Computer-assisted ultrasound quantification analysis in fatty liver model of rats

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Background: Early detection of nonalcoholic fatty liver disease (NAFLD) shows its significant efficacy in preventing the patients from liver failure. The ultrasonic image quantitative analysis software can assist to diagnose NAFLD in the clinical studies. In this study, we aim to explore new method to evaluate the value of computer-assisted ultrasound in diagnosis and classification of fatty liver via Image J software.

Methods: Forty Sprague-Dawley rats were randomly divided into control group (n=10) and model group (n=30). For model group, the rats received high fat diet and subcutaneous injection of carbon tetrachloride to establish nonalcoholic fatty liver model. Ultrasound and pathological examinations on rats were performed on 4, 8, and 12 weeks. Image J software was used to measure the liver grayscale value (LGV) and renal grayscale value (RGV). The difference between LGV and RGV (LRGV) was analyzed. The diagnostic performance of computer-assisted ultrasound quantification was evaluated by receiver operating characteristic (ROC) analysis.

Results: We compared ultrasonic quantization parameters between control and model groups and found that the LGV and LRGV were statistically different between the normal and light fatty livers, light and moderate fatty livers, as well as moderate and severe fatty livers, respectively (P<0.05). There was no significant difference in RGV among these groups (P>0.05). Kappa statistic and Bland-Altman analyses showed the consistency of ultrasonic examination and pathological examination was good in diagnosis of fatty liver.

Conclusions: This study indicated that the computer-assisted ultrasound quantification analysis, with high performance of NAFLD diagnosis like pathological examination, could provide a new and flexible non-invasive method for preclinical pharmacological research and basic research.

Keywords: Fatty liver; ultrasound; Image J; liver grayscale value (LGV); renal grayscale value (RGV)

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patients from liver cirrhosis and liver failure, and reducing the pain and burden from the patients (5).

At present, liver biopsy and pathological examination are the gold standards for diagnosis of NAFLD (7,8). However, there are still some limitations of these techniques for NAFLD diagnosis. For example, these examination methods are invasive, which will bring some complications that associated with the operative procedure in the patients. Meanwhile, the distribution of fatty liver is quite heterogeneous, and biopsy of liver tissue only accounts for one in one million of the liver tissue. This will bring concerns of tissue sampling variability and increase the false negative rate in patients (7,9). Therefore, additional approaches for noninvasive diagnosis of NAFLD with high efficacy are still desirable in the clinical settings.

Recent accumulated studies have shown that ultrasound, a non-invasively imaging technique with good stability, has become a widely-used method for diagnosis of NAFLD in the clinic (10,11). However, as limited by the specialist experience and instrumentation, the objectivity of diagnosis results is relatively low. In addition, there is no quantitative index for classification of results from NAFLD diagnosis. Although ultrasonic image quantitative analysis software has been employed to diagnose fatty liver in the clinical studies (12,13), the potential application of Image J software (Image J bundled with 64-bit Java 1.8.0_112, Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA) on the computer for analysis of ultrasonic images and obtaining quantitative grading diagnosis of NAFLD is still not fully applied in the clinic. Recent studies have revealed that this method can quantitatively evaluate the degree of fatty liver by comparing the gray scale median of liver and kidney accurately. Another advantage of this method is that it can analyze ultrasonic images offline, which can improve the efficiency and cost-performance value as it allows clinician to assess the ultrasound results with enough time.

In this study, we aim to explore proper quantitative ultrasonic index for classification of diagnosis of fatty liver via Image J software on the computer, and thus provide new approach to improve the accuracy and stability of ultrasonography for NAFLD diagnosis.

We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi.org/10.21037/apm-19-545).

