Development of non-invasive method for assessment of hepatic steatosis

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Abstract

Steatosis is a critical feature of liver disease and is considered to play a pivotal role in the progression of nonalcoholic fatty liver disease, as well as being a surrogate marker of metabolic syndrome. The purpose of this study was to develop a non-invasive diagnostic method for assessment of liver steatosis. It is well known that ultrasonic velocity depends on materials and temperature. For example, the ultrasonic velocity in water is 1530m/s at 37°C and 1534m/s at 39°C, while that in fat is 1412 m/s at 37°C and 1402 m/s at 39°C. On this basis, we thought that the percentage of fat in hepatic steatosis could be assessed by detecting changes of ultrasonic in the liver, caused by

warming. In order to confirm the effectiveness of this method, we obtained the ultrasonic velocity changes of tissue phantom including lard oil and the liver of living rabbit by ultrasonic warming, and then succeeded in 2-D imaging of ultrasonic velocity changes of the phantom and the liver of living rabbit. We named this the ultrasonic velocity-change method. The experimental results show the possibility that hepatic steatosis could be characterized using our novel, non-invasive method.

Key words: ultrasonic velocity change, hepatic steatosis, pulse shift, temperature

1. Introduction

Liver steatosis results from the accumulation of fatty droplets in the hepatocytes. There are several causes, such as alcohol consumption, viral hepatitis and metabolic dysfunction. Liver steatosis is a reversible, benign condition and a common abnormality occurring in approximately 30% of the general population in the United States [1]. However, in many cases, steatosis can be associated with inflammation (i.e. steato-hepatitis), which may result in liver fibrosis and can progress to cirrhosis, liver failure and hepatocellular carcinoma [2]. Recently, hepatocellular carcinoma also has been recognized as a complication of liver steatosis and emerging evidence suggests that cardiovascular disease may also be more common in patients with steatosis, even when adjusted for traditional risk factors [3, 4]. Liver biopsy is regarded as the gold standard for the assessment of steatosis. However, its use in clinical practice is limited because of its invasiveness. Therefore, various imaging methods, including ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (MRS), have been used for the non-invasive evaluation of

steatosis. US commonly detects changes in the liver parenchyma as increased echogenicity compared to the kidney and is the most frequently used tool in clinical practice. The sensitivity of US ranges from 60% to 94%, with a specificity between 88% and 95% [5-7]. The major drawbacks of ultrasonography are operator dependency and are affected considerably by increasing body mass. The sensitivity improves to 80% when liver fat exceeds 30%, but drops to 50% in morbid obesity or when the liver fat content is below 20% [8-10]. CT provides a better estimation of liver fat. Estimation of liver fat content is based on the comparison of hepatic and splenic attenuations and can predict moderate to severe degrees of steatosis with greater sensitivity when steatosis is above 33% of the liver parenchyma [11]. Both US and CT are considered qualitative tests, best suited to detect liver steatosis but not to quantify the amount of fat or to be used to follow disease progression or patients' responses to treatment. On the other hand, MRS has a very good correlation with the amount of liver fat estimated by liver biopsy [12]. The lower limit of MRS is considered to be 5% of the liver wet weight as hepatic triglycerides content [13]. MRS usually can be performed within 20 to 30 minutes but is currently limited to academic centers.

The purpose of this study was to develop a non invasive and inexpensive diagnostic method for assessment of liver steatosis. It is well known that the ultrasonic velocities of various materials have inherent values and vary with temperature. For example, the ultrasonic velocity in water is 1530 m/s at 37°C and 1534 m/s at 39°C, while that in fat is 1412 m/s at 37°C and 1402 m/s at 39°C. The rate of change of the ultrasonic velocity in water is +2 m/s at 37°C and that in fat is -4 m/s at the same temperature [14, 15]. The ultrasonic velocity increases in muscle and internal organs with a high percentage of water content, but decreases in fatty tissue. On this basis, we thought that the fat in

hepatic steatosis could be assessed by detecting ultrasonic velocity change of the liver caused by warming. In order to confirm the effectiveness of this method, we obtained the ultrasonic velocity changes of tissue phantom including lard oil and the liver of living rabbit by ultrasonic warming, and then succeeded in 2-D imaging of ultrasonic velocity changes of the phantom and the living rabbit liver. We named this the method ultrasonic velocity-change method. We wished to confirm whether this method can provide a quantitative evaluation when the liver fat content is between 5% and 20%. The experimental results showed the possibility that the assessment of hepatic steatosis could be achieved using our novel and noninvasive imaging method.

