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Research

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Viscoelasticity measured by shear wave elastography in a rat model of NAFLD: comparison with dynamic mechanical analysis

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) is rapidly becoming one of the most common liver diseases. Ultrasound elastography has been used for the diagnosis of NAFLD. However, clinical research on steatosis by elastography technology has mainly focused on steatosis with fibrosis or non-alcoholic steatohepatitis (NASH), while steatosis without fibrosis has been poorly studied. Moreover, the relationship between liver viscoelasticity and steatosis grade is not clear. In this study, we evaluated the degree of liver steatosis in a simple steatosis rat model using shear wave elastography (SWE).

Results: The viscoelasticity values of 69 rats with hepatic steatosis were measured quantitatively by SWE *in vivo* and validated by a dynamic mechanical analysis (DMA) test. Pathological sections were used to determine the steatosis grading for each rat. The results showed that the elasticity values obtained by the two methods followed the same trend, and the elasticity is significantly correlated with liver steatosis. The Pearson's correlation coefficients indicate that elasticity obtained by SWE is positively linear correlated with DMA ($r = 0.628$, $p = 7.85 \times 10^{-9}$).

Conclusion: SWE might have the feasibility to be introduced as an auxiliary technique for NAFLD patients in clinical settings. However, the viscosity results measured by the two methods are significantly different because the two methods work in different frequency bands.

Keywords: Nonalcoholic fatty liver disease, shear wave elastography, Dynamic mechanical analysis, Viscoelasticity

Background

Nonalcoholic fatty liver disease (NAFLD) is a main cause of chronic liver disease (CLD) and is rapidly becoming one of the most common liver diseases, with an approximate prevalence of 20%–30% of the total population in Western countries [1, 2]. It represents a spectrum of diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), which has a close relationship with liver cirrhosis and hepatocellular carcinoma [3-5]. NASH is critical in the development of liver fibrosis and liver failure and has become a new challenge in the field of liver disease research.

Many different techniques have been studied in the diagnostic of NAFLD. Magnetic resonance elastography (MRE) uses a modified phase-contrast method to image the propagation characteristics of the shear wave in the liver. Early researches of MRE suggest that it could accurately differentiate simple steatosis from NASH with or without fibrosis. However, MRI is costly and time consuming for use in routine clinical practice [6]. Recently, several ultrasound-based elastography techniques, including transient elastography (TE) [7-9], acoustic radiation force impulse (ARFI) [10] and shear wave elastography (SWE) [11], have been used for the noninvasive diagnosis of liver disease. Compared with liver biopsy or other imaging methods, ultrasound

elastography has the advantages of a lower cost and a smaller time commitment. As the procedure is non-invasive, it can supersede biopsy-related postoperative complications and potential diagnosis error due to a small sampling range [12].

The mechanical properties of liver tissue may be helpful in the diagnosis of liver diseases. Many studies had shown that liver stiffness measured by elastography techniques is strongly linked to liver fibrosis [13, 14]. The World Federation for Ultrasound in Medicine and Biology (WFUMB) recently produced guidelines which recommended the use of elastography techniques for distinguishing advanced fibrosis ($\geq F2$) from early fibrosis ($\leq F1$) [15]. However, the mechanism of hepatic steatosis development is different from that of fibrosis. Some researches on steatosis by elastography technology mainly focused on steatosis with fibrosis, while steatosis without fibrosis has been poorly studied. The relationship between steatosis severity and liver stiffness has not been determined [16-18]. Many studies have suggested that liver steatosis with the accumulation of lipid deposits may cause changes of viscoelasticity rather than stiffness. Zhu et al. proposed that the characterization of shear wave dispersion is significantly related to various oil percentages in gelatin phantoms [19]. Barry et al. showed that an increase in liver steatosis clearly increased the viscosity in a mouse liver model, *ex vivo* [20]. Deffieux et al. [21] found no obvious correlation of liver viscosity and dispersion curve slope with steatosis or disease activity. However, the relationship between liver viscoelasticity and steatosis grade is not clear.

