pattern of the duodenum and the generated TIC were consistent in all dogs.

The mean±SDs, and intraday, interday, and intraobserver SDs, CVs, and CIs for all measured perfusion parameters derived from CEUS of duodenum as well as hemodynamic parameters (HR and MAP) are summarized (Table 1). Intraday and interday CVs for TTP, PI, AUC, WiR, and WoR were less than 25% (range, 2.27-23.41%). Intraobserver CVs for all perfusion parameters ranged between 2.27 and 8.30%. Significant partial correlations were indicated between HR and 2 of 5 perfusion parameters, TTP ($p = 0.012$) and WiR ($p = 0.007$). A negative partial correlation was

indicated between HR and TTP ($r = -0.444$), while a positive partial correlation was shown between HR and WiR ($r = 0.471$). No significant partial correlations were observed between MAP and perfusion parameters.

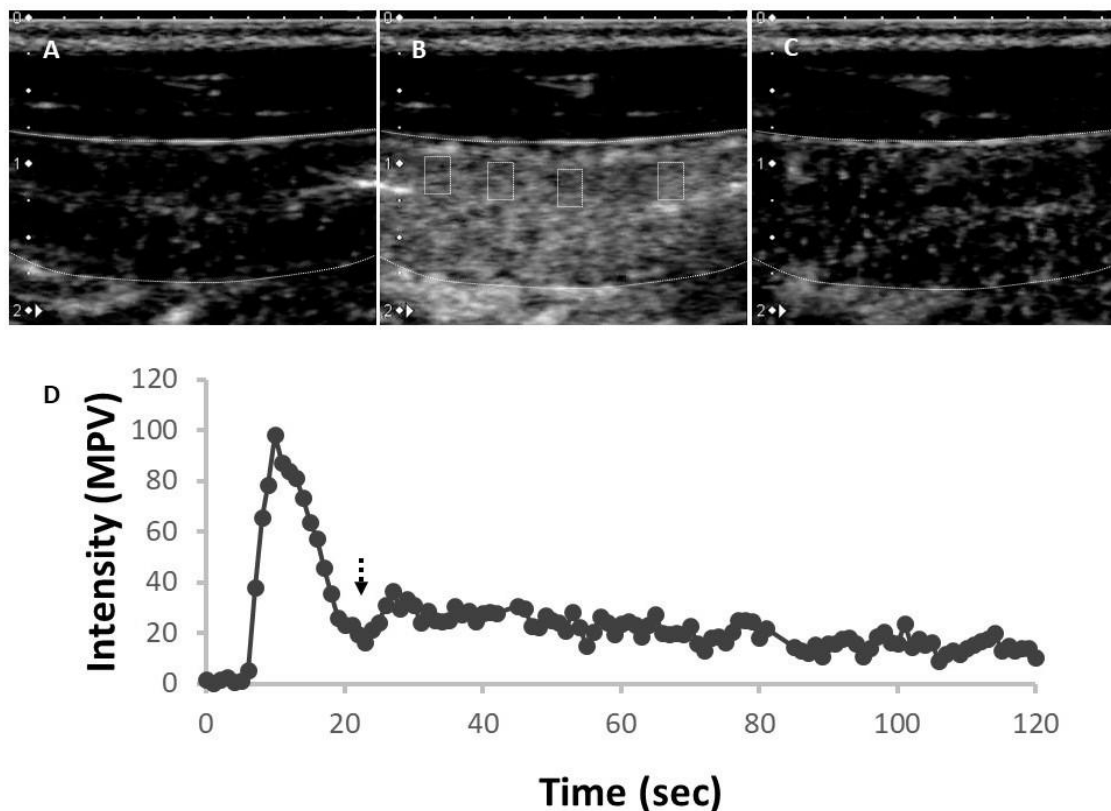


Figure 2. Sequence images of the duodenum (dashed outline) following Sonazoid® administration in one representative dog (A-C), and the generated TIC (D). (A) The image of the duodenum during the arrival time of contrast agent [6 sec in this dog]. (B) Homogeneous enhancement along the imaged duodenal segment at PI [10 sec in this dog]. Multiple ROIs (dashed box) were drawn at the same depth within the duodenal mucosa. (C) Contrast wash-out at the end of recording [120 sec]. (D) TIC showed a biphasic decrease. Rapid initial wash-in and wash-out (within 23 sec as shown here), followed by recirculation (dashed arrow).

Table 1

Intraday, interday, and intraobserver variabilities of perfusion parameters derived from the CEUS of duodenum and hemodynamic parameters

Variable	Mean±SD	Intraday			Interday			Intraobserver		
		SD	CV(%)	CI	SD	CV(%)	CI	SD	CV(%)	CI
Perfusion parameters										
TTP (sec)	4.40 ± 0.90	0.1	2.27	0.28	0.87	19.68	2.4	0.1	2.27	0.28
PI (MPV)	106.50 ± 11.50	13.71	12.87	37.97	9.12	8.57	25.27	4.61	4.33	12.78
AUC (MPV.sec)	3474.12 ± 816.53	221	6.36	612.17	519.98	14.97	1440.3	173.93	5.01	481.78
WiR (MPV/sec)	26.80 ± 6.50	1.59	5.92	4.4	6.27	23.41	17.37	2.22	8.3	6.16
WoR (MPV/sec)	-0.85 ± 0.09	0.05	6.44	0.15	0.08	9.11	0.21	0.05	6.44	0.15
Hemodynamic parameters										
HR (beat/min)	78.42 ± 15.94	25.83	32.94	71.56	4.95	18.31	39.78	NE	NE	NE
MAP (mmHg)	90.4 ± 12.25	4.95	5.48	13.71	8.59	9.5	23.78	NE	NE	NE

TTP = time to peak, PI = peak intensity, AUC = area under the curve, WiR = wash-in rate, WoR = wash-out rate, HR = heart rate, MAP = mean arterial pressure, MPV = mean pixel value, SD = standard deviation, CV = coefficient of variation, CI = 95% confidence interval for mean, NE = not examined.

4. DISCUSSION

This chapter evaluated the repeatability (intraday variability) and reproducibility (interday variability) of CEUS-derived perfusion parameters of duodenum in healthy sedated dogs. The results showed that the repeatability and reproducibility of CEUS in assessing duodenal perfusion of healthy sedated dogs were clinically acceptable, with the CV for all perfusion parameters (including TTP, PI, AUC, WiR, and WoR) being less than 25%.

Intraday and interday CVs for all perfusion parameters ranged from 2.27 to 23.41% (Table 1). In a study using a mouse tumor model, CVs ranging from 3.74 to 29.34% were considered acceptable for CEUS-derived perfusion parameters obtained from 3 repeated injections.⁴² In another previous study of quantitative CEUS for abdominal organs of healthy cats including the small intestine, a CV of less than 25% for repeated examinations within a short time interval was considered acceptable.³⁹ In human medicine, a study of renal perfusion using CEUS also considered a change of more than 25% between 2 measurements to be significant.⁴⁰ Referring to these studies, the cut-off value of the CV required for a perfusion parameter derived from CEUS to be considered acceptable is 25%.

Low intraobserver CVs (range, 2.27-8.30%) for all perfusion parameters were documented in my study. Intraobserver variability indicates the size of variation when a CEUS image is analyzed more than once consecutively by the same observer. Intra-reader variability was examined in a study evaluating the reproducibility of hepatic hemodynamics with CEUS in healthy volunteers and patients with liver cancer, and CVs

within a range of 5-15% were reported. In this study, a single reader was assigned to analyze all scans twice. The author considered the documented CVs to be almost perfect.⁴³

Variability of perfusion parameters derived from CEUS could be related to internal and external factors. Internal factors are correlated with the animal physiology such as cardiac output, blood pressure (BP), and HR. In the current study, significant partial correlations were observed between HR and two perfusion parameters, TTP and WiR. A low HR resulted in a longer TTP and lower WiR. Low intraday and interday CVs of MAP were indicated, but those of HR were high, possibly influenced by the physiological status, level of excitement, and sedative effect (Table 1). This could be the cause of the relatively higher interday CV for TTP and WiR in comparison with those for the other 3 parameters (PI, AUC, and WoR).

External factors influencing the variability of perfusion parameters might include the scanning and analysis processes. Continuous scanning of the duodenum was challenging due to its peristaltic movement. Therefore, during off-line analysis, ROIs were carefully placed in the mucosa to avoid noise and artifacts. The result of analysis by manual placement of ROI could be influenced by the human error, but it was better than automatic analysis at avoiding problems related to intestinal or respiratory motion. The software for automatic analysis utilized in the preliminary study of my institution was not able to filter out the influence of such motions. Furthermore, it was difficult to perform repeated scanning exactly in the same segment of the duodenum and place the ROI at the same depth. I have minimized the spatial variation by standardizing the transducer approach to obtain a longitudinal view of the duodenum.

Enhancement pattern and generated TIC of the duodenum, which depicted the

mural vascularization of the duodenal wall,^{44,45} in the current study are consistent with those of canine and feline intestines described in previous studies using CEUS.^{24,25,27,46} Recirculation which was detected after the first wash-out in my TIC, was the same as that described in healthy cats.⁴⁶ However, it was not described in the TIC of other previous studies on dogs,^{24,25,27} probably due to differences in the type of MB contrast agent, dosage, and image setting between the current and previous studies. Recirculation is likely to cause only a small increase in the intensity, often smooth and gradual, as most of the MBs are destroyed due to ultrasound beam exposure during the initial circulation.⁴⁷

Perfusion parameters derived from TIC provide a more objective way to evaluate hemodynamic changes in the duodenum. TTP, WiR, and WoR were correlated with the blood flow velocity, while PI and AUC were correlated with the blood volume within the corresponding ROI.²⁰ These parameters may change with chronic inflammation that causes a vascular rearrangement in the intestine due to physiological and pathological angiogenesis.^{29,30} Increases in the angiogenesis and microvascular density were investigated in the intestine of patients with IBD, resulting in increased regional blood flow. The blood flow was reported to increase only in the mucosa and submucosa, and remained unchanged in the muscularis layer.^{48,49} Even though differences between canine CE and human IBD have been reported,³¹ similar pathological changes in the intestinal perfusion could be seen.