2. Principle of ultrasonic velocity-change method

In general, biological tissues consist mainly of water and their ultrasonic velocities increase with rising temperature. On the other hand, the velocities of tissues containing fat decrease with rising temperature. The ratio of ultrasonic velocity change is +1.9 m/s in water but it is -4.9 m/s in fat, therefore, it may be possible to quantify the percentage of fat on the basis of the change in ultrasonic velocity with temperature. In the liver, the change in ultrasonic velocity with respect to temperature depends heavily on the percentage of fat [16]. Fig.1 shows the basic experimental setup for the ultrasonic velocity-change method, which consists of a water tank kept at constant temperature, a block containing fat dispersed in water, the ultrasonic linear array transducer and the warming ultrasonic transducer. The ultrasonic linear array transducer transmits and receives the ultrasonic pulse wave. The ultrasonic pulses emitted from the array transducer are reflected from the various boundaries of different acoustic impedance. As shown in Fig.1, the echo pulses reflected at the boundaries shift according to the

ultrasonic velocity change caused by the local temperature rise [17, 18] with the warming ultrasonic transducer.

The echo pulses reflected at the boundary surfaces I and II are received by the same transducer. After warming, the echo pulse becomes faster when it propagates in the water-rich domain and reflects back from boundary surface I ($\Delta\tau_1$), but becomes slower when it propagates in the fat containing domain and reflects back from boundary surface II ($\Delta\tau_2$). Consequently, the difference in shift may be expressed as $\Delta\tau_1 + \Delta\tau_2$. The pulse shift between the bi-border ($\Delta\tau_n$) distinguishes the latter shift ($\Delta\tau_{n-1} + \Delta\tau_n$) from the former ($\Delta\tau_{n-1}$). The round trip time of the echo pulse between the boundaries and the time difference are denoted by τ_n and $\Delta\tau_n$, respectively, so that the velocity change, Δv_n , of the warmed region may be represented by $\Delta v_n = v \frac{\Delta\tau_n}{\tau_n}$, where v is the ultrasonic velocity.

The echo waveform of each acoustic scan line was divided into appropriate sections with the width of the transmitted pulse. The cross-correlation between the corresponding section of the waveform data stored before and after warming was calculated to obtain the time difference, $\Delta\tau$, of the echo pulse shift induced by the temperature change. The ultrasonic velocity-change is constructed from $\Delta\tau$ and τ for each acoustic scan line.

The ultrasonic velocity change Δv by the temperature change ΔT is represented as follows,

$$\frac{\Delta v}{\Delta T} = \left(\frac{\Delta v}{\Delta T}\right)_w \cdot x + \left(\frac{\Delta v}{\Delta T}\right)_f (1 - x),$$

where x is the fat content, $(\Delta v/\Delta T)_w$ is the temperature dependence of ultrasonic velocity in water and $(\Delta v/\Delta T)_f$ is that in fat. As $(\Delta v/\Delta T)_w$ and $(\Delta v/\Delta T)_f$ can be determined in advance, the fat percentage x can be determined from the Δv and ΔT values of the object. Δv can be obtained by the ultrasonic velocity-change imaging method. ΔT is required for the calculation.