### Methods

#### Animal establishment and grouping

Forty male Sprague-Dawley rats (6–12 weeks, 175±25 g) were purchased from China Medical University, and maintained in the Experimental Animal Center at China Medical University under specific pathogen-free conditions. All the animal experiments reported herein were carried out in accordance with the approved guideline and approved by the committee on the Animal Care and Use of Institute of China Medical University. Noting that the reason for choosing rat here was because the rat has large mass of liver and kidney and it is more easily examined by ultrasound. Briefly, rats were randomly divided into control group (n=10) and model group (n=30) by an investigator who was blinded to this study. Rats in normal control group received basic food daily, while rats in model group received high-fat diet daily as well as subcutaneous injection of carbon tetrachloride solution (0.05 mL/kg, China National Pharmaceutical Group Corporation, Shenyang, China) from twice a week for 4 weeks to establish nonalcoholic fatty liver model (14-16).

#### Ultrasonic examination and parameter analysis

Before the ultrasonic examination, rats were fasted overnight. The chest and abdomen were depilated with 8% Na₂S solution (China National Pharmaceutical Group Corporation, Shenyang, China). After that, rats were anesthetized by an intraperitoneal injection of 4% chloral hydrate (China National Pharmaceutical Group Corporation, Shenyang, China), and then placed in the supine position. Ultrasound diagnostic instrument and probe (L12-5) (PHILIPS iU22, The Netherlands) were applied to examine the rat liver and kidney conditions in this study. Under MSK Sup, a total gain was 88 dB/DR50, the depth was 2.5 cm, resolution and sensitivity were fixed, and the time gain compensate (TGC) curve adjustment knob was placed in a straight line for ultrasonic examination. The rat liver was observed in the intercostal transverse longitudinally. Ultrasonic images of rat liver and kidney were taken and compared. After that, the images were imported to the computer through image conduction function of PHILIPS iU22, and opened through Image J software.
Two circular region of interests (ROIs) with 330 mm\(^2\) were applied on liver and kidney, respectively (Figure 1). One ROI was placed in the liver parenchyma (1.0±0.5 cm from skin). The other ROI was placed on the junction of the renal cortex medulla, which was away from renal column. The two ROIs were kept on the same vertical line. ROI, region of interest.

**Table 1** Comparison of pathological diagnosis result and ultrasonic examination using LGV parameter in rats

<table>
<thead>
<tr>
<th>Degree</th>
<th>Pathological diagnosis (n)</th>
<th>Ultrasound diagnosis (n)</th>
<th>P value</th>
<th>kappa value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFL</td>
<td>11</td>
<td>8</td>
<td>&gt;0.05</td>
<td>0.747</td>
</tr>
<tr>
<td>LFL</td>
<td>10</td>
<td>13</td>
<td>&gt;0.05</td>
<td>0.570</td>
</tr>
<tr>
<td>MFL</td>
<td>10</td>
<td>7</td>
<td>&gt;0.05</td>
<td>0.748</td>
</tr>
<tr>
<td>SFL</td>
<td>9</td>
<td>12</td>
<td>&gt;0.05</td>
<td>0.881</td>
</tr>
</tbody>
</table>

LGV, gray liver value; NFL, no fatty liver; LFL, light fatty liver; MFL, moderate fatty liver; SFL, severe fatty liver.

The normal, light fatty, moderate fatty, and severe fatty livers were evaluated by ultrasonic examination and Image J software. Two ROIs were placed on liver and kidney in an ultrasonic image by Image J software. One ROI was placed in the liver parenchyma. The other ROI was placed on the junction of the renal cortex medulla. The two ROIs were kept on the same vertical line. ROI, region of interest.

**Pathological examination**

The right lobe of livers (1 cm × 1 cm × 0.5 cm) were harvested and fixed in 4% Paraformaldehyde solution (China National Pharmaceutical Group Corporation, Shenyang, China). After paraffin-embedding and sectioning of tissues, the obtained slices (5 μm thickness) were stained with hematoxylin-eosin (H&E) staining. After that, five random slices were selected, and mounted with an aqueous mounting medium. The pathological grades were determined according to the proportion of fat infiltrated hepatocytes in the total number of hepatocytes (17): less than 30% was considered as light fatty liver disease (FLD); 30–60% was considered as moderate FLD; and more than 60% was considered as severe FLD.