The values of TTP, PI, AUC, WiR, and WoR in the current study may serve as a reference for future examination of duodenal perfusion in clinical practice. In addition, the 95% CIs provided in the current study encompass the actual range of the mean for each perfusion parameter in healthy dogs (Table 1). In other words, true alterations related to pathological changes can only be considered if the values decrease below or increase

above this interval. Further study in dogs with chronic intestinal disorders should be performed to confirm whether the evaluated parameters have adequate sensitivity and specificity.

TTP in the current study was shorter compared with a previous report²⁵ because I selected the arrival time of the contrast agent as the starting point instead of contrast injection. The method of measuring TTP in my study was determined to minimize the influence of systemic blood flow and/or the contrast injection speed and yielded better repeatability and reproducibility. The PI and AUC were higher than those recorded in the same report due to different scan settings.²⁵ Therefore, the reference value provided in the current study should be used only for examination with the same protocol.

This study had several limitations. First, this study only evaluated the repeatability and reproducibility of CEUS in assessing duodenal perfusion of sedated dogs. Therefore, the repeatability and reproducibility of this method in non-sedated dogs are unknown. A lower repeatability for CEUS parameters of hepatic vein was documented in conscious dogs when compared with sedated dogs.⁵⁰ Second, even though CEUS-derived perfusion parameters of duodenum demonstrated acceptable intraday and interday CVs in the current study, these results cannot guarantee that this technique is applicable for other sonographers and/or other ultrasound machines. Interobserver variability was also not assessed in this study. Third, the dogs enrolled in the current study did not present any symptoms or laboratory findings related to GI disorders, but the absence of inflammatory lesions could not be confirmed because the histopathological evaluation of the duodenum was not performed.

From the results, it can be concluded that quantitative CEUS was feasible in assessing duodenal perfusion in healthy sedated dogs. TTP, PI, AUC, WiR, and WoR

demonstrated adequate intraday, interday, and intraobserver CVs. Further study in dogs with chronic intestinal diseases is necessary to evaluate the clinical applicability of CEUS in assessing duodenal perfusion.

5. SUMMARY

In this chapter, I assessed the repeatability (intraday variability) and reproducibility (interday variability) of CEUS-derived perfusion parameters of duodenum in healthy sedated dogs. Intraday, interday, and intraobserver CVs of perfusion parameters including TTP, PI, AUC, WiR, and WoR were evaluated. The results showed that the repeatability and reproducibility of CEUS in assessing duodenal perfusion of healthy sedated dogs were clinically acceptable, with the CVs for all perfusion parameters being less than 25%. From the results, it can be concluded that quantitative CEUS was feasible in assessing duodenal perfusion in healthy sedated dogs. Further study in dogs with chronic intestinal disorders is necessary to evaluate the clinical applicability of CEUS in assessing duodenal perfusion.

CHAPTER 2

THE EFFECT OF SEDATION WITH A COMBINATION OF BUTORPHANOL AND MIDAZOLAM ON QUANTITATIVE CONTRAST-ENHANCED ULTRASONOGRAPHY OF DUODENUM IN HEALTHY DOGS

1. INTRODUCTION

A few studies on the characterization of intestinal perfusion using quantitative CEUS in healthy dogs have been reported.^{24,25,27} In addition, previous chapter indicated that the repeatability and reproducibility of this modality were clinically acceptable in assessing duodenal perfusion of healthy dogs.⁵¹ Thus, clinical application of this modality to detect changes of intestinal perfusion which is expected to occur related to inflammatory (i.e. CE) and neoplastic diseases (i.e. intestinal lymphoma) in dogs is subjected in the near future.

In veterinary studies, it is important to maintain animal cooperation during CEUS acquisition because animal movement interferes with the ROI placement. For this purpose, a sedative is sometimes required to restrain uncooperative animals. A combination of butorphanol and midazolam is commonly used to produce sedation and analgesia for minimally invasive diagnostic procedures in dogs.⁵² This combination was reported to have minimal effect on the cardiopulmonary system,⁵³ thus it is expected to be less likely in interfering with the CEUS interpretation. Moreover, it has a rapid onset and short duration of action that make it an ideal choice for the short-term procedure. Unfortunately, the effect of this combination on the perfusion parameters obtained from CEUS of canine intestine has not been reported. Therefore, the aim of chapter 2 was to investigate the effect of sedation with a combination of butorphanol and midazolam on the perfusion parameters derived from CEUS of duodenum in healthy dogs.

2. MATERIALS AND METHODS

2.1 Animals

Six beagle dogs (3 intact males and 3 intact females, aged 1-4 years, weighed 8.8-12 kg) were studied. The sample size was determined based on a previous report on the sedative effect of CEUS parameters in other organs.^{54,55} All dogs were healthy based on normal physical examination, blood count, and biochemistry (e.g. albumin, C-reactive protein (CRP), lipase). No dogs showed clinical signs related to GI diseases. Food was withheld for approximately 12 hrs before the experiment. All procedures in the current study were approved by the Hokkaido University Animal Care and Use Committee (Approval no. 16-0094).

Each dog underwent CEUS twice on the same day with at least 1-hr wash-out interval. Baseline CEUS was performed before sedation, whereas post-sedation CEUS was performed at approximately 15-30 min after sedative administration. The sedative drugs used in this study composed by butorphanol (Vetorphale® 5 mg/ml, Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and midazolam (Dormicum® 5 mg/ml, Astellas Pharma Inc., Tokyo, Japan). The mixture was administered intravenously at a dosage of 0.2 mg/kg and 0.1 mg/kg for butorphanol and midazolam, respectively. The sedative effect was confirmed by a decrease in blood pressure, lower jaw tone, and less response to sound and contact.^{53,56}

2.2 Ultrasonography

At first, B-mode ultrasound was performed to image duodenum in general and neither focal nor diffuse abnormalities were found in the duodenum of all dogs. CEUS

acquisition was subsequently undertaken using the same setting and technique as described in chapter 1. All CEUS was performed by one sonographer (K.N.). In addition, hemodynamic parameters including indirect blood pressure [(BP): systolic arterial pressure (SAP), diastolic arterial pressure (DAP), MAP and HR were monitored noninvasively during CEUS scanning using a patient monitor with an oscillometric BP device (BSM-5192, Nihon Kohden Co, Tokyo, Japan).

2.3 Quantitative analysis

Quantitative analysis of CEUS images was performed by using a method which has been described in detail in the first chapter.

2.4 Statistical analysis

Statistical analysis was done using statistical analysis programs (JMP pro 12.0.1, SAS Institute Inc., Cary, NC, U.S.A. and IBM SPSS Statistics V22.0, IBM Corp., Armonk, NY, U.S.A.). All parameters were evaluated for normal distribution using Shapiro-Wilk Test, and the results are expressed as the mean \pm SD. Values of all perfusion parameters before and after sedation were compared using paired student's *t* test for normally distributed values or using a non-parametric test (Wilcoxon rank-sum test) for nonnormally distributed values. Furthermore, the differences of these parameters before and after sedation were evaluated by using Bland-Altman analysis.⁴¹ Mean differences (bias), 95% CIs for bias, and limits of agreement (LoAs) were calculated. Bias before and after sedation was considered significant when the 95% CI did not contain 0. Results of the Bland-Altman analysis are summarized as least square mean (95% CI) of bias for each parameter.

The data of SAP, DAP, MAP, and HR were analyzed using mixed model for repeated measures with time (0, 5, 10, 15, 20, 25, 30 min) as fixed effect and dog as a random effect. The F test was performed to assess the effect of time on the values of repeated measures. Pairwise comparisons between times were performed by calculating least square means and using Bonferroni correction to adjust the multiple comparisons. In addition, the partial correlations between hemodynamic and perfusion parameters were analyzed. For partial correlation analysis, the values of SAP, DAP, MAP, and HR which were detected at the contrast agent injection were used. $P < 0.05$ were considered significant for all analyses.

3. RESULTS

All CEUS images obtained from 6 dogs were adequate for analysis. CEUS images before and after sedation of 1 representative dog at the AT and PI are presented (Figure 3A-D). The duodenum of this dog is similarly enhanced at PI in the awoken state and after sedative administration (Figure 3B, 3D, respectively). The TICs derived from CEUS of the representative dog are presented in Figure 4. In this representative dog, TICs before and after sedation show no different pattern.

Mean and SD of all perfusion parameters obtained from CEUS of duodenum before and after sedation are given in Table 2. Bland Altman analysis confirmed no significant difference in any of perfusion parameters before and after sedation (Figure 5A-E). The SAP was significantly lower at 25 min, while the MAP was significantly lower at 5, 10, 15, and 25 min when compared to baseline (time = 0, before sedative administration) (Figure 6A). DAP and HR did not show significant difference at any time point (Figure 6A, 4B). No significant partial correlations were found between perfusion and hemodynamic parameters.

