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Role of acoustic radiation force impulse imaging elastography in the assessment of steatohepatitis and fibrosis in rat models

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ABSTRACT

Acoustic radiation force impulse (ARFI) elastography is a non-invasive method for performing liver assessment via liver shear wave velocity (SWV) measurements. The aim of this study was to evaluate the performance of the ARFI technique in the diagnosis of nonalcoholic steatohepatitis (NASH) and fibrosis and to investigate the effect of steatosis and inflammation on liver fibrosis SWV measurements in a rat model of nonalcoholic fatty liver disease (NAFLD). The *ex vivo* right liver lobes from 110 rats were processed and embedded in a fabricated gelatin phantom, and the other lobes were used for histologic assessment. The SWV induced by acoustic radiation force was derived to evaluate liver stiffness. The experimental results showed that the liver SWV value could be used to differentiate non-NASH rats from NASH-presenting rats and NASH from cirrhosis, and these comparisons showed areas under the receiver operating characteristic curves (AUROC) of 0.951 and 0.980, respectively. The diagnostic performances of ARFI elastography in predicting severe fibrosis ($F \geq 3$) and cirrhosis ($F \geq 4$) showed AUROC values of 0.997 and 0.993, respectively. In rats with mild fibrosis (F0–F1), severe steatosis had a significant effect on the mean SWV values. In rats with significant fibrosis (F2–F4), severe lobular inflammation had significant effects on the mean SWV values. Our findings indicate that ARFI elastography is a promising method for differentiating non-NASH rats from NASH rats and for staging hepatic fibrosis in NASH. The presence of severe steatosis and severe lobular inflammation are significant factors for evaluating fibrosis stages.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is increasingly recognized as the hepatic manifestation of insulin resistance and the systemic complex known as metabolic syndrome. Currently, the prevalence of this condition is approximately 20% to 30% in the general population in affluent countries, and it has the potential to become a serious public health problem [1–2]. Nonalcoholic steatohepatitis (NASH), the essential link between simple steatosis and cirrhosis in the NAFLD spectrum, could be present in one-third of NAFLD cases. Among patients with NASH, end-stage NASH carries a high risk of cirrhosis, liver transplant, and hepatocellular carcinoma [3–5]. Therefore, the assessment of patients with NASH has important prognostic implications that should be considered when counseling and monitoring patients.

The gold standard for the diagnosis of NAFLD is liver biopsy. However, this method has important sample error issues, requires subjective interpretations and can be associated with rare but serious complications. Moreover, the decision to perform a liver biopsy is even more difficult because NAFLD patients present relatively mild liver damage [6]. In an attempt to identify alternatives to liver biopsy and to subcategorize patients with NAFLD *via* different prognoses and thereby promote better management decisions, several elastography imaging methods, including acoustic radiology force impulse (ARFI) elastography, transient elastography (TE), and magnetic resonance elastography (MRE), have been proposed to measure liver stiffness as a non-invasive alternative to liver biopsy and have been studied over the past decades [7–13].

ARFI elastography uses short acoustic impulses to generate localized displacements and then measures the shear wave velocity (SWV) in the tissue, providing information on the localized stiffness of the tissue [14–15]. This technique has been implemented in high-end ultrasound systems and is used in the clinical diagnosis of liver fibrosis [16–22]. Recently, studies have investigated fibrosis in the complex pathology of NAFLD or NASH by ARFI or

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TE technology [23–29]. The corresponding results indicate that several factors, including hepatic steatosis, inflammation and ballooning, potentially affect liver stiffness measurements in the evaluation of fibrosis [7,27,30–31]. However, the conclusions are conflicting. For example, Petta et al. concluded that the presence of severe steatosis should always be considered to avoid overestimations of liver fibrosis assessed by TE in NAFLD patients [30], whereas Wong et al. found that neither steatosis grade nor necroinflammation influenced the liver stiffness values in fibrosis patients with NAFLD [31].

In our previous study, we developed a rat model with different severity grades of steatosis and used ARFI elastography to determine the performance of SWV measurements for evaluating steatosis [32]. Subsequently, that work was extended to a NAFLD model, and a NAFLD scoring system was applied to evaluate the ability of ARFI to differentiate among NAFLD histologic subtypes and diagnose the fibrosis stages of NAFLD [33]. In the present paper, ARFI elastography was used to diagnose nonalcoholic steatohepatitis according to the NASH scoring system and to investigate the effect of steatosis and inflammation on liver fibrosis SWV measurements in a rat model.

