의학박사 학위논문

Evaluation of Hepatic Steatosis Using Acoustic Structure Quantification Ultrasound in a Rat Model: Comparison with Pathology and MR Spectroscopy

지방간 모델에서 초음파 음량구조정량화를 이용한 지방 정량화에 대한 연구: 병리 소견, 자기공명 영상 소견과의 비교

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이 동 호

A thesis of the Degree of Doctor of Philosophy in Medicine

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Abstract

Purpose: To assess the diagnostic performance of the focal disturbance (FD) ratio calculated by acoustic structure quantification (ASQ) technique in assessing the hepatic steatosis and to determine the significant affecting factors for FD ratio in a dietary-induced fatty liver disease rat animal model using histopathology as a reference of standard.

Methods: Thirty-two F344 male rats were fed a methionine/choline-deficient diet with a variable duration (i.e., 0 weeks for the control, a half-week, or 1, 2, 3, 4, 5, or 6 weeks; n=4). At the end of each diet period, ASQ ultrasound and MR spectroscopy (MRS) were performed. Then, the rat was sacrificed and histopathologic examination of the liver was performed for the reference of standard. To assess the diagnostic performance of the FD ratio in evaluating the degree of hepatic steatosis, receiver operating characteristic curve analysis was performed. The Spearman correlation coefficient was calculated to assess the correlation between the ordinal values, and multivariate linear regression analysis was used to identify significant determinant factors for the FD ratio.

Results: The FD ratio showed excellent diagnostic performance in assessing the degree of hepatic steatosis (area under the curve: 1.000 for 5-33% steatosis, 0.981 for >33-66% steatosis and 0.965 for >66% steatosis) and was comparable to MRS. There was a strong negative

linear correlation between the FD ratio and the estimated fat fraction on MRS (Spearman rho, -0.903; P<0.001). The multivariate linear regression analysis showed that the degree of hepatic steatosis (P<0.001) and fibrosis stage (P=0.022) were significant factors affecting the FD ratio.

Conclusions: The FD ratio could provide good diagnostic performance in assessing the degree of hepatic steatosis, with a strong negative linear correlation with the estimated fat fraction on MRS. The degree of steatosis and stage of fibrosis on histopathology were significant affecting factors for FD ratio.

Keywords: Hepatic Steatosis; Hepatic fibrosis; Acoustic Structure Quantification; MR spectroscopy

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LIST OF ABBREVIATION

NAFLD: Nonalcoholic fatty liver disease
NASH: nonalcoholic steatohepatitis
US: ultrasound
CT: computed tomography
MRI: magnetic resonance imaging
MRS: magnetic resonance spectroscopy
ASQ: acoustic structure quantification
FD ratio: focal disturbance ratio
IACUC: Institutional Animal Care and Use Committee
MCD diet: methionine/choline-deficient diet
ROI: region of interest
NAS: NAFLD activity score

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a condition of lipid accumulation in hepatocytes and is known to be strongly associated with the metabolic syndrome characterized by obesity, type 2 diabetes, dyslipidemia and atherosclerotic cardiovascular disease (1-4). NAFLD represents a broad spectrum of diseases, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which can further progress into fibrosis, cirrhosis and eventually end-stage liver disease requiring liver transplantation when not adequately treated (5). In addition, NAFLD is currently considered the most common cause of chronic liver disease in the Western world, with a prevalence of 30% in the general population (6, 7). Therefore, the early detection and assessment of NAFLD using quantitative measurement of hepatic steatosis are of crucial importance to achieve earlier treatment and prevent progression to cirrhosis.

Traditionally, liver biopsy is considered the gold standard for the diagnosis and quantification of hepatic steatosis (8). However, liver biopsy has intrinsic limitations, including potential sampling errors and complications such as bleeding (9). Moreover, due to the invasive nature of this procedure and its potential complication, repeated liver biopsy is often difficult to perform when disease monitoring is required. Therefore, there has been an urgent need for non-invasive methods for the diagnosis

and quantification of hepatic steatosis. For this purpose, ultrasound (US), computed tomography (CT) and magnetic resonance imaging with spectroscopy have been used. Among them, hydrogen 1 (^{1}H) magnetic resonance spectroscopy (MRS) has been considered an alternative, non-invasive standard to assess hepatic steatosis due to its high diagnostic accuracy and reproducibility (10, 11). Additionally, recently developed T2*-corrected multi-echo MR imaging such as IDEAL has been known to be an accurate and reproducible method for assessing the hepatic steatosis (12-14). However, MRS and MRI are expensive; therefore, it is also not easily repeated to monitor the degree of hepatic steatosis. The availability of MR can be another drawback because its availability is limited compared with other imaging modalities such as US or CT.

US can potentially be a good imaging modality that is minimally invasive and inexpensive and that can be performed repeatedly without risk to the patient. However, US has an intrinsic limitation for the quantification of hepatic steatosis, and the subjective nature of the examination is a major drawback of US.

Recently, acoustic structure quantification (ASQ), a commercially available quantification method provided by Toshiba medical system (Otawara, Japan), has been introduced in clinical practice for the evaluation of diffuse liver disease. In the ASQ technique, theoretical echo amplitude distribution is compared to real echo amplitude

distribution, and the theoretical echo amplitude distribution of the liver region is thought to be a function of the Reyleigh distribution based on the assumption that the speckle pattern is induced only by US interference of very small scattering objects, which are located closer than the US wavelength (15, 16). However, the real echo amplitude distribution of the normal liver parenchyma actually does not fit a Reyleigh distribution mainly due to the presence of small structures such as hepatic vessel walls that scatter the US. As the degree of steatosis increases, the real echo amplitude distribution can become more similar to the theoretical echo amplitude distribution (i.e., Reyleigh distribution) with the blurring of the small structures, including hepatic vessels wall (15, 16). According to the results of recent studies, the focal disturbance (FD) ratio, calculated by using ASQ technique, was significantly correlated with the degree of hepatic steatosis in both an animal model (15) and a human study (16).

However, previous studies did not assess the potential affecting factors, such as concurrent inflammation or fibrosis, for the value of the FD ratio calculated by ASQ on the evaluation of hepatic steatosis. For the widespread clinical application of the ASQ technique as a quantitative biomarker for the evaluation of hepatic steatosis, potential affecting factors for the FD ratio in the quantification of hepatic steatosis should be defined. In addition, the exact diagnostic performance of the FD ratio

for the assessment of hepatic steatosis must also be evaluated. Therefore, the purpose of our study was to assess the diagnostic performance of the (FD ratio calculated by ASQ technique in assessing the hepatic steatosis and to determine the significant affecting factors for FD ratio in a dietary-induced fatty liver disease rat animal model using histopathology as a reference of standard.