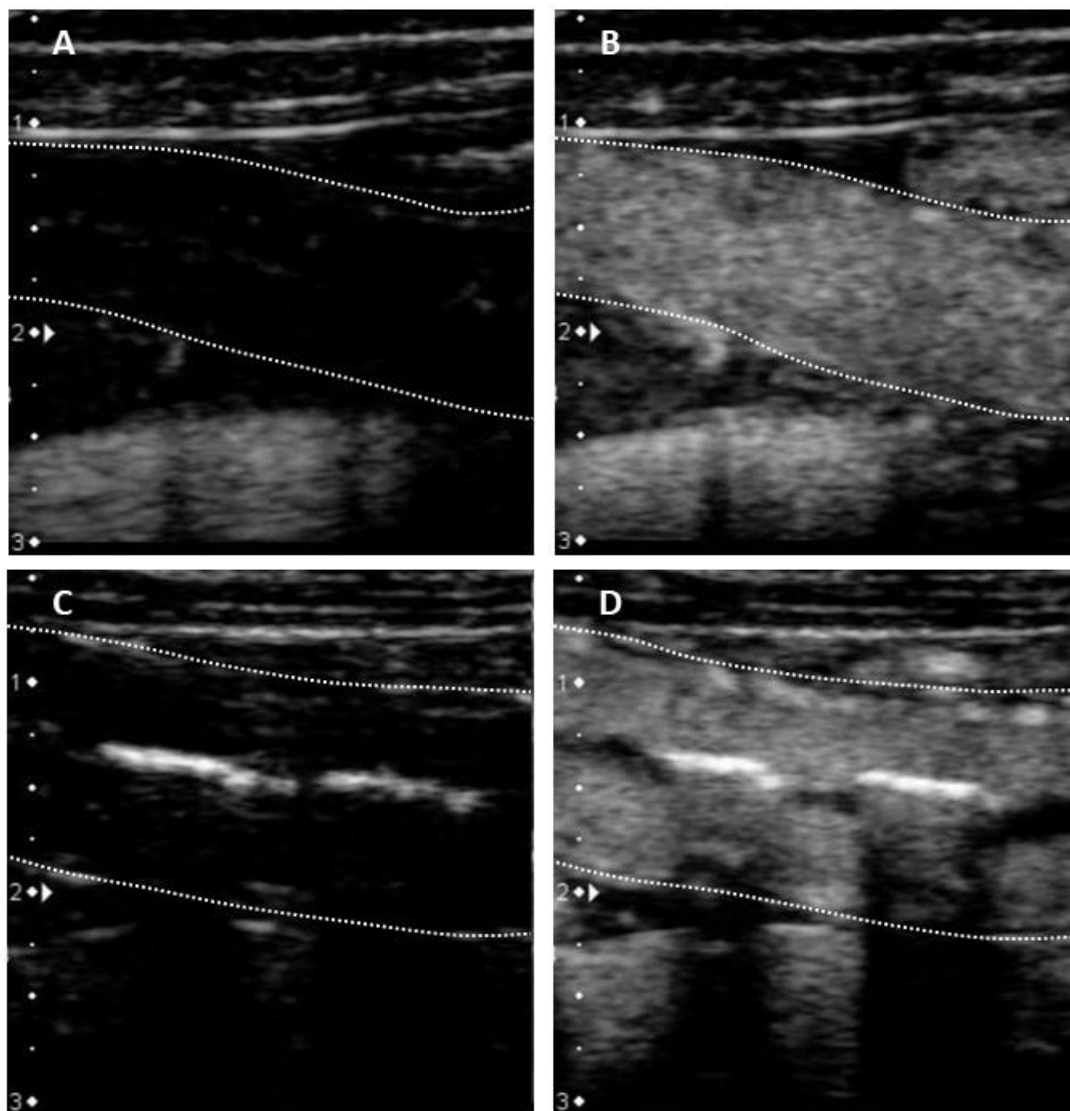


Figure 3. CEUS images of the duodenum at arrival time and peak enhancement of 1 representative dog before (A, B) and after sedation (C, D). Duodenum (dashed line) shows no enhancement at the arrival time of contrast agent in the dog before (A) and after (C) sedation. The duodenum is similarly enhanced at PI before (B) and after (D) sedation.

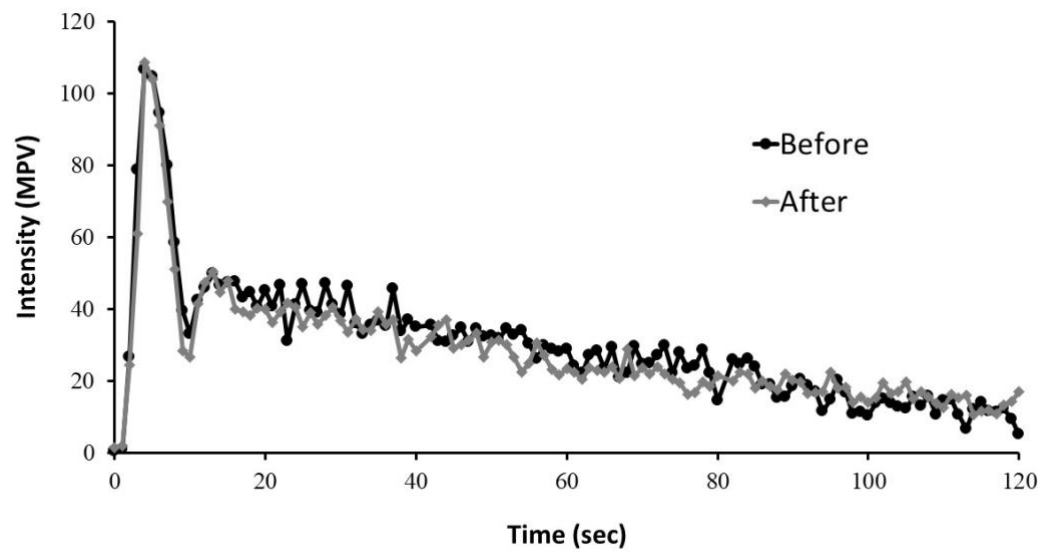


Figure 4. TICs derived from CEUS of 1 representative dog before and after sedation. In this dog, TICs before and after sedation are similar.

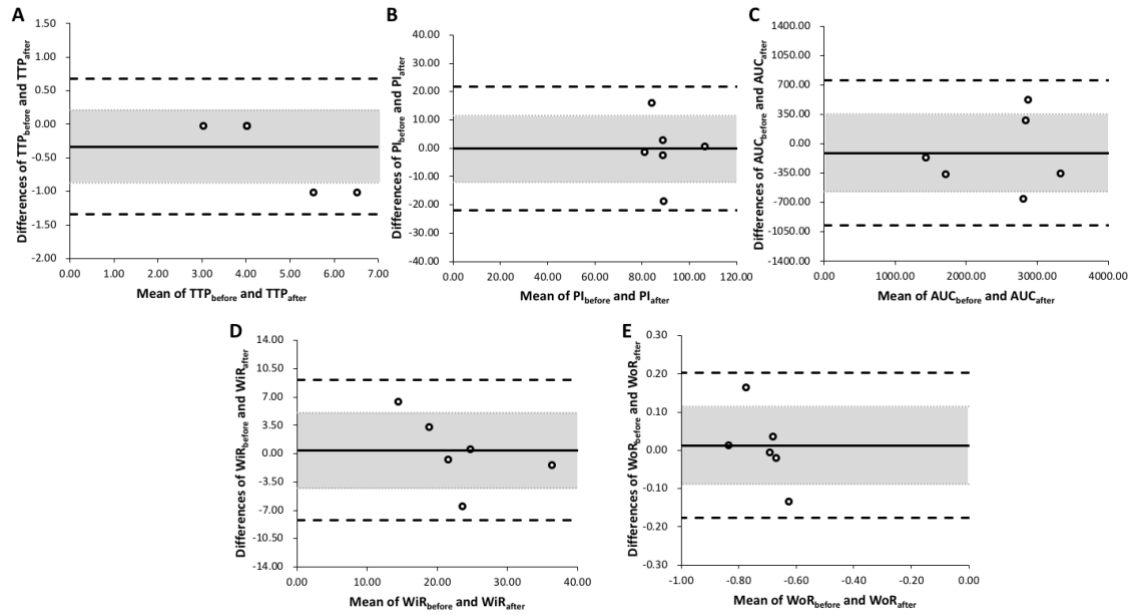


Figure 5. Bland-Altman plots of the differences among perfusion parameters before and after sedation with mean differences [(bias), continuous line], 95% CI of mean difference (shaded areas), and LoA (dashed line). (A) Bias (95% CI) between TTP_{before} and TTP_{after} was -0.33 (-0.88 to 0.21). (B) Bias (95% CI) between PI_{before} and PI_{after} was -0.12 (-11.84 to 11.56). (C) Bias (95% CI) between AUC_{before} and AUC_{after} was -108.07 (-572.53 to 356.40). (D) Bias (95% CI) between WiR_{before} and WiR_{after} was 0.44 (-4.20 to 5.07). (E) Bias (95% CI) between WoR_{before} and WoR_{after} was 0.01 (-0.09 to 0.12).

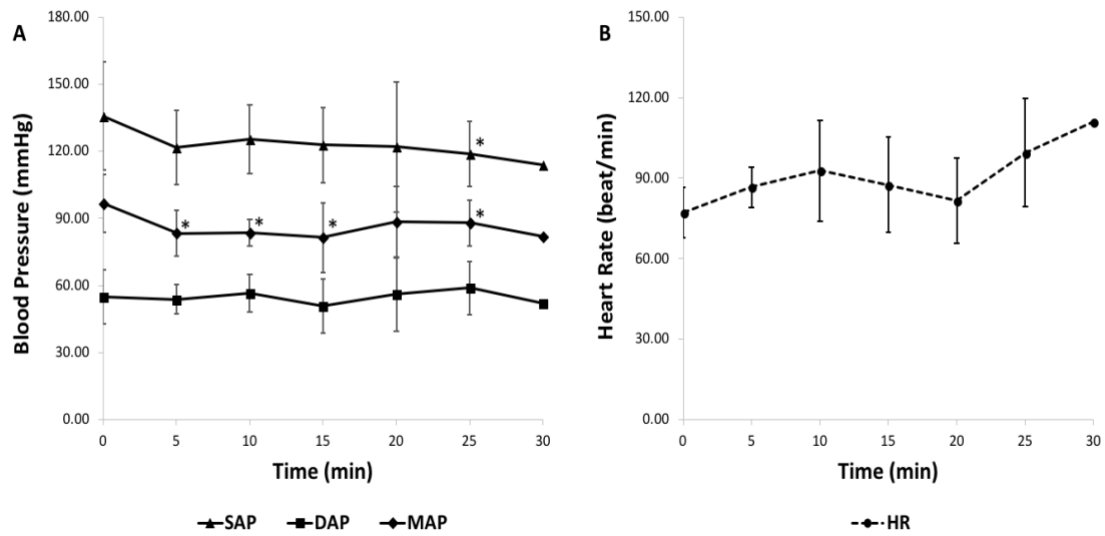


Figure 6. Blood pressure (systolic, diastolic, mean arterial pressure = SAP, DAP, MAP, respectively) (A) and HR (B) following the sedative administration of a combination of butorphanol and midazolam. The SAP is significantly lower at 25 min, while the MAP is significantly lower at 5, 10, 15, and 25 min when compared to baseline (time = 0, before sedative administration). DAP and HR do not show significant difference at any time point.