2. Materials and methods

2.1. Animal model

Male Sprague–Dawley rats (Guangdong Medical Laboratory Animal Center, Guangdong, China) weighing between 170 and 220 g were housed in sterile, isolated cages with a 10 h/14 h light/dark cycle under constant temperature (20–26 °C) and humidity (40–70%) conditions. A total of 110 rats were randomly divided into 4 groups. The first group ($n = 18$) was provided a standard diet with sterilized food and water. The second group ($n = 57$) was placed on a special high-fat emulsion diet (20% lard, 10% cholesterol, 2% sodium cholate, 0.5% propylthiouracil, and 30% fructose) that was provided once daily at 1 mL per 100 g rat weight for various periods (2, 4, 6, and 8 weeks) to induce different grades of steatosis. The third group ($n = 22$) was provided the same high-fat emulsion diet for 8 weeks. Furthermore, 50% carbon tetrachloride (CCl_4) in olive oil was injected subcutaneously twice weekly at 0.6 mL per 100 g rat weight for the first two weeks and at 0.3 mL per 100 g rat weight for the remaining weeks. The fourth group ($n = 13$) was provided a standard diet and injected with CCl_4 for 4 weeks. After gavage was complete, all rats were sacrificed by neck dislocation; the right lateral lobe of the liver was then harvested for ARFI measurements; the other lobes were used for histologic assessment. All procedures in this study were approved by the Animal Care Committee of Shenzhen University and the Guangdong Medical Laboratory Animal Center.

2.2. Liver histologic examination

The excised liver tissues were fixed in a 10% formalin solution for at least 24 h. After washing and dehydrating the tissue samples, they were embedded in paraffin and sliced to a thickness of 7 μm. The paraffin slices were stained with Oil Red O, hematoxylin & eosin (H&E), and Masson's trichrome by histopathology technicians before microscopy analysis (BX41, Olympus, Melville, NY, USA) by an expert pathologist (with 20 years of experience) who was blind to the study design, treatment groups, and ultrasound measurements. Histological scoring was performed according to the method of Brunt et al. [34]. The steatosis severity grade was evaluated based on the percentage of cells with fatty droplets: grade 0, none; grade 1, up to 33%; grade 2, 34%−66%; and grade 3, > 66%. Hepatocyte ballooning was scored on a 3-point scale: grade 0, none; grade 1, few balloon cells; and grade 2, many balloon cells/prominent ballooning. Intra-acinar (lobular) inflammation activity was evaluated as follows: grade 0, none; grade 1, 1 to 2 foci/field; grade 2, up to 4 foci/field; and grade 3, > 4 foci/field. Portal inflammation was graded as none, mild, moderate, or severe (0–3). The extent of fibrosis was evaluated to determine the unique zone 3 perisinusoidal fibrosis stage: stage F0, none; stage F1, perisinusoidal or periportal fibrosis; stage F2, perisinusoidal and portal/periportal fibrosis; stage F3, bridging fibrosis; or stage F4, cirrhosis. The NASH activity grade was calculated according to numerical assessments of the variables (steatosis, 0–3; lobular inflammation, 0–3; portal inflammation, 0–3; and ballooning, 0–2). The final pathologic diagnosis was denoted mild, moderate or severe [34].

2.3. Liver SWV measurement

The local liver SWV was measured by ARFI elastography, which has been referred to as ARFI quantification or point shear-wave elastography in the literature [35]. This technique focuses on a region of interest (ROI). Tissue in the ROI was mechanically excited by an excitation pulse, and then a series of A-line tracking pulses were transmitted to monitor the resulting tissue displacement. Normalized cross-correlation of the tracked echoes was used to estimate the tissue displacements. By measurements of the time to peak displacement at multiple lateral locations, the shear wave velocity of the tissue can be reconstructed [36].

In an *ex vivo* experiment, the right liver lobe was processed and embedded in a fabricated gelatin phantom (gelatin from porcine skin, G2500, Sigma-Aldrich, St. Louis, MO, USA) in a container ($11 \times 11 \times 7$ cm). ARFI elastography, implemented on an ultrasound system (Acuson S2000, Siemens, Erlangen, German) with an 8 MHz linear ultrasound transducer (9L4, Siemens, Erlangen, German), was performed by a sonographer (with 8 years of experience) who was blinded to the results of the histologic assessment. The measurements were obtained at a depth of 1.5–2 cm below the surface of the gelatin phantom where an area free of large blood vessels was chosen as the ROI. The SWV measurement was considered invalid when the screen displayed "xxx". Ten successful acquisitions were obtained in for each liver. The median value of the ten measurements was expressed as a rat's SWV value. Finally, the SWV values of all 110 rats were used for further statistical analyses.

2.4. Statistical methods

The rats in this study were grouped according to their pathologic grade. The relationship between the SWV values and the results of the pathologic analyses was evaluated using Spearman's rank correlation for univariate analysis or multivariate linear regression analysis for multivariate analysis. The diagnostic performance of the SWV parameter for discriminating among NASH severity levels and hepatic fibrosis stages was evaluated using non-parametric areas under the receiver operating characteristic curves (AUROC). The cutoff values were selected by maximizing the Youden index, and the corresponding sensitivity and specificity values were calculated with exact 95% confidence intervals. To investigate the effects of steatosis and inflammation on the ARFI assessment of fibrosis, the rats were first divided into two subgroups, namely, mild fibrosis (F0 and F1) and significant fibrosis (F2–F4) groups. In each subgroup, the rats were further divided into three subgroups, mild (grades 0 or 1), moderate (grade 2), and severe (grade 3), in accordance with their respective steatosis and lobular inflammation scores. Tukey's test, in conjunction with an analysis of variance (ANOVA), was performed to compare the mean SWV values among the three steatosis/inflammation subgroups in each fibrosis group.

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