Mechanical tests have been used as another method to assess tissue mechanical properties [22-25]. For example, the dynamic mechanical analysis (DMA) is considered a conventional “gold standard” method to assess the mechanical properties of tissues and can determine the viscoelastic properties of tissues. Many studies have used DMA tests to measure the mechanical properties of liver tissue for comparison of various ultrasound elastography technique results [26, 27].

Our previous study showed the feasibility of assessing the viscoelasticity of liver in a rat model of steatosis by the DMA test [25]. In this study, we used shear wave elastography to evaluate the viscoelasticity of rat liver with steatosis. A DMA test was performed by oscillatory shear test on a liver sample from the same rat model as before, and then parameters, including the elasticity and the viscosity, were subjected to comparative analysis with variation in the grade of liver steatosis.

Results

Histologic and biochemical characteristics

liver tissue sections from mice in each group after SWE measurements were stained with Oil Red O and examined under the light microscope. Fat droplets in liver tissue were increased significantly as the stages of steatosis increased. Fig. 1 shows representative histologic findings according to the severity of NAFLD.

Fig. 1 Typical specimens of five rat livers at different liver steatosis stages. (a-e) S0–S4.

Use of SWE and DMA for assessing the grade of steatosis

The elasticity and viscosity values of 69 rats for S0–S4 obtained by the two methods are shown in Fig. 2. For each stage, the rats in this stage were sorted according to their mean elasticity values obtained by DMA method. The mean elasticity and viscosity values for each stage are listed in Table 1. The mean values of elasticity obtained by SWE (from 0.86 ± 0.27 kPa to 1.05 ± 0.29 kPa) were slightly lower than those by DMA (from 1.12 ± 0.21 kPa to 1.39 ± 0.31 kPa). For viscosity, the values of DMA (from 5.40 ± 0.78 Pa*s to 6.27 ± 0.96 Pa*s) were significant greater than those of SWE (from 0.75 ± 0.10 Pa*s to 0.92 ± 0.18 Pa*s).

Table 1 The mean viscoelasticity parameters of all rat livers measured by SWE and DMA methods (n represents the number of rats. In SWE, 10 points in each rat liver were measured in vivo, and 3 liver samples were extracted in random from each rat in DMA, values are the mean $\pm$ standard deviation.)

Degree of liver steatosis	n	SWE		DMA	
		Elasticity (kPa)	Viscosity (Pa*s)	Elasticity (kPa)	Viscosity (Pa*s)
S0	17	0.86 ± 0.27	0.77 ± 0.15	1.12 ± 0.21	5.40 ± 0.78
S1	14	0.93 ± 0.23	0.76 ± 0.20	1.25 ± 0.26	5.77 ± 0.71
S2	19	1.05 ± 0.29	0.79 ± 0.14	1.39 ± 0.31	6.27 ± 0.96
S3	12	1.01 ± 0.20	0.75 ± 0.10	1.29 ± 0.18	5.97 ± 0.68
S4	7	0.90 ± 0.24	0.92 ± 0.18	1.31 ± 0.14	6.20 ± 0.54

Fig. 2 The mean and standard deviation of the elasticity and the viscosity in 69 rats for both methods. For each stage, the rats in this stage were sorted according to their mean elasticity values obtained by DMA method.

The box plots in Fig. 3 illustrate the variations of elasticity and viscosity of the two methods for each stage. The results of the ANOVA test with Tukey–Kramer multiple comparison tests found out the group pairs which had significant differences in elasticity or viscosity.

Fig. 3 Boxplot of (a) the elasticity and (b) the viscosity obtained by the two methods at five steatosis stages. The upper and lower boundaries of the boxes represent the 25th and 75th percentiles, lines within the boxes represent

medians, squares within the boxes represent means, and error bars represent ranges. *Asterisks* indicate the pairs having statistically significant differences in the Tukey–Kramer test after the ANOVA test. * $p < 0.05$, ** $p < 0.01$.

Correlation of SWE and DMA on the elasticity and the viscosity

Correlations between the SWE and DMA for the elasticity and viscosity are illustrated in Fig. 4. The Pearson's correlation coefficients indicate that elasticity obtained by SWE is positively linear correlated with DMA ($r = 0.628$, $p = 7.85 \times 10^{-9}$), while there is almost no correlation between viscosity obtained by SWE and DMA ($r = -0.01$, $p = -0.91$).