MATERIALS AND METHODS

Animals

This animal study was approved by our Institutional Animal Care and Use Committee IACUC No. 14-0176). Male F344 rats weighing 250-300 g were acclimatized for 1 week under standardized laboratory conditions with a 12-h light/dark cycle in a temperature-controlled room. Fatty liver disease was induced by feeding a methionine/choline-deficient (MCD) diet (Dyets, Bethlehem, PA) ad libitum for a half-week, or 1, 2, 3, 4, 5, or 6 weeks (n=4 per group), whereas the control group (n=4) was maintained on a standard diet (5, 17, 18). At the end of each diet period, US examination using the ASQ technique and ^{1}H-MRS were performed to assess the degree of steatosis. Immediately after the imaging studies, the rats were sacrificed, and the livers were excised for histopathologic examination.

US examination

All US examinations were carried out using a US scanner (Aplio XG; Toshiba Medical Systems, Otawara, Japan) with a 12-MHz linear transducer (PLT-1204BT) by author with 9 years of experience in liver US imaging. The display depth and transmit focus were set at 30 mm and 15 mm, respectively, and cross-sectional images of the hepatic

parenchyma were obtained initially. US images in the ASQ mode were then acquired five times for each animal, and regions of interest (ROIs) as large as possible were placed on the liver parenchyma, with care taken to avoid large hepatic vessels or artefacts (Figure 1). The FD ratio values were calculated by the system and displayed on the monitor and were recorded for further analysis.

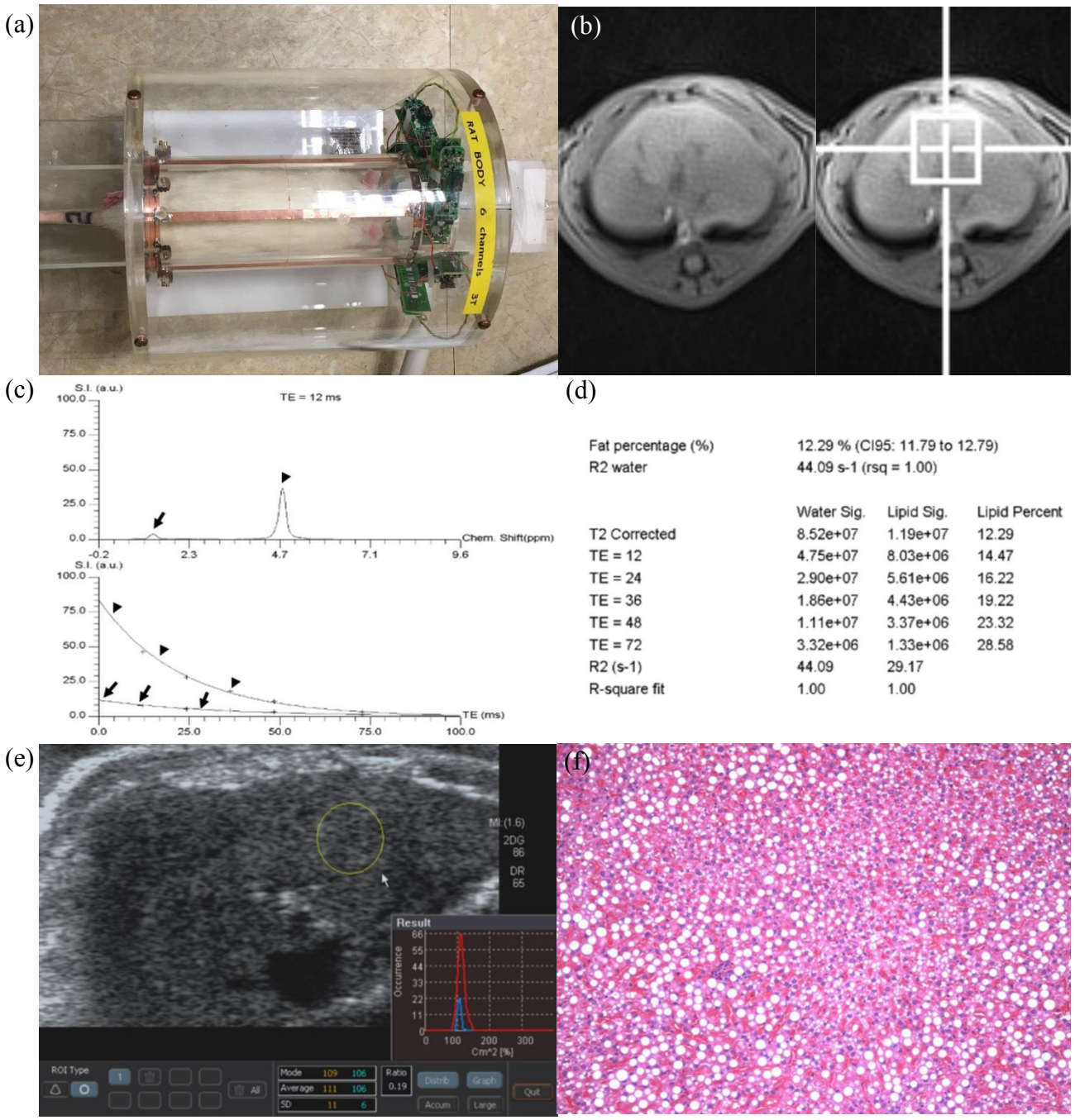


Figure 1. US examination using ASQ technique, ^{1}H MRS and histopathologic evaluation of a rat fed by MCD diet for 1 week.

(a) The setup of micro-coil and rat for MRS examination. Anaesthesia is induced by intraperitoneal injection of ketamine and rat is set in prone position into the dedicated micro-coil for MRS acquisition. (b) Left: Volumetric T1-weighted images of the liver were obtained and shown as axial image. Right: 10x10x10-mm^3 sized voxel was applied in central portion of liver parenchyma avoiding inclusion of the extrahepatic structures. (c) Top: representative spectrum of MRS for echo time (TE) of 12 msec. Arrow indicates fat peak and arrow head indicates water peak. Bottom: plot of echo time versus signal intensity for water and fat extrapolated from top graph to echo time of 0 msec. Arrow indicates extrapolated line for fat and arrow head indicates extrapolated line for water. a.u.=arbitrary units, S.I=signal intensity. (d) The estimated fat fraction in this exam is 12.29%. (e) ASQ examination obtained from a rat fed by MCD diet for 1 week. The ROI is placed in liver left lobe parenchyma avoiding large hepatic vessels or artefacts and the calculated FD ratio in this exam is 0.19. (f), Grade 2 steatosis (i.e., >33-66%) is seen on H & E staining.

Principles of Acoustic Structure Quantification and Calculation of FD Ratio

Acoustic structure quantification (ASQ) is a newly developed quantitative method that analyses the statistical information of the acquired echo signals. In ASQ technique, statistical analysis is based on the comparison of difference between theoretical and real echo amplitude distribution. Theoretical amplitude comes from Rayleigh distribution. A dispersion value from a Rayleigh distribution of a signal amplitude distribution of reception signals is calculated by performing statistical filtering process above, so as to express the calculated dispersion value in an image (1-3).