**Statistical analysis**

The data were statistically analyzed using SPSS statistical analysis software (version 13.0, SPSS Inc, Chicago, IL, USA). Data were presented as mean ± SD. One way analysis of variance (ANOVA) was used to compare the differences among different groups, and a P value <0.05 was considered statistically significant. Intra-observer agreement was evaluated using kappa statistic, and values in the ranges of 0.01–0.20, 0.21–0.40, 0.41–0.60, 0.61–0.80, and 0.81–1.00 were considered as slight, fair, moderate, substantial, and almost perfect agreement, respectively. The Bland-Altman analysis was applied to compare the ultrasonic examination and pathological examination for diagnosis of fat liver. Meanwhile, the maximum points of sensitivity and specificity were selected as the diagnostic thresholds according to the receiver operating characteristic (ROC) curve. Area under the curve (AUC) was used to evaluate the diagnostic performance of computer-assisted ultrasound quantification analysis in different groups.

**Results**

**Ultrasound results**

The normal, light fatty, moderate fatty, and severe fatty liver in rats were evaluated by ultrasonic examination and Image J software. Two ROIs were placed on liver and kidney on ultrasonic images, respectively, as to obtain the values of LGV and RGV. The LGV, RGV, and the difference between LGV and RGV (LRGV) were measured. When using LGV parameter to determine the grades of fatty liver, it showed that 8 rats with no fatty liver, 13 rats with light fatty liver, 7 rats with moderate fatty liver and 12 rats with severe fatty liver (Table 1). When using LRGV to determine the grades of fatty liver, it showed that 9 rats with no fatty liver, 14 rats with light fatty liver, 7 rats with moderate fatty liver and 12 rats with severe fatty liver (Table 1). When using LRGV to determine the grades of fatty liver, it showed that 9 rats with no fatty liver, 14 rats with light fatty liver, 7 rats with moderate fatty liver, and 12 rats with severe fatty liver (Table 1).
moderate fatty liver and 10 rats with severe fatty livers (Table 2). The representative ultrasonic images for different grades of fatty liver were shown in Figure 2.

### Comparison of ultrasonic quantization parameters among different groups

Next, the LGV, RGV and LRGV in different groups were statistically analyzed. As shown in Figure 3, the LGV was statistically different between normal and light fatty livers, light and moderate fatty livers, and moderate and severe fatty livers (P<0.05) (Figure 3A). There was no significant difference in RGV among these groups (P>0.05) (Figure 3B). The LRGV was statistically different between normal and light fatty livers, light and moderate fatty livers, and moderate and severe fatty livers (P<0.05) (Table 3).

### Pathological results

The pathological grades of FLD in each rat were examined by H&E staining. The results demonstrated that no fatty liver was observed in control group (n=10). While in model group, there were 1 rat with normal liver, 10 rats with light fatty liver, 10 rats with moderate fatty liver and 9 rats with severe fatty livers. The representative H&E images of normal, light fatty, moderate fatty livers, and moderate and severe fatty livers were presented in Figure 4, and the fatty cells in the liver were point out by yellow arrows. Meanwhile, at the end of the experiment (12 weeks later), the bodyweight of rats in each group was collected and shown in Table 4. The average bodyweight of rats in control group (241±22 g) was significantly lower than that in light fatty (302±29 g), moderate fatty (321±31 g), and severe fatty liver (354±29 g) groups (P<0.05), demonstrating that the bodyweight was associated with the grades of fatty liver in rats.