Fig. 4 Correlative analysis between the SWE and DMA about (a) the elasticity and (b) the viscosity at five steatosis stages.

Discussion

NAFLD is known to be a complex disorder and includes simple steatosis, NASH and fibrosis. In clinical situations, steatosis and fibrosis are usually interleaved together, and it is difficult to distinguish their separate effects on the mechanical properties of the liver tissue. Many clinical researchers of elastography technology have mainly focused on steatosis with fibrosis or NASH. Grimal et al. [28] investigated the relationship between liver tissue stiffness and histological inflammation score, hepatic fibrosis stage, ballooning score, steatosis analyzed by SWE in rats with NASH, and found that median liver stiffness values measured using SWE showed a stepwise increase with increasing steatosis grade ($p = 0.03$), the results were consistent with those of [29] that demonstrated that liver elasticity was effective in detecting NAFLD. However, Nightingale et al. [30] performed shear wave dispersion with a linear dispersion model to perform shear wave dispersion analysis in traditionally “difficult-to-image” subjects in 135 NAFLD patients. Nevertheless, viscoelastic parameters were not related to the steatosis stage. The presence of entirely different results in the relationship between liver viscoelasticity and steatosis stage may have many causal factors, such as different frequency ranges corresponding to different viscoelastic parameters, experimental conditions, machines and operators. Moreover, the role of

SWE measurement *in vivo* is more complicated because of the complex structure of the organism and variable factors. It also becomes difficult to evaluate when a patient has more than just a single disease in the clinical setting. Parker et al. [31] summarized numerous studies about ultrasound shear wave dispersion in healthy and steatosis livers and concluded that animal models and human populations need further investigation.

In this study, we used a rat model of liver steatosis, which avoided the confounder of liver fibrosis, thus we can focus on studying the effect of steatosis on viscoelasticity. Disease development in the soft tissue is always accompanied by a variety of mechanical properties of the tissues. The viscoelastic properties of tissue are promising for utilizing the inherent mechanical properties of tissue for grading disease. To this end, we performed liver viscoelasticity measurements using SWE in rat models with various degrees of steatosis severity. The DMA tests were used as a standard of reference, and the values of elasticity and viscosity obtained by SWE were compared to the degree of steatosis as evaluated by histological assessment. In this study, we used the Voigt model to obtain the viscoelasticity parameters in a rat model of NAFLD. We found that the values of elasticity obtained by SWE increased from stage S0 to S2 (Fig. 3(a)), which was similar to the trend of DMA. Interestingly, the values decrease slightly from stage S2 to S4, which is also similar to the DMA. Although the trend between the values of elasticity obtained by SWE is consistent with DMA, the p values of elasticity obtained by SWE were slightly greater than 0.05 and not statistically different among the five steatosis stages. The elasticity of DMA was statistically different between stages S0 and S2–S4. The values of elasticity obtained by SWE were relatively lower than those obtained by DMA. However, compared with the viscosity of DMA, the results of SWE were significantly lower, and there was no difference from S0 to S3. The mean value of S4 was higher than other stages (Fig. 3(b)). The trends of the five stages of viscosity obtained by SWE were not consistent with those obtained by DMA [19]. Although some phantom studies have shown that viscosity can provide relevant and independent information about the inherent state, until now, few researchers could determine the viscoelasticity parameters *in vivo* for the diagnosis of fatty liver disease.

Despite the results obtained by SWE based on shear wave dispersion, accurate staging of the grade of liver steatosis did not prove statistically significant. However, it is interesting to evaluate the values of viscoelasticity relationships between SWE and DMA. In Fig. 4, the mean values of SWE were highly correlated with those obtained by DMA; the correlation coefficient was 0.628, and $p = 7.85 \times 10^{-9}$, indicating significant linear correlation. However, the mean values of viscosity measurements obtained by SWE were relatively independent of those obtained by DMA with a correlation coefficient of -0.01 . This result suggests that the elasticity may provide useful information about the liver steatosis status obtained by SWE. There is little relationship between SWE and DMA in terms of viscosity, suggesting that it has a weak correlation with liver steatosis, which is consistent with previously reported results [21].