Theoretical echo amplitude is thought to be a function of the Rayleigh distribution based on the assumption that the speckle pattern is induced only by US interference of very small scattering objects, which are located closer than US wavelength (16). However, real echo amplitude of the liver parenchyma actually does not fit a Rayleigh distribution mainly due to the presence of small structures such as hepatic vessel walls that scatter the US. As hepatic steatosis progresses, liver parenchymal echogenicity increases and small structures such as blood vessel walls are obscured. Therefore, real echo amplitude of fatty liver might be more likely to be a theoretical echo amplitude and fit a Rayleigh distribution compared to normal liver echo amplitude.

In ASQ examination, when the examiner sets a comprehensive region of interest (ROI) on the image (hereafter, refed as a large ROI), several hundred of small ROIs (hereafter, small ROIs) are automatically set therein for the calculation of probability density function (PDF) (Figure 2). The average and variance of the echo amplitude in a small ROI is calculated for each small ROI, and essential parameter of C_m^2 (intensity or amplitude value) is estimated. Multiple results for small ROIs in a large ROI are displayed as an occurrence histogram of C^2 (red line in Figure 2) (15). Then, to calculate focal disturbance (FD) ratio, C_m^2 (ASQ value) is calculated by following equation (15) as first step:

$$C_m^2 = \sigma_m^2 / \sigma_R^2(\mu) = (\pi / (4- \pi))\ \sigma_m^2 / \mu^2$$

Where μ and σ^2 are the average and variance of the echo amplitude in a small ROI, and σ_m is the variance calculated from the limited samples less than $\mu + 4\sigma$. When the ratio C^2/C_m^2 is larger than threshold α, the result of C_m^2 is eliminated from the histogram (red line in Figure 2), but added to the alternative histogram (blue line in Figure 2) (15). As second step, the FD ratio can be calculated the ratio of the area under the curve (AUC) for these to histogram: FD ratio = [AUC (red) /AUC (blue)] (Figure 1). If the threshold α was set to 1.2, FD ratio = 0 when the obtained echo amplitude fits a Rayleigh distribution for PDF, and has a positive value in case of the presence of tiny structural changes (15). As the number of small ROIs with a high degree of deviation from the

Rayleigh distribution increases, the strength of the signal obtained from that ROIs would become more non-homogeneous. When hepatic steatosis occurs, hepatic parenchymal echogenicity increases due to the deposition of fat droplet and small hepatic vessel walls would become blurred. Therefore, echogenicity of fatty liver would be more homogeneous, and the number of focally inhomogeneous small-ROIs decreases. This can result in the decrease of FD ratio. With this manner, FD ratio calculated by ASQ technique can provide quantitative information regarding the change of echo-texture during the development of diffuse liver disease such as hepatic steatosis or fibrosis.

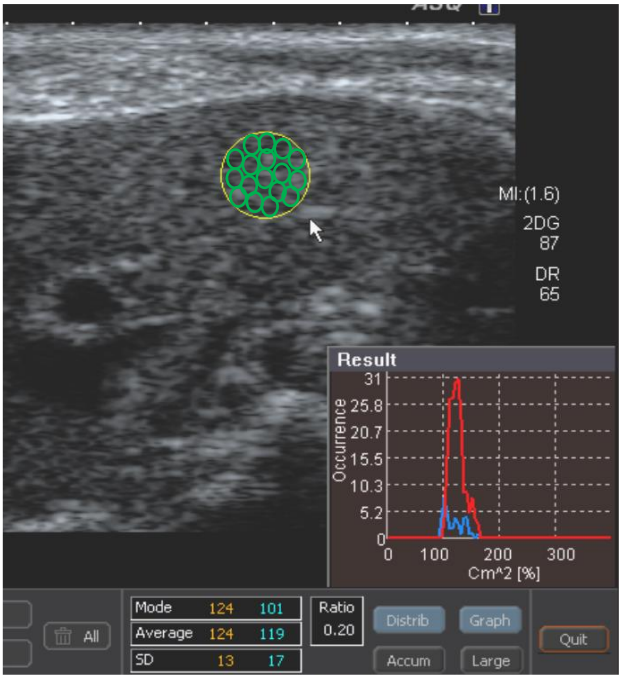


Figure 2. ASQ quantification using US image obtained from a rat fed by MCD diet for 1 week. When examiner set a large ROI (yellow circle) in liver left lobe parenchyma avoiding large vessels, several hundred of small ROIs (green circles) are automatically set within large ROI for the calculation of PDF, histogram and FD ratio.

^{1}H-MR Spectroscopy

All ^{1}H-MRS measurements were performed on a clinical 3.0T MR scanner (Trio Tim System; Siemens Healthcare, Erlangen, Germany) with a 5-cm inner diameter experimental dedicated 6 channel rat body coil (STARK CONTRAST MRI Coils Research, Erlangen, Germany). Scout images were acquired to localize the liver and surrounding structures, and volumetric T1-weighted images of the liver were obtained using the Radial VIBE sequence to avoid breathing motion artefacts. For the fat fraction spectroscopy measurement, the HISTO WIP (Work-in-Progress, not commercially released yet) sequence (echo times msec/repetition time msec, 12, 24, 36, 48 and 72/3000) was employed using a $10 \times 10 \times 10$-mm^3 voxel size located on the liver parenchyma to avoid inclusion of the extrahepatic structures. The hepatic fat fraction was calculated through T2 correction for both the water and lipid contents. Each T2 value of water and fat was calculated separately, and extrapolation of fat and water integrals for an echo time of 0 msec by using an exponential fit of the points acquired at five different echoes by automatic fitting of water (4.7 ppm) and the major fat peak of methylene (1.3 ppm) was done for fat quantification (10, 14). The hepatic fat fraction was calculated automatically and displayed as a percentage in Distal Imaging and Communications in Medicine format. Dedicated coil setup, T1-weighted volumetric image for localization and fat

quantification using MRS are given in Figure 1. ^{1}H-MRS examination was done five times for each animal, and mean value of hepatic fat fraction was calculated for further analysis.

Histopathologic examination

The excised liver specimens were fixed in 10% buffered formalin for 24 hours, dehydrated and embedded in paraffin. Next, 5-μm-thick sections were stained with haematoxylin and eosin and Masson's trichrome. The sections were evaluated by an experienced pathologist with 15 years of experience in liver pathology using the histological scoring system for NAFLD (2). In this scoring system, the degree of steatosis is classified into four groups: score 0, less than 5% steatosis; score 1, 5-33% steatosis; score 2, >33-66% steatosis; and score 3, more than 66% steatosis. Lobular inflammation (score 0-3) and hepatocyte ballooning (score 0-2) were assessed according to the histological scoring system for NAFLD (2). Thereafter, the final NAFLD activity score (NAS) which is defined as unweighted sum of the score of steatosis (0-3), lobular inflammation (0-3) and hepatocyte ballooning (0-2); thus ranging from 0 to 8 was determined (2). The stage of fibrosis (stage 0-4) was also evaluated and recorded.