3. Experimental verification for effectiveness of the imaging method

3.1 Preliminary experiment using tissue phantom

Before the experiment using living rabbits, we assessed different fat content of the tissue phantoms containing lard oil manufactured by OST Co., Ltd. (Tokyo, Japan). The lard-free phantom was composed of water, agar, talc, thickener and 1- propanol, and the proportions (by weight) of the water, agar, talc, thickener and 1- propanol were 85.54, 1.20, 3.60, 1.20 and, 8.46%, respectively. The lard phantom manufactured by adding sodium dodecyl sulfate and lard to the lard-free phantom. A conventional pulse echo system (5077PR, Olympus) was used to obtain the pulse shift in the lard-free phantom and the phantoms containing 10%, 20% and 30% lard. We measured the ultrasonic velocity in each phantom at 30°C. Then, we obtained the ultrasonic velocity change for each phantom at various temperatures between 30°C and 40°C. Fig. 2 shows that the ultrasonic velocity changes depend on the percentage of fat. Linear approximations of the velocity change rates of the lard-free phantom and the phantoms containing 10%, 20% and 30% lard gave values of 0.090, -0.001, -0.110 and -0.183, respectively. This shows that the fat contents of the phantoms were assessed quantitatively by ultrasonic velocity change under the given temperature change.

3.2 Experiment using living animal

Figure 3 shows the experimental system configuration which consists of the ultrasonic imaging equipment (Model:SSD-500,Aloka, Japan) with the 7.5 MHz linear array probe assembled with 128 channels , the high speed signal processing unit (PX-I, National Instruments Corporation, Austin, TX) that converts continuous RF signals of SSD-500 to digital signals at 10bits and 100MHz and records continuously these signals in the storage module, and the warming circular ultrasonic transducer (35mm in diameter, US-710, Ito Co., Ltd, Tokyo, Japan) driven by a continuous sine wave of 1MHz. The tip of the warming ultrasonic transducer has an attachment of silicone resin for good contact with the skin surface. The output of the warming transducer is 1 W/cm² and within the safety level of living tissue. We developed software for correction of the movement by breathing and heartbeat during acquisition of velocity change data.

Pathogen-free male Japanese White rabbits, about 1-year-old and weighing 3.0 to 3.5 kg, were obtained from Oriental Yeast (Tokyo, Japan). The rabbits were housed at a constant temperature, divided into two groups and given either 100 g/day of a standard diet (SD) (n=3, CR3 obtained from CLEA Japan Inc., Tokyo, Japan) or a high-fat and -cholesterol diet (HFD) (n=6), which consisted of SD supplemented with 12% corn oil and 1.50% (w/w) cholesterol, for 2-4 weeks [19, 20]. The rabbits were fasted for 24 hours before ultrasonography and sacrifice. Then, they were given an intravenous anesthetic, examined and laparotomized for blood and liver sampling. For removal of the liver, the portal vein of each animal was cannulated using an 18-gauge Teflon catheter and the liver was perfused with 100 ml of PBS to remove the blood. After harvesting, the liver was cut into pieces and the samples were either frozen or fixed in 4% paraformaldehyde. The experiments were conducted humanely in accordance with

the recommendations of the Guide for the Care and Use of Laboratory Animals of Osaka City University School of Medicine.

The ultrasonic velocity change in each phantom was measured in a constant temperature bath which permitted temperature variation from 32°C to 42°C. In the animal model, we performed warming for 60 seconds and stored the moving digital images for 10 seconds after switching off the warming source.

Paraformaldehyde-fixed specimens were cut into 5 µm thick sections and stained with H&E. Frozen sections (5 µm thick) were stained with Oil red O (Wako Pure Chemical Co., Osaka, Japan). The areas stained by Oil red O were measured using an image analyzer (Lumina Vision, Mitani Corporation, Tokyo, Japan) to quantify the areas of lipid deposition.

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured at the Special Reference Laboratories (Osaka, Japan). As biochemical parameters, levels of total cholesterol (TC), triglycerides (TG), and fatty acids (FA) in the livers were measured by Skylight Biotech (Akita, Japan) using the Folch method.

Analyses were performed using JMP-11 software (SAS Institute, Cary, NC). Linear regression analysis was performed between the change rate of the ultrasonic velocity and quantitative histologic analyses for fat accumulation and lipid extraction in each rabbit.