### Consistency of ultrasonic examination and pathological examination

The comparison of ultrasonic examination and pathological examination was conducted by kappa statistic and Bland-Altman analyses. The kappa values for these two examination in detection of normal, light fatty, moderate fatty, and severe fatty liver of rats were 0.747, 0.570, 0.748, 0.881 by using LGV parameter, respectively (Table 1), and 0.756, 0.676, 0.768, and 0.931 by using LRGV index, respectively (Table 2). The Bland-Altman analysis results were shown in Figure 5. The difference of pathological examination and ultrasonic examination did not exceed the maximum allowed difference (mean ±1.96 SD), implying the two examinations were with high consistency for diagnosis of fatty liver. The diagnostic performance of computer-assisted ultrasound quantification analysis was evaluated. When using LGV index for assessment, the sensitivity and specificity were 75% and 87.5% for the diagnosis of normal liver and light fatty liver respectively; and 77.8% and 85.8% for the diagnosis of light fatty liver and moderate fatty liver respectively; and 77.8% and 88.9% for moderate fatty liver and severe fatty liver respectively (Table 5). When using LRGV index for assessment, the sensitivity and specificity were 91.7% and 91.7% for diagnosis of normal liver and light fatty liver respectively; and 90.0% and 90.2% for light fatty liver and moderate fatty liver respectively; and 87.1% and 90.3% for moderate fatty liver and severe fatty liver respectively (Table 6).

### The diagnostic threshold of quantitative parameter

Based on ROC statistical analysis, the accuracy of the diagnostic test was evaluated by the area under the ROC curve (AUC). The sensitivity and specificity of ultrasound examination by using LGV and LRGV as index to compare the difference between different groups were calculated and plotted on the ROC curves (Figure 6). The AUC value of LGV in light FLD, moderate FLD, and severe FLD was 0.875, 0.808, 0.860, respectively (Table 5), and the LRGV in light FLD, moderate FLD, and severe FLD was 0.885, 0.909, 0.950, respectively (Table 6), which demonstrated high diagnostic performance of computer-assisted ultrasound quantification analysis in these groups.
Figure 2 The morphology of different pathological grades of fatty liver determined by ultrasound examination. (A) The normal liver; (B) the light fatty liver; (C) the moderate fatty liver; (D) the severe fatty liver. Yellow stars represented liver tissue and yellow triangles represented kidney tissue.

Discussion

As a noninvasive and reliable diagnosis technique, ultrasound has become a preferred method for diagnosis of fatty liver in the clinical practice, and with an overall sensitivity of 85% and specificity of 94% (11,18,19). Besides ultrasonography, there are some other noninvasive approaches, such as computed tomography (CT), magnetic resonance (MR), magnetic resonance elastography (MRE), that have been employed to detect fatty liver and grades (18). CT-based diagnosis of fatty liver is considered as accurate, however, as it requires radiation and is outperformed by dual-gradient echo MRI, CT is not recommended for the diagnosis of fatty liver (20). MR imaging is more sensitive than CT for hepatic steatosis diagnosis, and generally considered the effective radiologic modality for qualitative and quantitative assessment of fatty
The liver is a vital organ of the lipid metabolism. Fat metabolism disorder has a great influence for dynamic balance of lipids. When the fat storage volume exceeds more than 5% of the liver mass, fat will be stored in hepatocytes. If more than 33% fatty degeneration, it is called fatty liver. According to hepatocyte fat storage volume, the grades of fatty liver can be classified as light, mild and severe ones (25). Ultrasonic characteristics for fatty liver are due to hepatic fat deposition in the cells, obvious ultrasonic energy absorption and scattering effect. The sound energy is mainly absorbed by the former site of liver, and obviously attenuated in the back site of liver, which formed the so-called bright liver (26). However, the attenuation of hepatic ultrasound images was not obviously observed in this study. This may because the probe L12-5 had a strong ability of penetration into the liver. In addition, a mild fibrosis also can reduce the degree of attenuation.

Currently, a computer-aided diagnostic system was used to objectively measure and classify the different grades of FLD, such as gray-level co-occurrence matrix (27), support vector machine (28), probabilistic neural network (29), extreme learning machine framework (30). In our study, we combined ultrasound and Image J software to diagnose and classify the grades of the fatty liver in rats. Single index, such as LGV and RGV of ultrasound images, were used to assess the classification of FLD. We compared the rat model of fatty liver through computer-assisted ultrasound quantification and pathological diagnosis, and confirmed the feasibility and stability of quantification ultrasonography analysis in FLD diagnosis. More importantly, the Image J software on the computer showed its great ability to analyze ultrasonic images for fatty liver diagnosis and classification.