Statistical analysis

Categorical variables were compared using Fisher's exact test, and continuous variables were evaluated using the Kruskal-Wallis test. The hepatic fat fraction assessed on ^{1}H-MRS and FD-ratio calculated using the ASQ technique were expressed as means ± standard deviation throughout the study. Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic performance of the FD ratio calculated by the ASQ technique for the assessment of the degree of steatosis determined on histopathology. The area under the curve (AUC) was calculated, and the optimal cut-off value as well as corresponding sensitivity, specificity, positive predictive value and negative predictive value were also determined. The Spearman correlation coefficient was used to assess the correlation between the ordinal variables. To determine the factors significantly affecting the FD ratio, multivariate linear regression analysis was performed. All of the statistical analyses were performed using SPSS version 21 (SPSS, Chicago, IL, USA).

RESULTS

Induction of fatty liver disease by the MCD diet

Changes in histopathology, the estimated fat fraction on MRS and the calculated FD ratio by the ASQ technique among the rat animal groups are summarized in Table 1. Control rat liver showed no steatosis on either the histologic examination or the MRS. A half-week MCD diet induced grade 1 or 2 steatosis (mean estimated fat fraction on MRS: 6.99 ± 0.71%), and a one-week MCD diet caused grade 2 or 3 steatosis (mean estimated fat fraction on MRS: 18.9 ± 5.6%). After a two-week MCD diet, grade 3 steatosis on histopathology and more than 30% of the estimated fat fraction on MRS were achieved in most of the animals (Figure 1). The degree of lobular inflammation was also significantly increased as the duration of MCD diet was prolonged. Accordingly, the final NAFLD activity score (NAS) was significantly increased as the duration of the MCD diet was prolonged. Fibrosis was first observed after a half-week MCD diet, and stage 2 fibrosis was induced after 5 or 6 weeks of the MCD diet (Figure 3). The FD ratio calculated by the ASQ technique was significantly decreased along with the prolonged duration of the MCD diet.

Table 1. Changes in histopathology, the estimated fat fraction on MRS and the FD ratio calculated by the ASQ technique among the animal groups

		Control	0.5 wk	1wk	2wks	3wks	4 wks	5 wks	6 wks	P-value
Pathology	Steatosis* (0-3)	0;0;0;0	2;1;2;1	3;2;2;2	3;3;3;3	3;2;3;3	3;3;3;3	3;3;3;3	3;3;3;3	<0.001†
	Lobular inflammation* (0-3)	0;0;0;0	1;0;0;1	3;2;2;1	2;1;2;1	2;2;1;1	1;3;2;2	3;3;3;3	3;2;3;3	0.001†
	Hepatocyte ballooning* (0-2)	0;0;0;1	0;1;1;0	1;1;0;0	0;0;0;0	0;0;0;0	1;0;0;0	1;1;1;1	1;1;1;1	0.013†
	Fibrosis* (0-4)	0;0;0;0	1;1;1;1	1;1;1;1	1;1;0;1	0;1;1;0	1;1;1;1	2;2;2;1	1;1;2;2	<0.001†
	NAS*	0;0;0;1	3;2;3;2	7;5;4;3	5;4;5;4	5;4;4;4	5;6;5;5	7;7;7;7	7;6;7;7	<0.001†
Fat fraction on MRS (mean ± standard deviation, %)		1.66 ± 0.42	6.99 ± 0.71	18.9 ± 5.6	33.3 ± 5.1	33.7 ± 2.1	35.4 ± 3.1	34.7 ± 4.5	37.7 ± 2.0	<0.001
FD ratio (mean ± standard deviation)		0.648 ± 0.038	0.311 ± 0.016	0.192 ± 0.028	0.139 ± 0.022	0.163 ± 0.012	0.137 ± 0.010	0.110 ± 0.030	0.080 ± 0.011	<0.001

Note: *=grade and score of each rat animal in each group. Each group comprised four rats; MRS=magnetic resonance spectroscopy; FD ratio=focal disturbance ratio; ASQ=acoustic structure quantification; NAS=nonalcoholic fatty liver disease activity score which is defined as unweighted sum of the score of steatosis (0-3), lobular inflammation (0-3) and hepatocyte ballooning (0-2); thus ranging from 0 to 8.

†: P-values were determined by Fisher's exact test and other P-values were determined by Kruskal-Wallis test.