As shown in Table 1, the liver weights of the rabbits on the HFD were significantly higher than those of rabbits on the SD (Fig.4). More hepatocytes were positive for Oil red O staining in the HFD group than in the SD group (Fig.5). Furthermore, the hepatic TC content was significantly higher in the HFD group (HFD2w, 39.2 ± 3.4 mg/g; HFD4W, 50.9 ± 15.3 mg/g) than the SD group (2.7 ± 0.5 mg/g), although the hepatic

TG content was similar. These data indicate that, in this animal model, the lipid droplets in the hepatocytes are composed mostly of cholesterol. Simple regression analyses of the relationship between histology and biochemical parameters indicated that the area positive for Oil red O staining correlated significantly with the hepatic lipid, made up of TC, TG and free cholesterol ($r=0.808$, $p<0.01$).

We assessed the performance of the ultrasonic velocity-change method *in vivo* using the living rabbit model. The echo data of 100 lines in the central portion of the 256 lines constituting the B-mode image were employed for determination of the ultrasonic velocity change. The ultrasonic velocity change was calculated from the echo waveforms on each line. The average and the standard dispersion of values of ultrasonic velocity change values of 100 lines were calculated. Fig. 6 shows a high correlation between the rate of change of ultrasonic velocity and the histochemical quantification of lipid droplets ($r=0.80$, $p<0.001$).

4. Discussion

In this study, the novel principle of ultrasonic velocity change dependent on temperature was implemented successfully in an animal model of steatosis. If this system can be developed for clinical examination, it should provide a non-invasive, inexpensive and rapid method for detection of fat in the human liver. This study is the first to report the development of a new method for quantification of fat.

Although Horinaka et al. studied the first system using optical assistance for warming [17, 18], we used low frequency ultrasound for warming deeper tissue. The safety of the transducer used for warming has been confirmed and the ultrasonic transducer (1or2MHz) is suitable for warming the liver, which is a deep organ. We assume that

stable warming is possible at a range of 10 cm from the surface of the human body. Regarding the timing of the measurement of the change of ultrasonic velocity, we assessed this before and after the warming process. When we accumulated the data for warming, we found that measurement during cooling was quite reliable. Therefore, we carried out the measurement from the maximum temperature until the normal temperature was achieved. In the animal experiments, the temperature change in the liver was minimal. When the rabbits' livers were warmed by the ultrasonic transducer, the organs received no damage according to the normal alanine aminotransferase (ALT) value and comparison with sham operation.

The Dixon method of MRI and MRS are widely regarded as the most accurate non-invasive methods for the clinical assessment of hepatic steatosis [21-23]. However, MRI techniques and MRS are somewhat impractical because of their lengthy scan times, their reliance on the compliance of the patient, respiratory artifacts and particular locality, and they typically sample only a portion of the liver. This new methodology based on ultrasonography has the advantage of being non-invasive, with a short scan time, no respiratory artifacts in human patients, occupying little space and assessing a significant proportion of the liver.

Animal models may be critical for the evaluation of novel methodologies and, in this study, harvesting of the entire livers from the rabbits enabled accurate assessment of fat accumulation. This is not possible in humans because only small biopsy samples may be available [24, 25]. Although this new method was validated in the animal model by correlation with the post-mortem results, including histologic analysis and hepatic lipid extraction, additional clinical validation is required in patients, correlating the imaging results with examination of biopsy tissue. In addition, the reproducibility of this method

must be determined before the clinical utility can be ascertained. Although investigators usually remove the animal liver for analysis, there may be individual variation, such that the model is not suitable for the calibration of an imaging system. We developed the phantom consisting of lard as a physical model of fat accumulation. This phantom was used for optimization and comparison in this system.