<table>
<thead>
<tr>
<th>Degree</th>
<th>n</th>
<th>LGV</th>
<th>RGV</th>
<th>LRGV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFL</td>
<td>11</td>
<td>41.40±5.50</td>
<td>92.62±7.00</td>
<td>-49.22±10.71</td>
</tr>
<tr>
<td>LFL</td>
<td>10</td>
<td>83.22±13.07a</td>
<td>101.32±21.59e</td>
<td>-18.10±15.94a</td>
</tr>
<tr>
<td>MFL</td>
<td>10</td>
<td>94.49±10.89b</td>
<td>83.32±14.62f</td>
<td>11.17±9.02b</td>
</tr>
<tr>
<td>SFL</td>
<td>9</td>
<td>105.96±13.48c</td>
<td>75.87±17.48g</td>
<td>-33.12±8.17c</td>
</tr>
</tbody>
</table>

a, LFL vs. NFL, P<0.05; b, MFL vs. LFL, P<0.05; c, SFL vs. MFL, P<0.05; d, LFL vs. NFL, P>0.05; e, MFL vs. LFL, P>0.05; f, SFL vs. MFL, P>0.05. LGV, liver gray value; RGV, renal gray value; LRGV, the difference between liver and kidney; NFL, no fatty liver; LFL, light fatty liver; MFL, moderate fatty liver; SFL, severe fatty liver.
Figure 4 The pathological change of different grades of fatty liver in rats. H&E staining was used to stain the liver tissues, and the pathological grades were determined according to the proportion of fat infiltrated hepatocytes. The representative images were taken at 400× magnification. (A) The normal liver; (B) the light fatty liver; (C) the moderate fatty liver; (D) the severe fatty liver. The fatty cells in the liver were pointed out by yellow arrows.
This combined approach demonstrated a simpler and easier way with high sensitivity and specificity of performance when compared with the above methods which ultrasound images are evaluated via software-embedded in ultrasonic machine (31).

Previous studies showed that the diagnostic sensitivity of ultrasonic examination for moderate fatty liver was more than 90%, but only 30–64% for light fatty liver (19,24,32).

In this study, we demonstrated that the sensitivity and specificity of ultrasonic examination by Image J software analysis of LGV and RGV were both very high. In addition, the quantitative joint index (LRGV) was proved to effectively act as a classification index for the FLD diagnosis, and offline image analysis software was allowed for ultrasonic results analysis. The obtained technique could provide a high cost performance and stability method for FLD classification, and exhibit their advantages over other related techniques.

There were still several limitations in this study. Firstly, only a small number of samples were analyzed, and further investigations with large number are required to reinforce this study. It has been reported that the degree of liver fibrosis can affect the sensitivity of the diagnosis, and therefore application of related parameter can reduce the influence of individual differences, but it is not enough for the filter degree under different environment. Although the parameters of the depth, gain, time compensation and gain compensation for each ultrasonic operation are fixed, the measuring sites, focal length settings and scanning sections for animal examination have inevitable influences for the results. When ROI in ultrasonic image was selected by using Image J software, the ROI of RGV was placed directly below that of LGV. This would result in incorrect

Table 4 The bodyweight of rats at the end of the experiment

<table>
<thead>
<tr>
<th>Degree</th>
<th>n</th>
<th>Minimum (g)</th>
<th>Maximum (g)</th>
<th>Mean ± SD (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFL</td>
<td>11</td>
<td>204</td>
<td>276</td>
<td>241±22</td>
</tr>
<tr>
<td>LFL</td>
<td>10</td>
<td>261</td>
<td>349</td>
<td>302±29(^a)</td>
</tr>
<tr>
<td>MFL</td>
<td>10</td>
<td>285</td>
<td>375</td>
<td>321±31(^b)</td>
</tr>
<tr>
<td>SFL</td>
<td>9</td>
<td>294</td>
<td>400</td>
<td>354±29(^{a,d,e})</td>
</tr>
</tbody>
</table>

\(^a\), LFL vs. NFL, \(P<0.05\); \(^b\), MFL vs. NFL, \(P<0.05\); \(^c\), SFL vs. NFL, \(P<0.05\); \(^d\), SFL vs. LFL, \(P<0.05\); \(^e\), SFL vs. MFL, \(P<0.05\). NFL, no fatty liver; LFL, light fatty liver; MFL, moderate fatty liver; SFL, severe fatty liver.