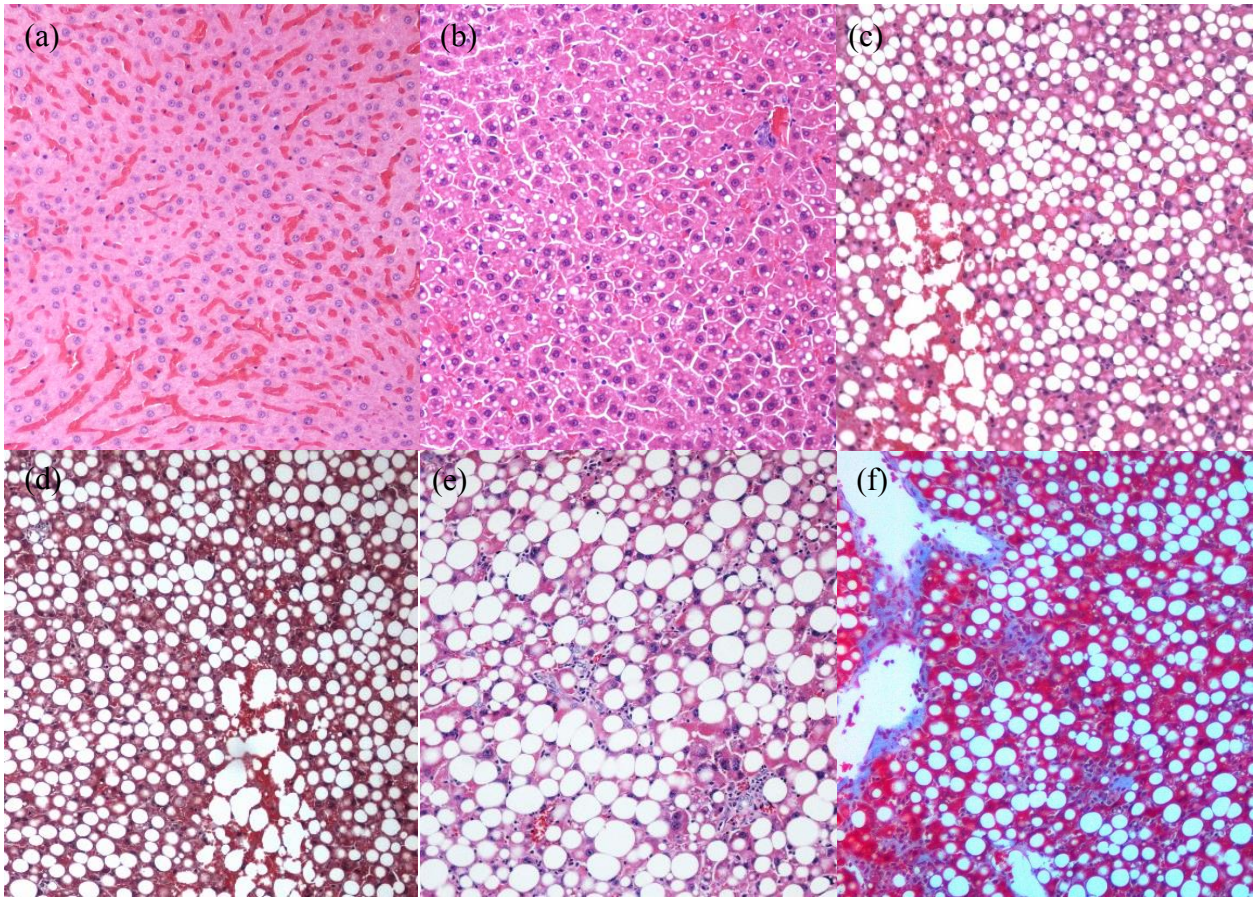


Figure 3. Histological changes (x 100) during the MCD diet. (a) Control group shows no steatosis on H & E staining. The estimated fat fraction on MRS was 1.62% and FD ratio calculated by ASQ technique was 0.667. (b) A half-week MCD diet induced score 1 steatosis (i.e., 5-33% of steatosis) on H & E staining. The estimated fat fraction on MRS was 6.38% and FD ratio calculated by ASQ technique was 0.316. (c and d) Two-week MCD diet induced score

3 steatosis (i.e., more than 66% of steatosis) on H & E staining (c), but no fibrosis on Masson's trichrome staining (d). The estimated fat fraction on MRS was 40.7% and FD ratio calculated by ASQ technique was 0.106. (d and e) Six-week MCD diet induced score 3 steatosis (i.e., more than 66% of steatosis) on H & E staining (d) and stage 2 fibrosis on Masson's trichrome staining (e). The estimated fat fraction on MRS was 38.1% and FD ratio calculated by ASQ technique was 0.064.

Diagnostic Performance of the FD ratio calculated by ASQ to assess the degree of hepatic steatosis

The diagnostic performance of the FD ratio and estimated fat fraction on MRS for assessing the degree of hepatic steatosis are summarized in Table 2. The area under the curve for the diagnosis of grade 1 (5-33% steatosis), grade 2 (>33-66% steatosis) and grade 3 steatosis (greater than 66% steatosis) were 1.000, 0.981 and 0.965, respectively, for the FD ratio calculated by the ASQ technique and 1.000, 0.994 and 0.962, respectively, for the estimated fat fraction on MRS (Figure 4). There was no significant difference between the FD ratio calculated by the ASQ technique and estimated fat fraction on MRS for the assessment of the degree of hepatic steatosis (P=1.000 for grade 1 steatosis, P=0.286 for grade 2 steatosis and P=0.954 for grade 3 steatosis).

Table 2. Optimal cut-off values of the FD ratio and estimated fat fraction on MRS for the diagnosis of hepatic steatosis

	Aim	Cut-off	AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
FD ratio	≥S1	0.330	1.000 (0.891-1.000)	100% (28/28)	100% (4/4)	100% (28/28)	100% (4/4)	100% (32/32)
	≥S2	0.218	0.981 (0.857-1.000)	92.3% (24/26)	100% (6/6)	100% (24/24)	75.0% (6/8)	93.8% (30/32)
	≥S3	0.180	0.965 (0.831-0.999)	95.0% (19/20)	83.3% (10/12)	90.5% (19/21)	90.9% (10/11)	90.6% (29/32)
Fat fraction on MRS	≥S1	2.26 %	1.000 (0.891-1.000)	100% (28/28)	100% (4/4)	100% (28/28)	100% (4/4)	100% (32/32)
	≥S2	7.22 %	0.994 (0.879-1.000)	96.2% (25/26)	100% (6/6)	100% (25/25)	85.7% (6/7)	96.9% (31/32)
	≥S3	20.7 %	0.962 (0.828-0.998)	100% (20/20)	91.7% (11/12)	95.2% (20/21)	100% (11/11)	96.9% (31/32)

Note: FD ratio=focal disturbance ratio; MRS=magnetic resonance spectroscopy; S1=steatosis of 5-33% on histopathology; S2=steatosis of >33-66% on histopathology; S3=steatosis > 66% on histopathology; AUC=area under the curve; CI=confidence interval; PPV=positive predictive value; NPV=negative predictive value.

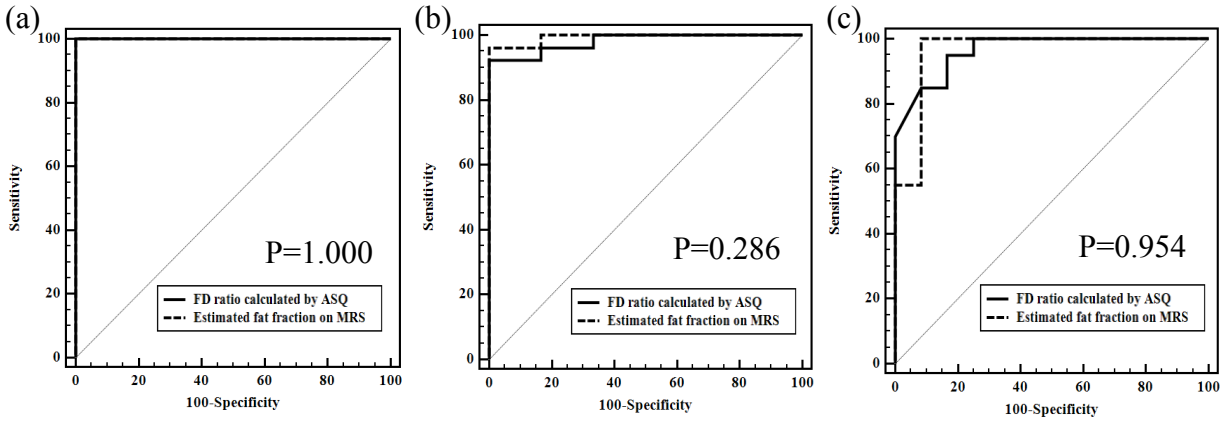


Figure 4. ROC curves of the FD ratio calculated by the ASQ technique and estimated fat fraction on MRS for the diagnosis of grade 1 steatosis (a), grade 2 steatosis (b) and grade 3 steatosis (c). There was no significant difference between the FD ratio and estimated fat fraction on MRS to diagnose the different degrees of hepatic steatosis.

Correlation between the FD ratio calculated by ASQ and estimated fat fraction on MRS

There was a strong negative linear correlation between the FD ratio calculated by ASQ technique and the estimated fat fraction on MRS (Figure 5). The Spearman rho correlation coefficient was -0.903 (95% confidence interval: -0.952 to -0.810; $P<0.001$).