Our study has several limitations. A major limitation was the inability to measure the temperature directly and noninvasively in the rabbit and human livers. Although the change rate of ultrasonic velocity could be obtained without precise temperature data, the accurate measurement of the deep body temperature contributed to improving quantitation and safety. There are methods for estimating deep body temperature that rely on calculation of thermal diffusion in the bloodstream and the coefficient of heat conduction from the border between the liver and the kidney, which does not accumulate fat. Safety is an important consideration for future human application of this method in preclinical or clinical studies. According to the American Institute of Ultrasound in Medicine (AIUM), no significant adverse biological effects were observed due to temperature increases of less than or equal to 2°C above normal for exposure durations up to 50 hours. The experiments in vivo using the living rabbit model were made under 1W/cm² of warming transducer. The maximum temperature increase in the livers of the rabbits is estimated to be 2.0 °C. The intensity of ultrasonic for warming can be set lower because the ultrasonic velocity change can be detected even under a temperature change of 0.5°C. Another limitation is the influence of the respiration artifact in the animal model. We carried out the experiments with the rabbits under anesthesia and observed the respiration and heartbeat artifacts. In order to use this technique on the living body, the images must be constructed in the presence of

respiration and heartbeat. In this evaluation of the ultrasonic velocity change method, we developed new software for image construction and this enabled clear images to be obtained from the anesthetized rabbits. The experimental results showed that the ultrasonic velocity change-images clearly displayed fat accumulation. The software is based on template matching using Zero-mean Normalized Cross Correlation [26]. However, image construction for humans will be simpler than the animal model because patients can hold their breath during measurement.

Subcutaneous fat did not affect the measurement in the rabbit experiment but, in patients, this produces individual differences in terms of the depth of measurement. Even though subcutaneous fat did not affect the image constructed from the pulse signal, it may affect the warming process. The ultrasonic wave can warm a wide area when the output of the wave form is changed but it can be focused on the measurement area for precision warming.

5. Conclusion

We have evaluated the ultrasonic velocity-change method as a new methodology and analyzed the strong correlation with conventional fat quantitation methods in a living rabbit model of non-alcoholic steatohepatitis. Our experimental results provide evidence that the ultrasonic velocity-change method enables accurate quantification of liver fat in the living rabbit and has potential for the quantitative assessment of hepatic steatosis in clinical practice.

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Figure legends

Figure 1. Principle of the ultrasonic velocity change imaging method

Left, schema of the position of the ultrasonic transducers for imaging and heating.

Right, the sequential of pulse shift.

I, II: tissue boundaries.

Figure 2. Measurement of ultrasonic velocity by the Pulser-Receiver in the physical model.

Figure 3. Application of the ultrasonic velocity change imaging system to an animal model.

A. An image of the experiments with the rabbit under anesthesia. B. Schema of the system applied to the animal model.

Figure 4. Histochemical observation of hepatic steatosis by oil red O staining. SD, standard diet; HFD, high fat diet. The black bar is 200 μ m.

Figure 5. Macroscopic image of hepatic steatosis in the removed liver. SD, standard diet; HFD, high fat diet.

Figure 6. Measurement of the rate of change of ultrasonic velocity in the animal model using our system. Open circles, standard diet; filled circles, high fat diet.

Table 1. Characteristics of the rabbit model

	BW [g]	LW [g]	Area [%]	TC [mg/g]	TG [mg/g]	Lipid [mg/g]
SD-1	3900	68	1.16	2.8	19.5	24.9
SD-2	3100	60	0.68	2.2	12.3	16.9
SD-3	3400	77	2.83	3.1	37.3	42.9
HFD2W-1	3200	86	2.20	43.1	27.3	76.9
HFD2W-2	3200	99	8.87	36.8	19.7	63.1
HFD2W-3	3200	85	6.52	37.7	23.6	67.6
HFD4W-1	3300	104	4.69	33.7	13.9	56.1
HFD4W-2	3200	108	9.61	56.0	43.8	107.7
HFD4W-3	3300	102	8.37	63.1	21.3	93.4

SD, standard diet; HFD, high fat diet; BW, body weight; LW, liver weight; TC, total cholesterol; TG, triglycerides.

Area, measurement of the positive area in Oil red O staining.

The values of TC, TG, and lipid are given for 1 gram of the liver.

Lipid, total amount of the TC, TG and free cholesterol.

Figure 1

Ultrasonic array transducer

Figure 2

Figure 3

A

B

Figure 4

Figure 5

Figure 6