The viscosity results measured by the two methods are significantly different, because

the measurements depend heavily on the frequency bands. It is focused to investigate why the elasticity values measured by SWE have the same trend with the DMA but without a statistical difference among the five steatosis stages. There may be several reasons for this result. First, DMA has much lower frequencies than SWE, which may lead to different vibrations in microstructures of liver tissue. Also, the frequency ranges of the shear wave measured using SWE may not reveal the actual viscoelastic properties of the tissue[32]. In DMA tests, the frequency ranged from 1 to 41 Hz, while the shear wave frequency of SWE ranged from 160 to 380 Hz. Specifically, the excised liver has no blood circulation in the DMA tests. Blood flow can affect the viscoelasticity measurement of the liver by SWE [33]. In addition, the limited size of the species utilized and ultrasound imaging quality considerations can also affect the results.

Conclusion

In this study, we compared the measurement of viscoelastic parameters, including elasticity and viscosity, by DMA tests and SWE methods in a rat model of NAFLD at five steatosis stages. The results showed that the elasticity values from the two methods followed the same trend, suggesting that elasticity has a significant correlation with liver steatosis. To some extent, elasticity values measured by SWE can reflect liver steatosis processes. SWE might have the feasibility to be introduced as an auxiliary technique for NAFLD patients in clinical settings.

Materials and Methods

Animal model

A total of 69 male Sprague-Dawley (SD) rats (weighing 200 ± 20 g, Guangdong Medical Laboratory Animal Center, Foshan, China) were used in this study. They were maintained under Specific Pathogen Free (SPF) conditions (relatively constant temperature: 20–26°C humidity: 30–50%, 10 h: 14 h light/dark rhythm alternately) with a high-calorie diet. To induce hepatic steatosis in different stages (S1–S4), a high-calorie diet for various numbers of days were supplied to SD rats at a dose of 1 mL/100 g rat weight once a day for sixty days. The control group (S0) was fed with a regular diet. Rat livers were harvested for DMA tests and histologic assessment after the rat had been used for *in vivo* ultrasound measurements. The histopathologic examination ultimately determined the hepatic steatosis stage. The number of animals in each group was stated in Table 1. Animal care and experiments were approved by the Animal Care and Use Committee of School of Medicine in Shenzhen University and Guangdong Medical Laboratory Animal Center.

In vivo SWE measurement

In this experiment, the rat was placed in the supine position on the experimental table after anesthesia at a relatively constant temperature of 24°C–26°C. The rat abdomen was shaved and coated with ultrasonic gel, and then an ultrasound B-mode image was used for localization of the liver and a region of interest (ROI) (size: 5mm×15mm) in the median lobe of the liver, devoid of large vessels, was chosen for measurement. After the ROI of the liver was selected, the operator kept the probe stable and switched to the SWE mode to finish the measurement. To obtain a relatively accurate result, for each rat, measurements were repeated ten times in ten locations, and the calculated elasticity and viscosity values were the mean of ten valid measurements.

The SWE measurement was performed on the Verasonic vantage 256 systems (Verasonics Inc., Kirkland, WA, USA) with a linear array transducer L11-4v (Philips Healthcare, Andover, MA). The measurement procedure is similar to that used in the previous study [34]. First, a B mode image of the liver was obtained to provide imaging guidance for selecting the measurement locations, then a region of interest (ROI), 5 mm axially × 15 mm laterally, was selected for measurement. A total of 20 array elements were used in one excitation, which consisted of three successive push beams to create a Mach cone [35]. These three push beams were focused at -5 mm, 0 mm, and 5 mm deep relative to the center of the ROI with a time interval of 50 microseconds. The push beam at each depth was generated by an 80 microsecond tone burst at a center frequency of 4.4 MHz. Second, the system was switched immediately to the plane wave imaging mode after the push wave transmission. All elements were set to fire 50 detect pulses with a center frequency of 6.25 MHz and a pulse repetition frequency of 10 kHz. Next, the data post-processing steps include: (1) In-phase quadrature (IQ) data within the ROI was processed with a 2D correlation algorithm to obtain particle velocity [36]; (2) a 3×3 median filter was used to the liver tissue to remove spike noise, cubic spline interpolation with a factor of five was conducted to reconstruct the displacement profiles to increase the temporal resolution; (3) low-pass filtering with a cut off frequency of 1000 Hz was used to reduce jitter after the reverberation frames were removed, then a Fourier transform was used to estimate the phase at each frequency; (4) then a directional filter was applied to reduce the artifacts from reflected shear waves at boundaries, a linear regression algorithm was used to calculate the shear wave phase velocities at each frequency (160–380 Hz) by estimating the average phase difference [37]; (5) finally the values of viscoelasticity were estimated by MATLAB (Mathworks, R2014a) with a nonlinear fitting method using Equation 1 based on a least-squares criterion.