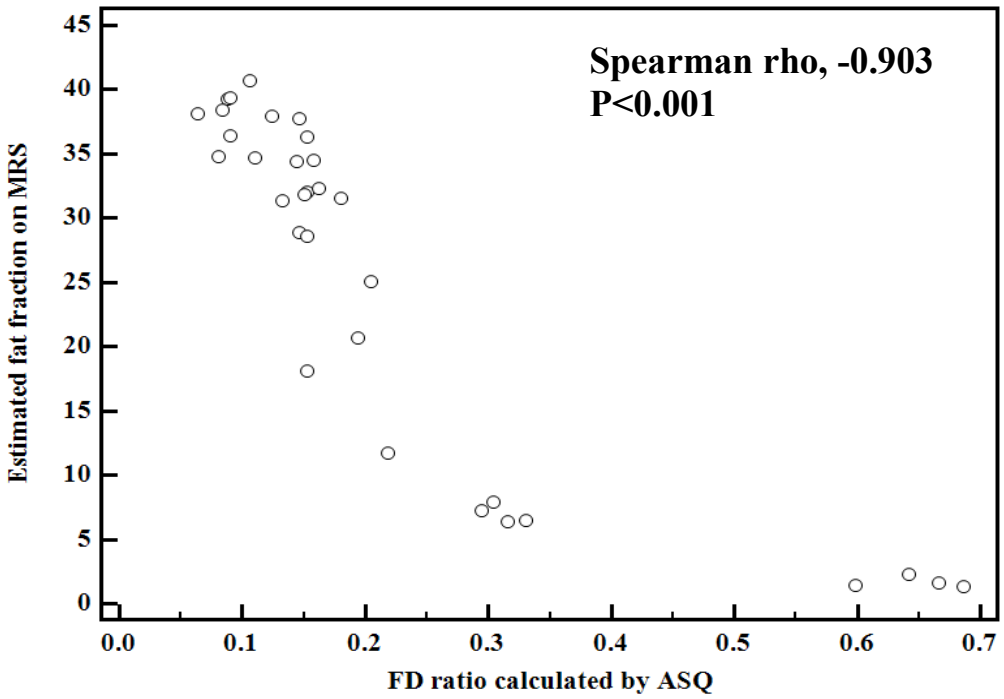


Figure 5. Scatter plot showing a strong negative linear correlation between the FD ratio calculated by the ASQ technique and the estimated fat fraction on MRS.

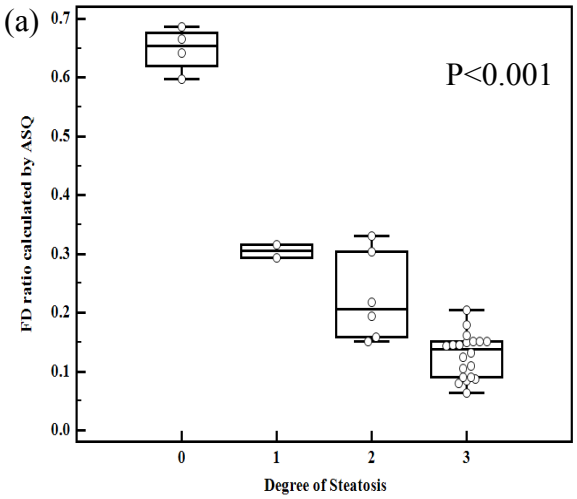
Factors affecting the FD ratio calculated by the ASQ technique

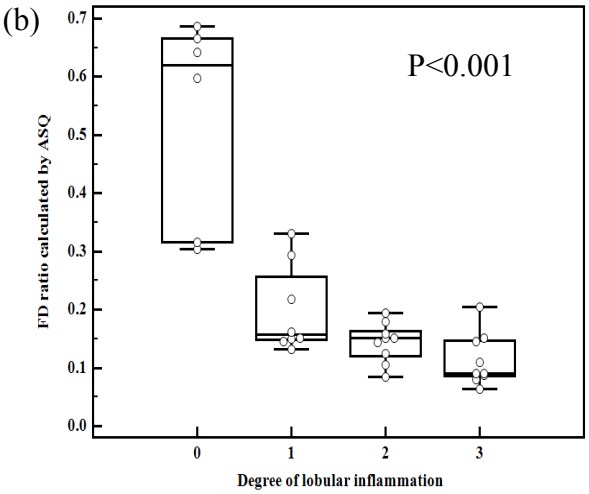
The FD ratio calculated by the ASQ technique significantly decreased as the degree of steatosis, lobular inflammation, and stage of fibrosis increased on histopathology (Figure 5). The FD ratio was also significantly decreased with the increase in NAS (Figure 5). By contrast, the degree of hepatocyte ballooning was not significantly associated with the FD ratio. The factors affecting the FD ratio calculated by the ASQ technique are summarized in Table 3. The multivariate linear regression analysis showed that the degree of hepatic steatosis and stage of fibrosis were factors significantly affecting the FD ratio calculated by the ASQ technique. Lobular inflammation, hepatocyte ballooning and NAS did not significantly affect the value of the FD ratio. The FD ratio calculated by the ASQ technique in 20 rats with grade 3 steatosis (i.e., more than 66% steatosis) was significantly decreased along with the increase in fibrosis stage (P=0.018) (Figure 6).

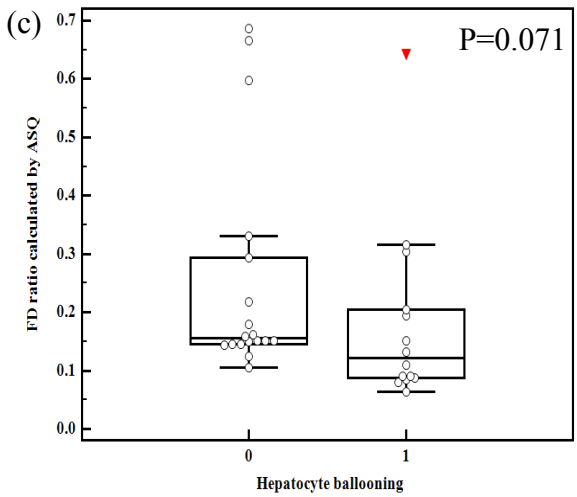
Table 3. Factors affecting the FD ratio calculated by the ASQ technique.

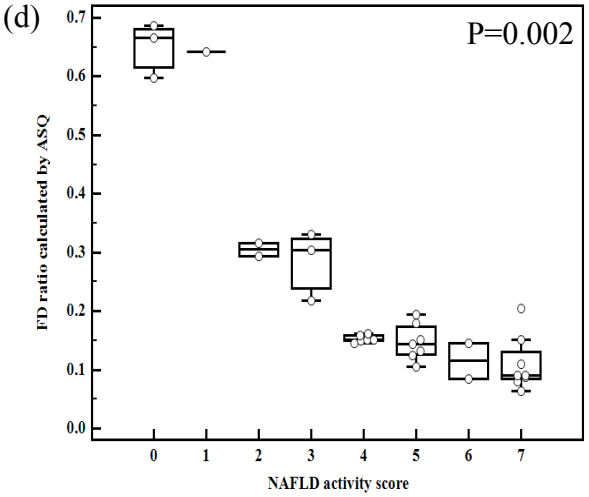
Characteristic	Univariate			Multivariate		
	Coefficient	95% CI	P-value	Coefficient	95% CI	P-value
Stage of fibrosis	-0.18	-0.26 to -0.10	<0.001	-0.06	-0.07 to -0.03	***0.022***
Hepatocyte ballooning	-0.07	-0.20 to 0.06	0.266			
Degree of steatosis	-0.16	-0.18 to -0.13	<0.001	-0.15	-0.18 to -0.12	***<0.001***
Lobular inflammation	-0.12	-0.16 to -0.08	<0.001	-0.02	-0.05 to 0.01	0.435
NAS	-0.07	-0.09 to -0.06	<0.001	0.02	-0.01 to 0.04	0.523