Table 2

Mean and standard deviation of CEUS-derived perfusion parameters of duodenum before and after sedation

Variable	Before Sedation	After Sedation
TTP (sec)	4.67 ± 1.51	4.33 ± 1.03
PI (MPV)	89.28 ± 11.07	89.15 ± 9.88
AUC (MPV.sec)	2536.18 ± 747.43	2428.11 ± 814.14
WiR (MPV/sec)	22.89 ± 8.82	23.32 ± 6.47
WoR (MPV/sec)	(-)0.72 ± 0.11	(-)0.71 ± 0.06

Data are expressed as mean±SD (n = 6). TTP = time-to-peak, PI = peak intensity, AUC = area under curve, WiR = wash-in rate, WoR = wash-out rate, MPV = mean pixel value.

4. DISCUSSION

In chapter 2, CEUS-derived perfusion parameters of duodenum in healthy dogs including TTP, PI, AUC, WiR, and WoR were evaluated before and after sedation using a combination of butorphanol and midazolam. No significant changes were observed in these parameters. This finding indicated that the combination could be a good option for sedation prior to CEUS of duodenum in uncooperative dogs.

Butorphanol produces an analgesic effect by its action at κ and μ receptors of central nervous system,⁵⁷ whereas midazolam interacts with gamma-aminobutyric acid (GABA) receptor to produce muscle relaxation.⁵² The combination of butorphanol and midazolam was reported to promote light to moderate sedation with small changes in cardiopulmonary functions.⁵³ Moreover, their rapid onset and short duration of action make them a safe and ideal option for a short-term procedure like CEUS. For this reason, the combination was selected for current study with the expectation that it would have less influence on the duodenal perfusion parameters.

The effect of butorphanol-midazolam on CEUS parameters of the canine duodenum was first examined in the current study. The use of butorphanol as a single sedative agent was suggested to cause neither significant change on the CEUS parameters of canine spleen nor those of feline kidney, that make this agent was selectable to be utilized during CEUS scanning of both organs.^{54,55} Butorphanol-midazolam, however, was reported to provoke a reduction of intrarenal blood flow in healthy dogs by Doppler ultrasound.⁵⁸ The latter finding was in contrast to my result, but the direct comparison could not be made. It was not only because duodenum and kidney may respond differently to this

combination, but also due to the difference in dosage. The dosage of midazolam used in the study above was twice of the dosage I injected to dogs in current study. In my study, midazolam was administered at a dosage of 0.1 mg/kg which was reported to produce adequate sedation in healthy beagles when combined with 0.2 mg/kg of butorphanol.⁵⁶

Autoregulation of intestinal blood supply might play a role to maintain duodenal perfusion, despite the reduction of systemic blood pressure which more likely caused by midazolam as it depresses the sympathetic nerve activity and causes a decrease in systemic vascular resistance.^{59,60} The autoregulation occurs to preserve nutrient and oxygen supply toward the absorption sites.⁶¹ In my study, ROI was placed in the mucosa which was found to have more potent local circulatory control mechanism than muscularis layer.⁶² A previous study reported that blood flow and blood volume remain unchanged in the intestinal mucosa of hypovolemic dogs, even though a decrease in the superior mesenteric artery (SMA) flow was detected together with an altered cardiac output and MAP.⁶³ Reduced blood flow towards splanchnic organs including intestine was suspected in dogs after administration of midazolam as a single anesthetic agent,⁶⁴ whereas butorphanol was suggested to cause a decrease in the intestinal blood flow of halothane-anesthetized ponies.⁶⁵ There was neither previous report explaining how butorphanol-midazolam in a sedative dosage affect canine splanchnic blood flow nor the blood distribution within intestinal wall, but considering the result of the previous studies, butorphanol-midazolam might cause changes in the SMA flow that induced autoregulation in the duodenum of dogs in current study. Apart from these mechanisms, very small changes of duodenal perfusion which could only be detected by a highly accurate technique such as radiolabel tracking might only cause a negligible effect on the CEUS-derived perfusion parameters.

This study had a number of limitations. First, this study only evaluated the combination of butorphanol and midazolam. The sedative or anesthetic agent which have a large influence on cardiovascular system was not examined as a positive control, so the effect of cardiovascular changes induced by such agents on CEUS-derived perfusion parameters of duodenum is unknown. In a previous study, dexmedetomidine was reported to cause prolonged arrival time of contrast agent and time to peak enhancement, as well as a lower wash-in rate on CEUS evaluation of canine small intestine.⁶⁶ Dexmedetomidine causes peripheral vasoconstriction resulting in transitory hypertension and bradycardia at the initial phase after administration and followed by lower cardiac output and reduction in perfusion of peripheral organs.⁶⁷ Second, healthy and relatively young dogs were subjected in my study. In clinical practice, however, dogs with chronic intestinal diseases such as steroid-responsive enteropathy and intestinal lymphoma are mostly middle-aged dogs.^{2,68,69} Butorphanol-midazolam was reported to cause mild to moderate effect in healthy and young dogs but may cause a deeper effect in sick and old animals.^{53,58} Therefore, this combination must be used with caution in a clinical setting. Moreover, the inter-sonographer variability was not examined in the current study.

From the results of current study, it can be concluded that a combination of butorphanol and midazolam did not cause significant changes in CEUS-derived perfusion parameters of duodenum in healthy dogs. This finding indicated that the combination could be a good option for sedation prior to CEUS of duodenum in uncooperative dogs.

5. SUMMARY

In this chapter, I investigated the effect of sedation with the combination of butorphanol and midazolam on the CEUS-derived perfusion parameters of duodenum in healthy dogs including TTP, PI, AUC, WiR, and WoR. No significant changes were observed in these parameters, even though significant differences were detected in hemodynamic parameters (SAP and MAP) before and after sedation. This finding indicates that the combination could be a good option for sedation prior to CEUS of duodenum in uncooperative dogs.

CHAPTER 3

THE EVALUATION OF DUODENAL PERFUSION BY CONTRAST- ENHANCED ULTRASONOGRAPHY IN DOGS WITH CHRONIC ENTEROPATHY AND INTESTINAL LYMPHOMA

1. INTRODUCTION

Diagnosis of CE in dogs requires histopathological evaluation of intestinal biopsies to confirm the presence of inflammation and exclude intestinal lymphoma.⁷ Unfortunately, intestinal biopsies have to be postponed in dogs with debilitated conditions due to anesthetic risk often associated with hypoalbuminemia. Furthermore, evaluation of CE is paramount in determining disease severity and therapeutic response as dogs with CE are currently managed by a sequence of treatment trials involving dietary change, antibiotics, and anti-inflammatory or immunosuppressive agents. Modalities which are currently utilized to guide the treatment plan such as clinical scoring, clinicopathological findings, and B-mode ultrasound are either non-specific for intestine or lack of correlation with therapeutic response.^{11–15} Endoscopy together with histopathology are a gold standard to assess the intestinal inflammatory activity, but are relatively invasive and cumbersome for repeated evaluation. Due to these reasons, there remains a need for an alternative modality for the diagnosis and evaluation of CE in dogs.

In human medicine, changes in the post-contrast enhancement pattern and CEUS-derived perfusion parameters of the intestine in patients with IBD were documented and showed good correlation with endoscopic and histopathological features.^{19,70} As CE in dogs has been known to share some common pathogenesis with human IBD,^{3,31} I hypothesized that changes of intestinal perfusion as assessed by CEUS can be useful in the diagnosis and evaluation of CE in dogs.

Previous chapters have shown that CEUS were feasible to assess duodenal perfusion in healthy dogs. Further studies are warranted to investigate the clinical applicability of CEUS in the diagnosis and evaluation of dogs with chronic intestinal

diseases. Therefore, the study of chapter 3 aimed to (1) determine presence of changes in the duodenal enhancement pattern and perfusion parameters in dogs with CE and intestinal lymphoma when compared to control, (2) evaluate differences of duodenal enhancement pattern and perfusion parameters between dogs with CE and intestinal lymphoma, and (3) examine the correlation between perfusion parameters and clinicopathological findings, clinical score, and histopathological findings in CE dogs.

2. MATERIALS AND METHODS

2.1 Animals

This chapter was designed as a cross-sectional study. Dogs presented to Hokkaido University Veterinary Teaching Hospital between September 2013 and November 2017 with a history of chronic persistent or recurrent GI signs (≥ 3 weeks) were prospectively enrolled. Only dogs with histopathologic evaluation of the duodenum were included. Dogs with histopathologic diagnosis of lymphoplasmacytic or eosinophilic duodenitis were included in CE group,⁷ while those with an infiltration of neoplastic lymphoid cells in the duodenum were included in intestinal lymphoma group.⁶⁹ In addition, client-owned dogs underwent gastroduodenoscopy or laparotomy for GI signs due to diseases other than CE and intestinal lymphoma (e.g. foreign body, gastric diseases) without histopathological lesion in the duodenum were recruited as control. All procedures in this study were approved by Hokkaido University Animal Care and Use committee, and the informed consent was obtained from all owners of dogs involved in this study.