Ex vivo DMA experiment

Each rat liver was harvested immediately for *ex vivo* DMA test after the *in vivo* ultrasound measurement. The livers were soaked with saline solution to prevent desiccation of liver specimens and kept in the refrigerator at a temperature of 4°C to preserve freshness.

Fig. 5 The dynamic mechanical analysis (DMA) experimental platform.

Fig. 1 shows the DMA experimental instrument (the ElectroForce3200 Series, Boss Corp., Eden Prairie, Minnesota, USA) operating at a parallel plate shear mode. Two to three liver specimens were taken from each rat, and then each specimen was cut by a scalpel into the same shape (size: 15mm×5mm×4mm, thickness: 4 ± 1 mm) to match the parallel plates. During the experiment, the liver specimen was fixed to the fixture in the water tank which was filled with a saline solution at a body temperature of $38.1 \pm 0.2^\circ\text{C}$. Sandpaper was attached between the parallel plates to prevent the liver specimen from slipping. The motion controller was rotated slowly to ensure sufficient contact between the parallel plates. After the sample was placed on the parallel plates, the sample slowly received a precompression of 2–3 g to ensure sufficient contact between the plate and the sample and avoid slippage. A sinusoidal torque was engendered by the machine to make the liver specimen generate stress and strain. Then, the dynamic mechanical properties, including storage modulus G' and loss modulus G'' were directly obtained from the system during the test. The phase velocities at all frequency points in the range of 1–41 Hz with an interval of 5 Hz were substituted in Equation 3 and Equation 3, and then shear elasticity μ and shear viscosity η of every sample were estimated by non-linear curve-fitting.

Methods

In this study, it is assumed that the liver consists of viscoelastic and isotropic homogenous material. The relationship between the shear wave velocity and frequency was derived from the Voigt model. The equation is as shown below [38]:

$$c_s(\omega) = \sqrt{\frac{2(\mu^2 + \omega^2\eta^2)}{\rho(\mu + \sqrt{\mu^2 + \omega^2\eta^2})}} \quad (1)$$

where c_s is the shear velocity, ω the angular frequency, ρ the mass density, μ and η are the shear elasticity and shear viscosity.

The principle of DMA is performed as previously described [25]. Briefly, for homogeneous biological tissue, the sinusoidal shear strain $\varepsilon(t) = \varepsilon_0 e^{j\omega t}$ induces a corresponding sinusoidal stress $\sigma(t) = \sigma_0 e^{j(\omega t + \delta)}$. The complex shear modulus G^* is defined as the ratio of stress to the strain:

$$G^*(\omega) = \frac{\sigma(t)}{\varepsilon(t)} = \frac{\sigma_0 e^{j(\omega t + \delta)}}{\varepsilon_0 e^{j\omega t}} = \frac{\sigma_0}{\varepsilon_0} (\cos \delta + j \sin \delta) = G'(\omega) + jG''(\omega) \quad (2)$$