Figure 5 Comparison of ultrasonic examination and pathological examination by Bland-Altman analysis. A represented pathological examination and B represented ultrasonic examination.

This combined approach demonstrated a simpler and easier way with high sensitivity and specificity of performance when compared with the above methods which ultrasound images are evaluated via software-embedded in ultrasonic machine (31).

Table 5 The diagnostic performance of LGV

<table>
<thead>
<tr>
<th>Degree</th>
<th>AUC</th>
<th>Threshold</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFLD</td>
<td>0.875</td>
<td>52.9</td>
<td>75.0</td>
<td>87.5</td>
</tr>
<tr>
<td>MFLD</td>
<td>0.808</td>
<td>94.9</td>
<td>77.8</td>
<td>85.8</td>
</tr>
<tr>
<td>SFLD</td>
<td>0.860</td>
<td>101.1</td>
<td>77.8</td>
<td>88.9</td>
</tr>
</tbody>
</table>

LGV, liver gray value; AUC, area under the curve; LFLD, light fatty liver disease; MFLD, moderate fatty liver disease; SFLD, severe fatty liver disease.

Table 6 The diagnostic performance of LRGV

<table>
<thead>
<tr>
<th>Degree</th>
<th>AUC</th>
<th>Threshold</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LFLD</td>
<td>0.885</td>
<td>−28.4</td>
<td>91.7</td>
<td>91.7</td>
</tr>
<tr>
<td>MFLD</td>
<td>0.909</td>
<td>10.9</td>
<td>90.0</td>
<td>90.2</td>
</tr>
<tr>
<td>SFLD</td>
<td>0.950</td>
<td>24.5</td>
<td>87.1</td>
<td>90.3</td>
</tr>
</tbody>
</table>

LRGV, the quantitative joint index (liver to kidney); AUC, area under the curve; LFLD, light fatty liver disease; MFLD, moderate fatty liver disease; SFLD, severe fatty liver disease.

In this study, we demonstrated that the sensitivity and specificity of ultrasonic examination by Image J software analysis of LGV and RGV were both very high. In addition, the quantitative joint index (LRGV) was proved to effectively act as a classification index for the FLD diagnosis, and offline image analysis software was allowed for ultrasonic results analysis. The obtained technique could provide a high cost performance and stability method for FLD classification, and exhibit their advantages over other related techniques.

Figure 5 Comparison of ultrasonic examination and pathological examination by Bland-Altman analysis. A represented pathological examination and B represented ultrasonic examination.
Figure 6 Evaluation of the accuracy of the diagnostic test by ROC curve. (A) The ROC curve of LGV and LRGV between NAFLD group and LFLD group; (B) the ROC curve of LGV and LRGV between LFLD group and MFLD group; (C) the ROC curve of LGV and LRGV between MFLD group and SFLD group. ROC, receiver operating characteristic; LGV, liver grayscale value; NAFLD, nonalcoholic fatty liver disease; LFLD, light fatty liver disease; MFLD, moderate fatty liver disease; SFLD, severe fatty liver disease.

calculation of LRGV as LGV and RGV came from the same sound beam. Therefore, the LRGV would be greatly influenced by different ultrasonic instrument, different section, abdominal gas and pseudomorphism.

Conclusions

In conclusion, our study indicated that both LGV and LRGV can be used as the index for diagnosis of fatty liver in rats. LRGV can also be used to as the index for classification diagnosis. Computer-Assisted Ultrasound Quantification Analysis may provide a new and flexible non-invasive method for preclinical pharmacological research and basic research.

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Footnote

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/apm-19-545

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/apm-19-545). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All the animal experiments reported herein were carried out in accordance with the approved guideline and approved by the committee on the Animal Care and Use of Institute of China Medical University.

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