2.2 Ultrasonography

Food was withheld for a minimum of 6 hrs prior to duodenal imaging. At first, the duodenum was imaged using B-mode ultrasound to assess the wall thickness, layering, echogenicity, presence of corrugation, as well as focal or segmental lesion.^{4,71-73} Normal duodenal wall thickness was determined to be ≤ 5.1 mm for dogs < 20 kg, ≤ 5.3 mm for dogs 20-29.9 kg, and ≤ 6 mm for dogs > 30 kg.⁷² Mild thickening was defined as up to 8 mm, moderate thickening as 8-20 mm, and severe thickening as > 20 mm.⁴ Duodenal layering was categorized as normal (all layers were identified and within normal limits),

present but altered (all layers could be discriminated but the relative thickness of one or more layers was abnormal), or effaced (layers were not visible).^{4,74} Echogenicity of duodenal mucosa was assessed as normal, predominantly hypoechoic, or predominantly hyperechoic;⁴ also the presence of hyperechoic mucosal striations.⁷⁴

For CEUS scanning, all dogs were either imaged with and without sedation using a combination of butorphanol (Vetorphale® 5 mg/ml, Meiji Seika Pharma Co, Ltd, Tokyo, Japan) and midazolam (Dormicum® 5 mg/ml, Astellas Pharma Inc, Tokyo, Japan) at a dosage of 0.2 mg/kg and 0.1 mg/kg for butorphanol and midazolam respectively. The technique and setting for CEUS have been mentioned in detail in the first and second chapters.

2.3 Quantitative analysis

The quantitative analysis of CEUS images was performed using the same method as mentioned in the first and second chapters.

2.4 Clinicopathological marker, clinical score, and histopathological score

Clinicopathological markers including plasma albumin level and CRP concentration were evaluated at the time of CEUS. Furthermore, clinical score was determined by the attending clinician based on the canine chronic enteropathy clinical activity index (CCECAI).² On the basis of total CCECAI score, dogs in CE group were further classified into remission (CCECAI 0-3) and symptomatic (CCECAI > 3). I determined 3 as a cut-off value because total CCECAI score of 0-3 was categorized as insignificant disease.² In addition, a single board-certified pathologist evaluated and assigned histopathological score of duodenum for dogs with CE based on standards

established by the world small animal veterinary association (WSAVA) GI standardization group.⁷

2.5 Statistical analysis

Statistical analysis was performed using a statistical analysis software (JMP pro 12.0.1, SAS Institute Inc., Cary, NC, U.S.A.). All data were evaluated for normal distribution using Shapiro-Wilk test and presented as median and range. CEUS parameters of control, remission CE, symptomatic CE, and intestinal lymphoma groups were tested using one-way ANOVA followed by post-hoc Tukey-Kramer test, or Wilcoxon/Kruskal-Wallis test followed by post-hoc Steel-Dwass test. Correlations between perfusion parameters and albumin, CRP, CCECAI and WSAVA score of dogs in CE group were analyzed using Spearman's correlation coefficient. Statistical significances were defined as $p < 0.05$.

3. RESULTS

3.1 Animals

A total of 33 dogs were included in CE (n = 26) and intestinal lymphoma (n = 7) groups. Dogs in CE group were classified as remission CE (n = 16) and symptomatic CE (n = 10). Dogs in the intestinal lymphoma group were diagnosed based on histopathological evaluation of duodenum obtained from gastroduodenoscopy (n = 6) and laparotomy (n = 1). In addition, 14 dogs with GI signs but normal duodenal histopathological findings were recruited as control. Signalment, albumin, CRP, CCECAI, and WSAVA score of all dogs are summarized in Table 3. Age and body weight of dogs from all groups were not significantly different. Twenty (43%) of all dogs were sedated before CEUS, while the other 27 dogs (57%) underwent CEUS with manual restraint.

3.2 B-mode ultrasound findings

B-mode ultrasound findings of all dogs are summarized in Table 4. All dogs in remission CE group showed normal wall thickness and layering. Nine dogs in symptomatic CE group showed normal wall thickness, 1 dog had mild thickening, but all dogs showed normal layering. Four dogs with lymphoma exhibited normal wall thickness; 3 with normal layering, while 1 showed thickened muscularis layer. The remaining 3 dogs with lymphoma exhibited mild thickening; 1 with normal layering, and another 2 with thickened muscularis wall. Corrugation was observed in 1 dog from the remission CE group, 1 dog from the symptomatic CE, and 5 dogs from the intestinal lymphoma group.

Changes in duodenal echogenicity were also observed in majority of dogs with CE and intestinal lymphoma groups. Predominantly hypoechoic duodenal mucosa was

captured in 7, 1, and 4 dogs of remission CE, symptomatic CE, and intestinal lymphoma groups, respectively. While predominantly hyperechoic mucosa was observed in another 5, 9, and 3 dogs with remission CE, symptomatic CE, and intestinal lymphoma groups, respectively. Hyperechoic mucosal striations were found in 5, 6, and 2 dogs with remission CE, symptomatic CE, and intestinal lymphoma, respectively.

3.3 CEUS findings

CEUS images from all dogs were adequate for analysis. The enhancement pattern of the duodenum after contrast injection was subjectively similar between dogs in the CE and intestinal lymphoma groups when compared to control. Enhancement of the duodenum started from perivisceral vessels, subsequently continued toward the mucosa and include all layers of the duodenum (Figure 7C, 7F, 7I, 7L). The muscularis layer was subjectively less enhanced when compared to mucosa. The enhancement of the submucosa and serosa not included into analysis since these layers are thin and inherently hyperechoic on the ultrasound. Among the representative dogs of each group, the maximum enhancement in the representative dogs of symptomatic CE and intestinal lymphoma groups are subjectively more prominent in comparison to representative dogs of control and remission CE groups (Figure 7C, 7F, 7I, 7L).

For quantitative analysis, 4 ROIs could be drawn in the duodenal mucosa of 28 dogs. Three ROIs could be drawn in 6 dogs, 2 ROIs in 9 dogs, and only 1 ROI in 4 dogs. TIC created from averaged MPV data of each group showed similar pattern with rapid wash-in and gradual wash-out. TIC of symptomatic CE and intestinal lymphoma groups show higher peak when compared to remission CE and control groups (Figure 8). All perfusion parameters derived from TIC were summarized in Table 5. PI was significantly

higher in symptomatic CE compared to control (Table 5, Figure 9B, $p = 0.048$). AUC was significantly higher in symptomatic CE when compared to control and remission CE (Table 5, Figure 9C, $p = 0.0094$, $p = 0.034$, respectively). A positive correlation was detected between CCECAI score and PI (Figure 10, $\rho = 0.55$, $p = 0.0033$), but not with the other perfusion parameters (TTP, AUC, WiR, and WoR). No significant differences in perfusion parameters were detected between intestinal lymphoma and symptomatic CE, remission CE, nor control groups. No significant correlations were found between perfusion parameters and albumin, CRP, nor WSAVA score.

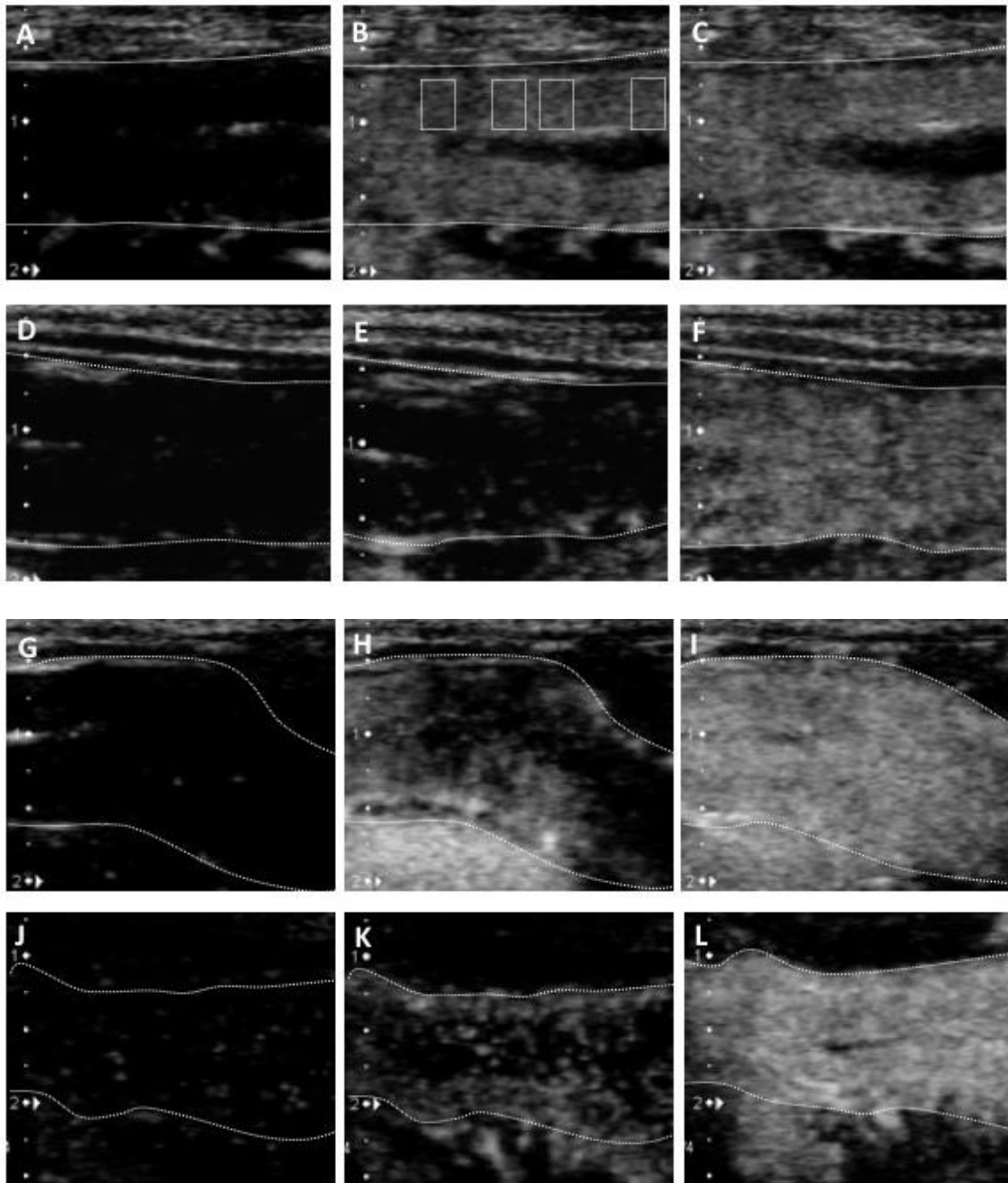


Figure 7. Representative sequential images of the duodenum (dashed line) after contrast injection in dogs of control (A-C), remission CE (D-F), symptomatic CE (G-I), and intestinal lymphoma (J-L) groups. Duodenum of all representative dogs just before the arrival time (A, D, G, J). Duodenum of all representative dogs soon after contrast injection (B, E, H, K). Duodenum of all representative dogs during maximum enhancement (C, F, I, L).