Where ω is angular frequency, δ is a phase difference, σ_0 and ε_0 are the shear stress amplitude and strain amplitude, respectively. The real part of the Equation 2, $G'(\omega)$ is the storage modulus relating to the elasticity parameter, μ , while the imaginary part $G''(\omega)$ is the loss modulus relating to the viscosity η . To quantitatively analyze the viscoelastic properties of the tissues, the complex modulus is expressed by $G^* = \mu + j\omega\eta$, which is derived from the Voigt model [39]. Therefore, the storage moduli and loss moduli of the liver are expressed as follows:

$$G' = \mu \quad (3)$$

$$G'' = \omega\eta \quad (4)$$

20 Histological assessment

The rest of each rat liver tissue was fixed in the 10% neutral formalin liquid, and conventional methods of dehydration, paraffin-embedded sections and Oil Red O staining were conducted by histopathology technicians in the Guangdong Medical Laboratory Animal Center. Two slices, with a thickness of 7 μm , from each rat were used for the histologic examination. These pathological slices were observed under an optical microscope by pathologists who had no knowledge of the *in vivo* ultrasound measurement. Stages of steatosis were determined by quantifying percentages of hepatocellular macrovesicular steatosis: 0–4% (S0); 5–25% (S1); 26–50% (S2); 51–75% (S3); >76% (S4). Fig. 1 shows typical liver specimens for the five steatosis stages.

1 **Statistical analysis**

2 All 69 rats were divided into five groups separated by histological grade (S0–S4). For
 3 each steatosis grade, the viscoelasticity parameter values of all the rats at that grade
 4 were grouped. The mean parameter value of each rat was then used to estimate the
 5 population mean of each steatosis group. If the value exceeded more than 1.5 times the
 6 interquartile range in its group, it was eliminated as an outlier and the corresponding
 7 measurement was excluded [25]. The relationship between the SWE and DMA on the
 8 elasticity and the viscosity was analyzed using the Pearson correlation coefficient and
 9 linear regression. All data were represented as means $\pm$ standard deviation (SD) and
 10 analyzed by using the two-tailed student t-test, analysis of variance. The one-way
 11 analysis of variance (ANOVA) with the Tukey–Kramer multiple comparison tests was
 12 used to compare the liver viscoelasticity parameters among the steatosis stages.
 13 Differences were considered significant when $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$,
 14 respectively.

15 **Abbreviations**

16 NAFLD: nonalcoholic fatty liver disease; NASH: non-alcoholic steatohepatitis; SWE:
 17 shear wave elastography; DMA: dynamic mechanical analysis; MRE: magnetic
 18 resonance elastography; TE: transient elastography; ARFI: acoustic radiation force
 19 impulse; SD: Sprague-Dawley; SPF: Specific Pathogen Free; ROI: region of interest;
 20 IQ: in-phase quadrature; ANOVA: analysis of variance.

21 **Authors' contributions**

22 Zhaoke Pi and Haoming Li designed the method and drafted the original manuscript;
 23 Mengke Wang, Yanrong Guo, Xianfen Diao, and Jie Zeng participated in the
 24 experiments and analyzed the data; Siping Chen, Hui Xia, Guoqiang Liu, Xinyu Zhang
 25 and Xin Chen provided supports for the research, designed the method, and revised the
 26 manuscript. All authors read and approved the final manuscript.

27 **Availability of data and materials**

28 The data used to support the findings of this study are available from the corresponding
 29 author upon reasonable request.

30 **Ethics approval and consent to participate**

31 Animal care and experiments were approved by the Animal Care and Use Committee
 32 of School of Medicine in Shenzhen University, China (Approval No.: 20160110).

Consent for publication

All authors give their consent for publication.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Typical specimens of five rat livers at different liver steatosis stages. (a-e) S0–S4.

Figure 2

The mean and standard deviation of the elasticity and the viscosity in 69 rats for both methods. For each stage, the rats in this stage were sorted according to their mean elasticity values obtained by DMA method.

Figure 3

Boxplot of (a) the elasticity and (b) the viscosity obtained by the two methods at five steatosis stages. The upper and lower boundaries of the boxes represent the 25th and 75th percentiles, lines within the boxes represent medians, squares within the boxes represent means, and error bars represent ranges. Asterisks indicate the pairs having statistically significant differences in the Tukey–Kramer test after the ANOVA test. * $p < 0.05$, ** $p < 0.01$.

Figure 4

Correlative analysis between the SWE and DMA about (a) the elasticity and (b) the viscosity at five steatosis stages.

Figure 5

The dynamic mechanical analysis (DMA) experimental platform.