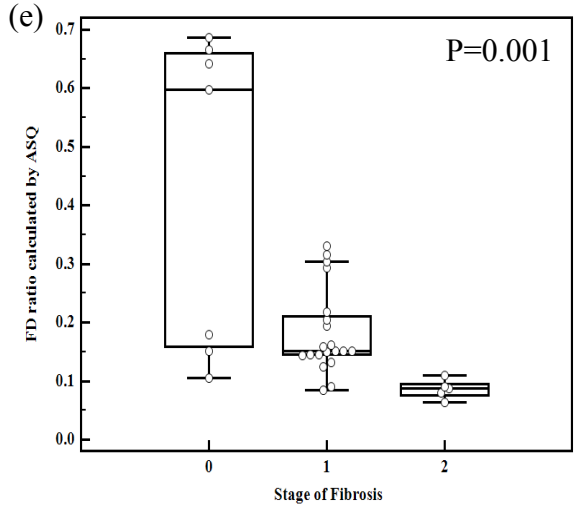
Note: FD ratio=focal disturbance ratio; ASQ=acoustic structure quantification; NAS=nonalcoholic fatty liver disease activity score.


(a)
P<0.001
FD ratio calculated by ASQ
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3
Degree of Steatosis


(b)
P<0.001
FD ratio calculated by ASQ
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3
Degree of lobular inflammation


(c)
P=0.071
FD ratio calculated by ASQ
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
Hepatocyte ballooning


(d)
P=0.002
FD ratio calculated by ASQ
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3
4
5
6
7
NAFLD activity score


(e)
P=0.001
FD ratio calculated by ASQ
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
Stage of Fibrosis

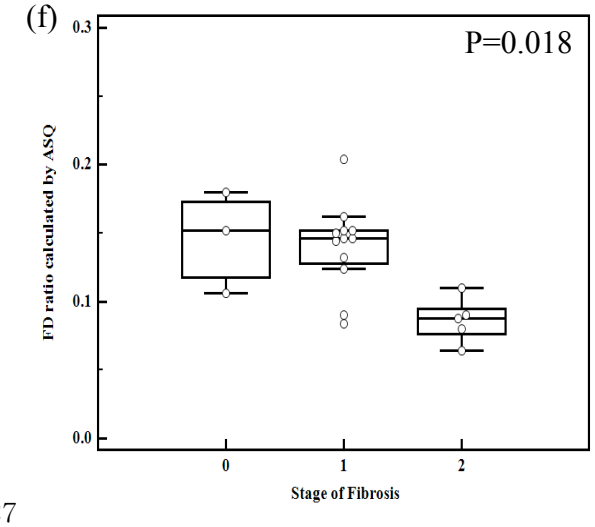

(f)
P=0.018
FD ratio calculated by ASQ
0.0
0.1
0.2
0.3
0
1
2
Stage of Fibrosis

Figure 6. Box plots showing the distribution of the FD ratio calculated by the ASQ technique according to the degree of steatosis (a), degree of lobular inflammation (b), degree of hepatocyte ballooning (c), final NAFLD activity score (d), and stage of fibrosis (e, 32 rats in total; f, 20 rats with grade 3 steatosis). Central box represents interquartile range (25 to 75 percentile) and the middle line represents the median value of FD ratio in each animal group. The vertical line extends from the minimum to the maximum value, excluding outliers which appear as dots.

DISCUSSION

In our study, the diagnostic performance of the FD ratio calculated by the ASQ technique for the assessment of the degree of hepatic steatosis seemed to be good and might be comparable to that of the estimated fat fraction on MRS, using the histopathology as a reference of standard. In addition, there was a strong negative linear correlation between the FD ratio and the estimated fat fraction on MRS.

Recently, Kuroda et al. reported that the FD ratio calculated by the ASQ technique was significantly correlated with the fat accumulation on histopathology in a leptin-deficient mouse model (15). However, in that study, the authors did not evaluate the diagnostic performance of the FD ratio in predicting the degree of hepatic steatosis on histopathology. In addition, the number of examined animal was small (n=9) in the study performed by Kuroda et al. Son et al. also reported that the FD ratio calculated by the ASQ technique showed a strong negative correlation with the estimated fat fraction on MRS in their patient cohort with fatty liver disease, and may be used for the detection of hepatic steatosis greater than 10% in living donor liver patients (16). However, in their study, there was a lack of histopathologic analysis; therefore, they could not evaluate the relationship between the FD ratio calculated by the ASQ and the changes in histopathology. Our study clearly showed both a significant association of the FD ratio calculated by the ASQ technique

with the degree of hepatic steatosis and a good diagnostic performance of the FD ratio in the assessment of the degree of hepatic steatosis. However, as we used a 12-MHz linear transducer for small animal in this study, applicability of ASQ technique using a 5-MHz convex human abdominal transducer should be evaluated for clinical use of this technique. Recently, Son et al reported that ASQ technique using a 5-MHz convex transducer may be valuable for the evaluation and quantification of hepatic steatosis (16), and given that, we would expect ASQ using a 5-MHz convex transducer can also be a good method for the evaluation of hepatic steatosis in patients group. As FD ratio calculated ASQ technique can provide good diagnostic performance in assessing the degree of hepatic steatosis, ASQ might be used for the evaluation of fatty liver disease patients. In addition, as ASQ technique can easily be repeated with no risk to patients and relatively low cost compared to MR, FD ratio calculated by ASQ technique can also be used for monitoring of fatty liver disease after the initial diagnosis.

As hepatic steatosis progresses, hepatic parenchymal echogenicity increases, and wall of small hepatic vessel or bile duct can be obscured. Therefore, pixels with large variance could be decreased, and the decreased number of pixels with large variance resulted in the decrease in FD ratio calculated by ASQ: this theoretical background of FD ratio calculation by using ASQ technique can explain our study result.