I, L). Four ROIs were drawn in the duodenal mucosa for quantitative analysis (B).

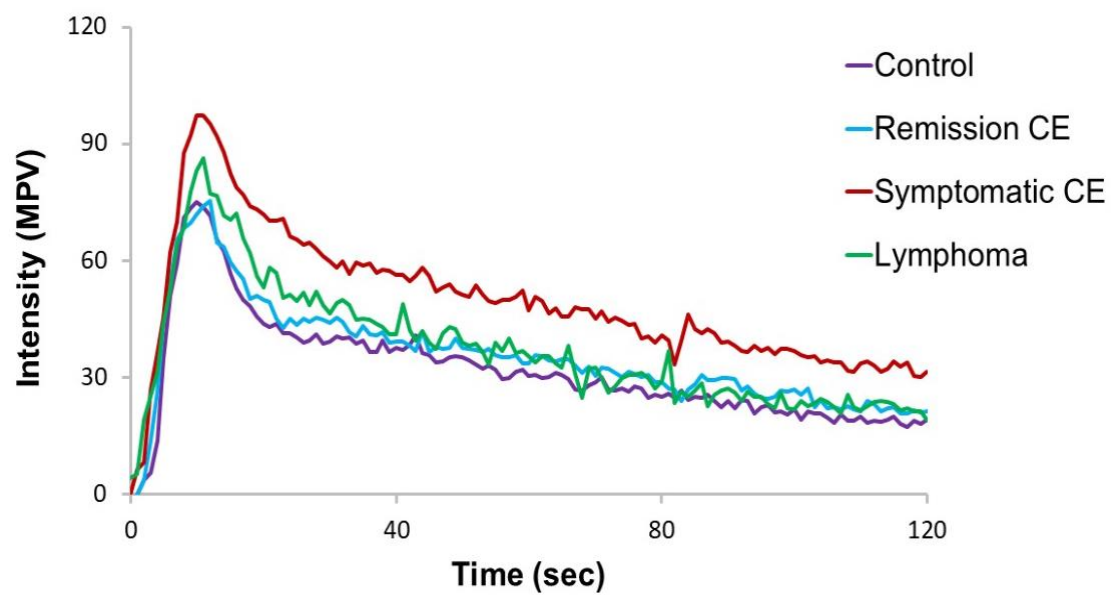


Figure 8. The averaged TICs of control (n = 14), remission CE (n = 16), symptomatic CE (n = 10), and intestinal lymphoma (n = 7).

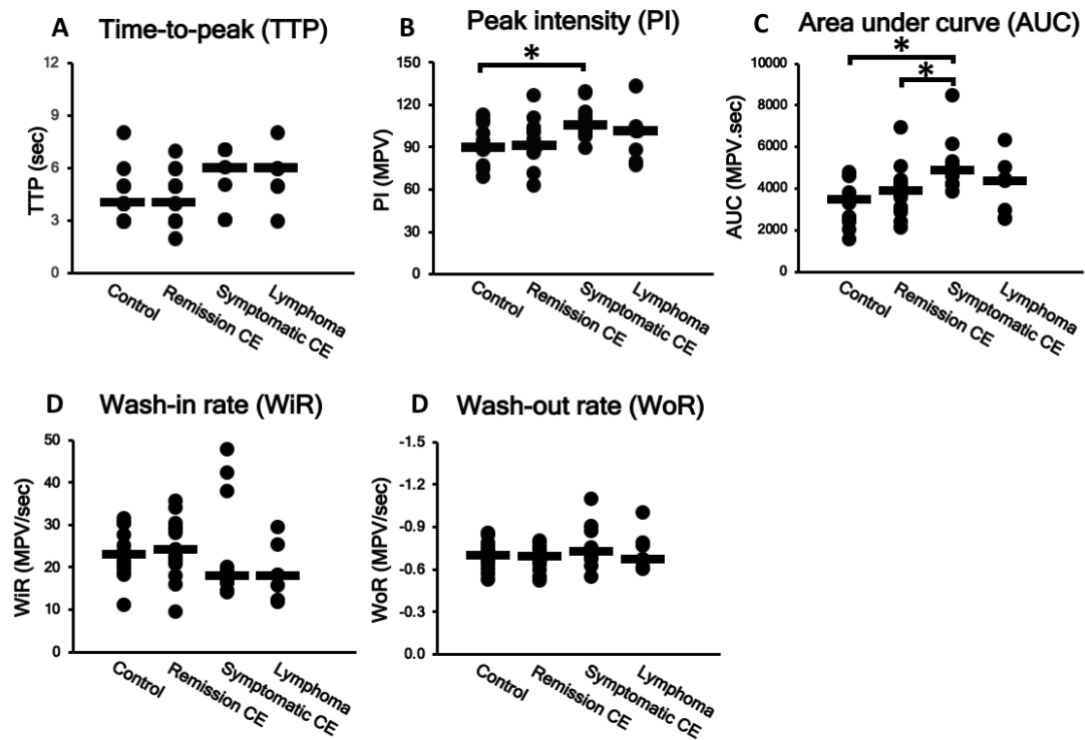


Figure 9. Scatter plot of perfusion parameters of control (n = 14), remission CE (n = 16), symptomatic CE (n = 10), and intestinal lymphoma groups (n = 7): (A) time-to-peak (TTP), (B) peak intensity (PI), (C) area under the curve (AUC), (D) wash-in rate (WiR), (E) wash-out rate (WoR). The floated bar represent median. Asterisk (*) indicates significant difference among groups (p < 0.05).

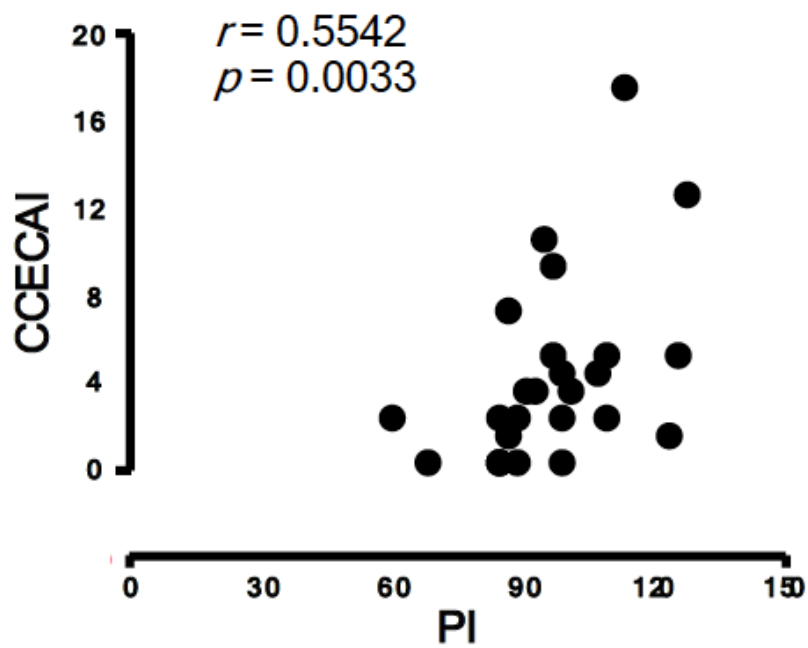


Figure 10. Correlation between peak intensity (PI) and canine chronic enteropathy clinical activity index (CCECAI). Spearman's rho (ρ) and p value are indicated.

Table 3. Signalments, laboratory markers, clinical score, and histopathological score of control, chronic enteropathy, and intestinal lymphoma groups

Variable	Control (n = 14)	Chronic Enteropathy		Intestinal Lymphoma (n = 7)	Overall P value [§]
		Remission (n = 16)	Symptomatic (n = 10)		
Signalment					
Age (years old) [†]	8.0 (2.0-14.0)	7.0 (5.0-12.0)	9.0 (7.0-13.0)	10.0 (7.0-12.0)	0.2685(K)
Body weight (kg) [†]	4.0(1.7-16.1)	4.2 (1.7-11.5)	3.9 (2.2-8.2)	5.7 (4.0-9.7)	0.0211(K)
Sex	2 M, 5 CM, 7 SF	2 M, 2 F, 7 CM, 5 SF	1 M, 2 F, 4 CM, 3 SF	1 M, 4 CM, 2 SF	NE
Breed	Chihuahua (4)	Chihuahua (3)	Miniature Dachshund (5)	Miniature Dachshund (3)	NE
	Miniature Dachshund (4)	Boston Terrier (2)	Boston Terrier (2)	French Bulldog (1)	
	Pomeranian (2)	Yorkshire Terrier (2)	Chihuahua (1)	Jack Russel Terrier (1)	
	Toy Poodle (2)	Italian Greyhound (2)	Welsh Corgi (1)	Pug (1)	
	Miniature Pinscher (1)	Japanese Spitz (1)	Yorkshire Terrier (1)	Shiba dog (1)	
	Mix (1)	Miniature Dachshund (1)			
		Miniature Schnauzer (1)			
		Papillon (1)			
	Shih Tzu (1)				
		Welsh Corgi (1)			
Laboratory marker					
Albumin [†] (RI: 2.6-4.0 µg/dL)	2.6 (1.6-5.1)	2.7 (1.3-3.7)	1.7 (1.2-3.8)	1.9 (1.4-2.7)	0.0414(A)
CRP ^{†,‡} (RI: 0-1 mg/dL)	0.2 (0.0-12.0)	0.1 (0.0-1.8)	2.0 (0.0-20.0)	1.9(0.3-4.2)	0.0465(K)
Clinical score					
CCECAI ^{†,¶}	3.0 (0.0-15.0) ^{ab}	1.5 (0.0-3.0) ^a	6.0 (4.0-17.0) ^b	10.0 (8.0-18.0) ^b	<0.0001(K)
Histopathological score					
WSAVA [†]	NE	3.5 (1.0-7.0)	4.5 (1.0-7.0)	NE	0.1830(T)

M = Male, F = Female, CM = Castrated male, SF = Spayed female, NE = Not examined, RI = Reference interval, CRP = C-reactive protein,

CCECAI = Canine Chronic Enteropathy Clinical Activity Index, WSAVA = World Small Animal Veterinary Association

[†]Values are presented as median (range)

[§]Based on One-way ANOVA (A), Wilcoxon/Kruskal-Wallis (K), or student's *t* test (T)

[¶]Values with different superscript letters indicate significant differences among groups based on post-hoc analysis (Tukey-Kramer or Steel-

Dwass

Table 4. B-Mode ultrasound findings of duodenum in control, chronic enteropathy, and intestinal lymphoma dogs

Variable	Control (n = 14)	Chronic Enteropathy		Intestinal Lymphoma (n = 7)
		Remission (n = 16)	Symptomatic (n = 10)	
Wall thickness				
Normal	14	16	9	4
Mild	0	0	1	3
Moderate, severe thickening	0	0	0	0
Wall layering				
Normal	12	16	10	4
Present but altered	2	0	0	3
Effaced	0	0	0	0
Echogenicity of mucosa				
Normal	11	4	0	0
Predominantly hypoechoic	2	7	1	4
Predominantly hyperechoic	1	5	9	3
Striation	1	5	6	2
Corrugation				
Presence	2	1	1	5
Absence	12	15	9	2
Focal or segmental lesion				
Presence	0	0	0	0
Absence	14	16	10	7

Table 5. Perfusion parameters of control, chronic enteropathy, and intestinal lymphoma groups

Variable	Control (n = 14)	Chronic Enteropathy		Intestinal Lymphoma (n = 7)	Overall P value‡
		Remission (n = 16)	Symptomatic (n = 10)		
TTP (sec) [†]	4.0(3.0-8.0)	4.0(2.0-7.0)	6.0(3.0-7.0)	6.0(3.0-8.0)	0.1049(K)
PI (MPV) [†]	89.9(68.5-112.2) ^a	90.9(61.8-125.9) ^{ab}	105.4(89.3-128.8) ^b	100.5(76.7-132.4) ^{ab}	0.0426(A)
AUC (MPV.sec) ^{†,§}	3448.9 (1559.5-4736.9) ^a	3862.3 (2094.5-6899.0) ^a	4847.9 (3824.3-8462.8) ^b	4343.7(2526.8-6237.0) ^{ab}	0.0124(K)
WiR (MPV/sec) [†]	23.0(10.8-31.4)	24.0(9.3-35.5)	17.8(13.8-47.7)	17.9(11.7-29.4)	0.2285(K)
WoR (MPV/sec) [†]	(-)0.7(0.5-0.9)	(-)0.7(0.5-0.8)	(-)0.7(0.5-1.1)	(-)0.7(0.6-1.0)	0.5304(K)

TTP = time to peak, PI = peak intensity, AUC = area under the curve, WiR = wash-in rate, WoR = wash-out rate, MPV = mean pixel

value

[†]Values are presented as median (range)

[‡]Based on One-way ANOVA (A) or Wilcoxon/Kruskal-Wallis (K)

[§]Values with different superscript letters indicate significant differences among groups based on post-hoc analysis (Tukey-Kramer or Steel-Dwass)

4. DISCUSSION

In my study, CEUS-derived parameters, PI and AUC which represent regional blood volume of duodenal mucosa were significantly higher in dogs with symptomatic CE compared to control. This finding suggests that PI and AUC could differentiate chronic inflamed duodenum in symptomatic CE dogs from normal duodenum and might be useful for the diagnosis and evaluation of CE in dogs. However, CEUS-derived parameters were not different between dogs with CE and intestinal lymphoma groups which precludes the use of these parameters to differentiate both diseases.

Subjective observation of duodenal contrast enhancement pattern after contrast injection showed no obvious differences in dogs with remission CE, symptomatic CE and intestinal lymphoma dogs when compared to control dogs. The duodenal enhancement depicted in all of dogs was in accordance to the physiology of intestinal blood flow where the blood being carried through small branches of splanchnic arteries that penetrate the surface and muscular coat of duodenum toward an extensive submucosal network of small arteries, subsequently pass through mucosal arterioles network into the microvascular bed of mucosa.⁷⁵ My result is in contrast with people with IBD where post-contrast enhancement pattern differed among patients with symptomatic and remission status of the disease.^{19,70} Main affected intestinal layer in human IBD prone to be submucosa that result in prominent enhancement of the corresponding layer in patients with symptomatic status. Whereas patients with remission status showed complete inward enhancement or low even absence of enhancement due to progressive fibrosis and reduction of mural vascularization.⁷⁰ In addition, the distribution of blood supply among

layers did not change as all dogs showed less contrast enhancement in the muscularis layer when compared to mucosa. The muscularis layer receives less blood supply due to less metabolic demand.⁶¹

PI and AUC are CEUS-derived parameters representing regional blood volume within the certain ROI. PI represents the maximum volume of blood filling in the vessels within the ROI, while AUC represents the summation of blood volume within the ROI during the period of analysis. My findings of increased duodenal PI and AUC in dogs with symptomatic CE correspond to an increase in blood supply with perpetuating chronic inflammation of the duodenum. Studies in human and mice reported that an increase of inflammatory cells influx as well as nutrient supply toward a chronic inflamed tissue were suggested to induce proliferation of endothelial cells and capillary remodeling, which result in an expansion of microvascular bed in the corresponding tissue.^{30,76,77}

The increase in AUC might also be due to a prolonged enhancement of duodenal mucosa which was resulted from retained MBs within tortuous microvasculature in the duodenal mucosa. In people with celiac disease, chronic inflammation of intestinal mucosa causes replacement normal capillary architecture by a network of microvasculature with increased tortuosity and arteriovenous shunts.^{78,79} The microvascular tortuosity in related to chronic inflammation was also discussed in a CEUS study of canine pancreatitis.⁸⁰

Although PI and AUC of dogs in the intestinal lymphoma group were expected to be increased when compared to control, but no significant differences were observed in this study (Figure 4B-C). This could be due to the small number of dogs with intestinal lymphoma in my study. An increase of blood supply toward cancerous growth has been considered as a result of angiogenesis that support the survival and proliferation of

cancers.⁸¹ An inhomogeneous hyperintensity was documented among various enhancement pattern of intestinal lymphoma in human as assessed by contrast-enhanced computed tomography.^{82–84} Meanwhile, mild to moderate enhancement was a common feature in the contrast-enhanced magnetic resonance imaging of intestinal lymphoma.^{85–87} These reports suggest hypervascularization of intestinal lymphoma in people, however, it was not observed in all cases. It is unclear if dogs with intestinal lymphoma undergo a similar angiogenesis process, but an increase of microvascular density was reported in the lymph nodes of dogs with nodal lymphoma.⁸⁸

There was no significant difference in any of perfusion parameters between CE and intestinal lymphoma groups. This result indicates that a change of intestinal perfusion may not facilitate the differentiation between chronic inflammation and neoplastic infiltration of the intestine. Nevertheless, it might also be due to limitations of the evaluated parameters in my study that could not detect different pattern of vascularization occur in different pathological condition of the intestine. From this finding, CEUS-derived perfusion parameters might differentiate chronic inflammation from normal duodenum but could not distinguish inflammation and neoplasia.

CCECAI score was significantly correlated with PI, but not with the other perfusion parameters. The correlation indicated that PI could be useful to evaluate severity of CE in dogs. However, in my study, CEUS evaluation was limited to duodenal segment. Whereas laboratory markers (i.e. albumin, CRP) and CCECAI score could be influenced by pathological condition in other parts of the GI tract. This might contribute to the lack of correlation between other perfusion parameters and CCECAI. Moreover, CEUS-derived perfusion parameters did not seem directly correlated with the degree of morphological change and inflammatory cells infiltration in the duodenum examined by

WSAVA scoring system.⁷ Further analysis on the microvascular architecture of the duodenal biopsy specimens is warranted to confirm this finding.

Some limitations are present in my study. The possibility that some of the control dogs suffering from CE cannot be completely ruled out because these controls were enrolled based on the presence of GI signs with absence of histopathological abnormalities in the duodenum. Another limitation was the use of sedation during CEUS scanning in almost half of dogs currently involved. According to results of chapter 2, sedation using a combination of butorphanol and midazolam did not influence CEUS-derived perfusion parameters of duodenum.⁸⁹ Duodenal perfusion could be maintained despite the reduction of systemic blood pressure after administration of butorphanol-midazolam possibly due to intestinal autoregulation system.⁸⁹ It remains possible that diseased dog responded differently to sedation when compared to healthy dogs. However, this cannot be confirmed since hemodynamic parameters (i.e. cardiac output, HR, BP) were not continuously recorded during CEUS scanning.

In conclusion, CEUS-derived perfusion parameters, especially PI and AUC, could detect changes of duodenal perfusion in dogs associated with CE. These parameters are useful for the diagnosis and evaluation of CE in dogs.

5. SUMMARY

In this chapter, I determined that duodenal enhancement pattern as assessed by CEUS was subjectively not different between remission CE, symptomatic CE, intestinal lymphoma, and control dogs. I clarified that CEUS-derived parameters; PI and AUC, which represent the regional blood volume of duodenal mucosa were significantly higher in dogs with symptomatic CE in comparison to control. AUC was also significantly higher in symptomatic CE compared to remission CE. In addition, there was a significant correlation between PI and CCECAI score. These findings suggest that PI and AUC could distinguish chronic inflammation from normal duodenum and may be useful for the diagnosis and evaluation of CE in dogs. However, CEUS-derived parameters were not different between dogs with CE and intestinal lymphoma groups which precludes the use of these parameters to differentiate both diseases.