Traditionally, increased liver parenchymal echogenicity compared with renal parenchyma on B-mode US imaging has been regarded to be indicative of the presence of hepatic steatosis, and the hepatorenal ratio has been subsequently suggested for the quantitative assessment of the degree of hepatic steatosis (19-21). However, the assessment of hepatic parenchymal echogenicity is subjective in nature, and the quantitative evaluation of hepatic steatosis can scarcely be performed with B-mode US imaging alone. Furthermore, whether the hepatorenal ratio is sufficient for the quantification of hepatic steatosis as an alternative to MRS, which has been considered the standard of reference for the non-invasive diagnosis of hepatic steatosis, remains questionable (16). Although one study reported that the hepatorenal ratio showed an excellent correlation with the degree of steatosis as assessed by MRS (22), other studies have emphasized the need for an additional process such as a standardized or artificial neural network to reach the sufficient diagnostic performance of the hepatorenal ratio for the assessment of hepatic steatosis (21, 23). Controlled attenuation parameter (CAP) obtained from transient elastography has been known to be another promising tool for the non-invasive evaluation of hepatic steatosis (24, 25). CAP measures the ultrasound beam attenuation by hepatic fat at the central frequency of the transient elastography M probe (26), and could assess the hepatic steatosis quantitatively using different physical

background from ASQ used in this study. However, when comparing the ASQ from CAP, ASQ evaluation can easily be cooperated with the routine B-mode examination as ASQ uses the same transducer used in routine B-mode, and this could be a merit of ASQ over CAP.

In addition to the degree of steatosis, the stage of fibrosis on histopathology was also a significant determinant factor of the FD ratio calculated by the ASQ technique in our study. As hepatic fibrosis develops and progresses, speckles of the liver parenchyma change from homogeneous to heterogeneous (27). The presence of fibrosis can also change the liver parenchymal echogenicity and cause the blurring of small structures such as hepatic vessels and the bile duct, especially in initial stage. Therefore, the presence and stage of hepatic fibrosis can also affect the value of the FD ratio calculated by the ASQ technique. In our study, the FD ratio calculated by the ASQ technique was significantly different among the different stages of fibrosis in 20 rats with grade 3 steatosis on histopathology (i.e., more than 66% steatosis) and clearly showed the effect of fibrosis on the value of the FD ratio. Therefore, when the FD ratio calculated by the ASQ technique is planned to be used as the quantitative biomarker for the degree of hepatic steatosis, the possibility of concurrent hepatic fibrosis should be considered because it can affect the value of the FD ratio. The combination of shear-wave elastography may help the assessment of concurrent hepatic fibrosis as

shear-wave elastography can evaluate the degree of hepatic fibrosis and presence of cirrhosis accurately (28-31), and thereby can have a potential to improve the diagnostic performance of the FD ratio for the assessment of hepatic steatosis. However, to evaluate whether the combination of elastography can enhance the accuracy of the FD ratio calculated by the ASQ technique for the assessment of the degree of hepatic steatosis, further study with a larger population size and a prospective design is warranted.

Our study had some limitations. Because the most advanced stage of fibrosis in our rat animal model was stage 2, we could not assess the effect of more advanced stages of fibrosis or cirrhosis based on the value of the FD ratio calculated by the ASQ technique, and this could be a limitation of our study. In addition, the number of rats with mild to moderate steatosis could be considered relatively small compared with those with severe steatosis (8 rats with mild to moderate steatosis vs. 20 rats with severe steatosis), and this could be a limitation for the statistical power. Indeed, small number of animal with mild degree steatosis could limit the clinical value of our study, as exact quantitative assessment of hepatic steatosis among the patients with mild fatty liver might be important in clinical practice, especially for the selection of candidate for living liver donor. In addition, as the degree of hepatic steatosis on histopathologic examination in our study was classified into grade with

somewhat wide ranges, our results might be optimistic, particularly regarding the comparison of diagnostic performance between ASQ technique and MRS. Use of special staining such oil-red O staining with digital image analysis has been known to be more accurate for quantitative assessment of hepatic steatosis on histopathology compared to estimation with H & E staining used in our study (32). As we did not use this special staining, this could be another limitation.

In conclusion, the FD ratio calculated by the ASQ technique could provide good diagnostic performance for the assessment of the degree of hepatic steatosis with a strong negative linear correlation with the estimated fat fraction on MRS, and the degree of steatosis and stage of fibrosis on histopathology are factors that significantly affect the FD ratio calculated by the ASQ technique.

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국문 초록

서론: 이 연구에서는 음량구조정량화기법 (Acoustic structure quantification, ASQ)을 이용한 초음파 검사에서 구한 국소변이 (focal disturbance ratio, FD ratio) 값이 지방 정량화에 사용될 수 있는가와 FD ratio 에 영향을 주는 인자를 식이 유도 지방간 동물 모델에서 병리조직 검사 결과를 표준으로 알아보는 것을 목적으로 하였다.

방법: 250-300 그램의 몸무게를 가진 rat 32 마리를 메티오닌-콜린 결핍 식이 (Methionine-Choline deficiency diet)을 이용하여 지방간 모델을 유도하였다. 식이 기간은 0.5, 1, 2, 3, 4, 5, 6 주로 7 그룹으로 나누었으며, 한 그룹은 대조군으로 정상 식이를 유지하였고 각각의 그룹에는 4 마리의 rat이 속하도록 하였다. 각각 식이 기간의 마지막 날 ASQ 기법을 이용한 초음파 검사와 자기공명 분광 기법 검사를 시행하였으며, 영상 검사 직후 rat 을 안락사 시킨 후 간을 적출하여 병리조직학적 검사를 시행하였다. ASQ 기법을 이용한 초음파 검사에서는 FD ratio 를 측정하여 기록하였으며, 자기공명 분광 기법 검사에서는 추정 지방 분율을 구하였다. FD ratio 와 추정 지방 분율이 병리조직학적 검사에서 평가한 각각 지방간의 등급을 예측하는 진단능을 평가하기 위해 ROC 분석을 시행하였다. ASQ 기법을 이용한 초음파 검사에서 얻은 FD ratio 와 자기 공명 분광 기법 검사에서 얻은 추정 지방 분율 사이의 상관 관계를 평가하기 위해 Spearman 상관 계수를 구하였다. 이 후 FD

ratio 에 영향을 미치는 병리조직학적 요인을 찾기 위해 multivariate linear regression 분석을 시행하였다.

결과: ASQ 기법을 이용한 초음파 검사에서 얻은 FD ratio 는 병리조직 검사에서 결정된 지방간 등급의 예측에 있어서 훌륭한 진단능을 보여주었다 (area under the curve: 1.000 for 5-33% steatosis, 0.981 for >33-66% steatosis and 0.965 for >66% steatosis). FD ratio 와 자기 공명 분광 기법 검사에서 얻은 추정 지방 분율 사이에는 강한 음의 선형 상관 관계가 있었다 (Spearman 상관계수, -0.903; P<0.001). 병리조직학적 요인 중 지방간의 정도와 섬유화의 정도가 FD ratio 에 유의한 영향을 주는 요인이었다.

결론: ASQ 기법을 이용한 초음파 검사에서 얻은 FD ratio 는 지방간의 정량적 평가에 있어서 좋은 진단능을 가지고 있으며, 자기 공명 분광 기법 검사에서 얻은 추정 지방 분율과 강한 음의 상관관계를 가지고 있다. 병리 검사에서 평가된 지방간의 정도와 섬유화의 정도가 FD ratio 를 결정하는 주요 요인이었다.

주요어 : 지방간, 음량구조정량화기법, 자기공명분광기법, 간섬유화

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