GENERAL CONCLUSION

The aim of my study was to establish the clinical usefulness of CEUS in the assessment of canine duodenal perfusion. Findings in my current study indicated that perfusion parameters derived from quantitative analysis of CEUS images, PI and AUC, could detect changes of duodenal perfusion in associated with chronic inflammation. This finding suggests that these parameters provide potentially useful information for the diagnosis and evaluation of CE in dogs.

In first chapter, I clarified that the repeatability and reproducibility of CEUS-derived perfusion parameters which include TTP, PI, AUC, WiR, and WoR were clinically acceptable in assessing duodenal perfusion of healthy sedated dogs. The intraday, interday, and intraobserver CVs for all perfusion parameters mentioned above being less than 25%.

In second chapter, I confirmed that TTP, PI, AUC, WiR, and WoR derived from CEUS of duodenum in healthy dogs before and after sedation using a combination of butorphanol and midazolam were not significantly different. This finding indicated that the combination could be a good option as sedative drugs to maintain dog cooperation during CEUS of duodenum.

In third chapter, I identified that CEUS-derived parameters especially PI and AUC, which represent the regional blood volume of duodenal mucosa, being significantly higher in dogs with symptomatic CE compared to control. I also detected a significant correlation between PI and CCECAI score. This finding suggests that PI and AUC could distinguish actively chronic inflammation from normal duodenum and could be useful for the diagnosis and evaluation of CE in dogs. However, CEUS-derived parameters were

not different between dogs with CE and intestinal lymphoma.

Further study to confirm the usefulness of CEUS in the monitoring of dogs with CE is warranted. Longitudinal study by performing CEUS before and after treatment may detect changes of duodenal perfusion in associated with therapeutic response in dogs with CE. Moreover, the evaluation of other parameters (i.e. heterogeneity of enhancement) is suggested as the CEUS parameters in my study could not discriminate CE and intestinal lymphoma. Furthermore, the evaluation of microvascular structure by histopathology in CE dogs may support the hypothesis of microvascular reconstruction and further understand the mechanism of prolonged enhancement in chronic inflamed intestine associated with CE.

In conclusion, from this study I could establish the quantitative CEUS method to assess duodenal perfusion in dogs. This modality is repeatable and reproducible in healthy sedated dogs and not influenced by sedation using a combination of butorphanol and midazolam. The perfusion parameters derived from CEUS especially PI and AUC could discriminate chronic inflamed duodenum in associated with CE from normal duodenum.

ENGLISH SUMMARY

Application of contrast-enhanced ultrasonography to diagnose and evaluate chronic intestinal diseases in dogs

Chronic intestinal signs (i.e. diarrhea, vomiting, weight loss) in dogs can be very challenging to diagnose and manage as these signs can be caused by various entities including inflammatory diseases and neoplasia. Chronic enteropathy (CE) is the most frequent inflammatory disease of the intestine in dogs which often difficult to discriminate from intestinal lymphoma. In addition, there is still lack of an appropriate biomarker for the evaluation of therapeutic response during management of CE. Histopathology of intestinal biopsy remains the gold standard to exclude intestinal lymphoma and characterize the inflammatory progression, but it is relatively invasive and impractical for repeated examination. Due to above reasons, there remains a need for an alternative modality to diagnose and evaluate CE in dogs.

Contrast-enhanced ultrasonography (CEUS) which utilize microbubble contrast agent allows qualitative and quantitative evaluation of organ perfusion. The use of CEUS to evaluate intestinal perfusion in veterinary practice is still limited. In human medicine, CEUS has been utilized to evaluate intestinal perfusion of patients with inflammatory bowel disease (IBD) and revealed change in enhancement pattern as well as quantitative perfusion parameters compared to healthy controls. CE in dogs has been known to share some common pathogenesis with human IBD. Thus, evaluation of intestinal perfusion by CEUS is expected to provide valuable information for the diagnosis and evaluation of CE in dogs.

Before clinical application of CEUS to evaluate intestinal perfusion, the

assessment of its repeatability and reproducibility is important to determine the feasibility and the best parameter that allow detection of pathological changes in the intestine. As this modality is aimed for serially repeated assessment such as evaluation of disease severity and treatment response, it demands a clear discrimination between physiological changes or measurement errors and changes related to disease severity or the treatment response. Furthermore, it is important to maintain animal cooperation during CEUS acquisition because animal movement interferes with the image analysis. For this purpose, a sedative is sometimes required to restrain uncooperative animals. To my knowledge, repeatability and the effect of sedation on the CEUS of canine intestine are still limited.

Considering the above background, I conducted research on the application of CEUS to evaluate intestinal perfusion focusing on duodenum within 3 chapters. In first chapter, I have clarified that the repeatability (intraday variability) and reproducibility (interday variability) of CEUS-derived perfusion parameters which include time-to-peak (TTP), peak intensity (PI), area under curve (AUC), wash-in and wash-out rate (WiR and WoR respectively) were clinically acceptable in assessing duodenal perfusion of healthy sedated dogs. The intraday, interday and intraobserver coefficients of variation (CVs) for all perfusion parameters mentioned above being less than 25%.

In second chapter, I confirmed that TTP, PI, AUC, WiR, and WoR derived from CEUS of duodenum in healthy dogs were not significantly different in awaken state and after sedation using a combination of butorphanol and midazolam. This finding indicated that the combination could be a good option for sedation prior to CEUS of duodenum in uncooperative dogs.

In third chapter, I identified that CEUS-derived parameters especially PI and AUC, which represent the regional blood volume of duodenal mucosa, were significantly higher

in dogs with symptomatic CE compared to control. I also detected a significant correlation between PI and CCECAI score. This finding suggests that PI and AUC could distinguish chronic inflammation from normal duodenum and could be useful for the diagnosis and evaluation of CE in dogs. Unfortunately, no CEUS-derived parameters were significantly different between CE and intestinal lymphoma groups that preclude the use of these parameters to differentiate both diseases.

In conclusion, from this study I could establish the quantitative CEUS method in the assessment of duodenal perfusion. This modality is repeatable and reproducible in healthy dogs and not influenced by sedation using a combination of butorphanol and midazolam. The perfusion parameters derived from CEUS especially PI and AUC could discriminate chronic inflamed duodenum in associated with CE from normal duodenum.

JAPANESE SUMMARY (要旨)

Application of contrast-enhanced ultrasonography to diagnose and evaluate chronic intestinal diseases in dogs

(犬の慢性腸疾患の診断と評価における造影超音波の応用)

下痢、嘔吐あるいは体重減少などの慢性消化器症状は、炎症性疾患や腫瘍性疾患を含む様々な原因によって引き起こされるため、診断および治療管理が非常に難しい。慢性腸症（CE）は、犬で最も頻繁に認められる炎症性の消化器疾患であり、消化器型リンパ腫との鑑別がしばしば困難である。さらに、CEの治療反応を評価するための適切なバイオマーカーは存在しない。腸生検組織の病理学的検査は、リンパ腫の除外および炎症の程度を確認するためのゴールドスタンダードであるが、比較的侵襲的な検査であるため繰り返し実施することは現実的ではない。これらの理由から、犬のCEを診断及び評価するための非侵襲的な新たなモダリティが必要とされている。

マイクロバブル造影剤を使用する造影超音波検査（CEUS）は、臓器灌流の定性的および定量的評価を可能にする。しかし、犬の腸管灌流を評価するためにCEUSを使用した報告はほとんどない。ヒトでは、炎症性腸疾患（IBD）患者の腸管灌流を評価するためにCEUSが利用されており、健常対照と比較して増強パターンおよび定量的灌流パラメータが変化することが明らかになっている。犬のCEの病態は、ヒトIBDといくつかの共通点を持つことが知られている。したがって、CEUSによる腸管灌流の評価は、犬CEの診断及び評価のための貴

重なる情報になることが期待される。

CEUS を慢性腸疾患症例に適応する前に、最適な CEUS パラメーターの決定ならびにその再現性の評価が必要である。腸における CEUS は、疾患重症度や治療反応性の評価を目的としているため、生理学的変化や測定誤差と疾患に関連する変化を明確に区別する必要がある。さらに、動物の動きが CEUS の画像解析に干渉するため、動物の協力を維持することが重要である。そのため、非協力的な動物に対して時には鎮静剤が必要とされる。私の知る限りでは、犬の腸の CEUS における鎮静薬使用の効果と検査再現性は検討されていない。

上記の背景を踏まえて、私は十二指腸に焦点を当てた CEUS の適用に関する研究を 3 章構成で行った。第 1 章では、鎮静下の健康犬において、time-to-peak (TTP)、peak intensity (PI)、area under curve (AUC)、wash-in rate (WiR)、および wash-out rate (WoR) を含む CEUS パラメータが臨床的に許容できる再現性(日内変動性および日間変動性)を有することを明らかにした。上記の全てのパラメータの coefficient of variation (CV)は 25%未満であった。

第 2 章では、健康犬の十二指腸における CEUS パラメータ (TTP、PI、AUC、WiR および WoR) は、ブトルファノールとミダゾラムを併用した鎮静による影響を受けないことを確認した。この知見は、非協力的な犬において CEUS を適用する際に、鎮静処置が有効であることを示した。

第 3 章では、症候性 CE の犬の CEUS パラメータ、特に十二指腸粘膜の局所血流量を反映する PI および AUC が、病理学的に正常な十二指腸を持つ犬と比較して有意に高いことを見出した。また、PI と犬慢性腸症臨床スコア

(CCECAI) は有意に相関することを示した。この知見は、PI および AUC が、十二指腸における慢性活動性炎症と正常な十二指腸を識別できることを示唆しており、CE 犬の診断および評価にとって有益な情報となり得る。しかしながら、いずれの CEUS パラメータにおいても CE と消化器型リンパ腫との間で有意差は認められず、両者を区別することは困難であると考えられた。

結論として、本研究では犬の十二指腸灌流評価における定量的 CEUS 法を確立した。十二指腸における CEUS は、健康な犬では良好な再現性があり、ブトルファノールとミダゾラムを用いた鎮静の影響を受けないことが確認された。CEUS パラメータ、特に PI および AUC は、十二指腸において慢性活動性炎症と正常な状態を識別することが可能である。

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