Preliminary Feasibility Study on Diffusion Kurtosis Imaging to Monitor the Early Functional Alterations of Kidneys in Streptozocin-Induced Diabetic Rats

Zhong-Yuan Cheng, MD#, Ping-Kang Chen, MS#, You-Zhen Feng, MD#, Xiao-Qiao Chen, MS, Long Qian, PHD, Xiang-Ran Cai, MD

Rationale and Objectives: The aim of this study was to investigate the potential of diffusion kurtosis imaging (DKI) to assess the early renal functional undulation of diabetic mellitus (DM).

Materials and Methods: Fifty-seven Sprague-Dawley (SD) rats were randomly divided into two groups and eventually 48 rats were included in this study: the normal control (CON) group and diabetic mellitus (DM) group. Weeks 0, 4, 8, and 12 after the diabetes model was successfully established, all the rats were scanned on the 3.0T MRI. The DKI derived parameters of renal parenchyma, including fractional anisotropy (FAco, FAme), mean diffusivity (MDco, MDme), and mean kurtosis (MKco, MKme) were measured. Their alteration over time was analyzed and then correlated with urine volume (UV), blood urea nitrogen (BUN), and serum creatinine (Scr) using Pearson correlation analysis. Finally, hematoxylin and eosin (H&E) staining was performed on the kidneys of the two groups.

Result: There was a decreasing trend in FA, MK, and MD values over time in diabetic rats. Also, the gradually worsening histological damage of kidneys was noted over time in diabetic rats. The cortical FA and MK values and medullary FA, MK and MD values of diabetic rats were significantly lower than those of controls at most time points after DM induction. In addition, negative correlations were revealed between the BUN and FAco (r = -0.43, p = 0.03) or FAme value (r = -0.49, p = 0.01). The cortical MK value was moderately correlated with UV (r = -0.46, p = 0.03) and BUN (r = -0.55, p = 0.01).

Conclusion: The preliminary findings suggest that DKI might be an effective and sensitive tool to assess the early changes of renal function impairment in diabetic rats. The FA values of the cortex and medulla and the MK value of the cortex are sensitive markers in detecting renal injury in diabetic rats.

Key Words: Diffusional kurtosis imaging; Magnetic resonance imaging; Diabetes mellitus; Diabetic nephropathy; Rat; Renal injury.

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INTRODUCTION

Diabetic nephropathy (DN), as a serious kidney-related complication of diabetes mellitus (DM), is becoming a worldwide public health challenge. The latest meta-analysis, which included 30 studies involving 79,364 adults with type 2 DM (T2DM) revealed that its prevalence was as high as 21.8% in China (1). Also, DN which occurs in 30% to 40% of all patients with diabetes, is the leading cause of end-stage renal disease (ESRD) (2). In clinical practice, several measures have been adopted for the early identification and prevention of DN, for instance circulating biomolecules (3), urine exosomes markers, urine proteomics,
metabolomics and so on (4). However, most of these biomarkers are still in research stage and not yet widely used clinically (3,4).

DN is characterized by structural and functional alternation of the kidneys, and one of its earliest clinical characteristics is microalbuminuria (5). Some laboratory parameters, such as serum creatinine and the estimated glomerular filtration rate (eGFR) are used for the diagnosis, prognosis and follow-up of DN patients. But these parameters are neither sensitive nor specific (6). Kidney biopsy is considered the gold standard for the diagnosis of DN. However, biopsy is invasive, time-consuming, prone to sampling error and carries a risk of complications (7). Therefore, an effective and noninvasive method with high sensitivity is desperately needed to identify DN and facilitate subsequent follow-ups after treatment.

Diffusion kurtosis imaging (DKI), a novel technique of magnetic resonance diffusion-weighted imaging (DWI), which is based on water molecules’ non-Gaussian distribution behavior, has been successfully applied to normal kidney tissue (8,9). IgA nephropathy (10) and renal injury with hyperuricemia (11). All of these studies suggested that this method can be used to evaluate renal changes in a non-invasive manner and to acquire additional microstructural information. Notwithstanding, the animal model of DN has been investigated by many functional magnetic resonance imaging techniques, including diffusion tensor imaging (DTI) (12), intravoxel incoherent motion imaging (IVIM) (13) and blood oxygen level-dependent (BOLD) (14,15). Recently, Zhou et al. has confirmed DKI may be valuable for the noninvasive detection of early DN (16). Taking into account the uniqueness of the DKI imaging principle and the good performance of previous research, we assume that DKI can be another appropriate method in detecting early renal dysfunction in DN. To fulfil this hypothesis, the rat model of DM was first successfully established, and then the early renal function in DM rats was monitored by using DKI technology at specific time points.

MATERIALS AND METHODS

Animal Model and Study Design

The animal protocol was approved by the Institutional Research Ethics Committee of our university. The process of our experiment was conducted according to the Institutional Guidelines of Experimental Animal Care and Use. Fifty-seven male Sprague-Dawley rats (6–7 weeks, 150–160 g) were used for the experiment (Experimental Animal Breeding Co., Ltd.) with the license number of SCXK (Yue 20140007).

The animals were age-matched and divided into two groups: normal control and DM. The rats in the DM group received an intraperitoneal injection of streptozocin [STZ; Sigma; dissolved in 0.1 mol/l citric acid-sodium citrate buffer (pH 4.6)] at a dose of 55 mg/kg after fasting for 8 hours. The controls received an equal volume of 0.1 mol/l citric acid-sodium citrate buffer (pH 4.6). Three days after the injection, animals with blood glucose levels of 16.7 mmol/L or less were excluded from the study.

The diabetic rats and the controls were then randomly subdivided into four subgroups. During the study, the rats were imaged longitudinally at four specific time points (0, 4, 8, and 12 weeks after injection). Before MRI scanning, the rats were placed in metabolic cages to collect 24 hours’ worth of urine samples while the body weight and plasma glucose levels were recorded. In addition, serum creatinine (Scr) and blood urea nitrogen (BUN) were tested from tail vein samples at each time point following the MRI scanning. To reduce breathing movement artefacts, the abdomen of rat was wrapped in gauze.

Image Acquisition and Postprocessing

All MRI scanning was performed on a clinical 3.0T whole-body MRI scanner (Discovery 750, GE Medical Systems, Milwaukee, WI) equipped with an HD wrist array upper coil. During imaging, the rats were kept in narcosis with 0.3% sodium pentobarbital, 2 ml/kg intraperitoneally and placed in a prone and head advanced position.

DKI sequence was acquired in the coronal plane using single-shot echo planar imaging. Diffusion encoding was applied in 25 directions with three b values (0, 500, and 1000 s/mm²). The sequence parameters were as follows: repetition time = approximately 3000 ms; echo time= 75.2 ms; slice thickness=2.0 mm; gap = 0.2 mm; matrix: 96 £ 96; field of view = 8.0 cm £ 7.2 cm; bandwidth =167kHz; NEX=2.0; number of slices =7; the acquisition time was 5 minutes and 36 seconds.

All DKI data were described by Equation (1) using a constrained linear least-square (CLLS) method (15,16):

\[
\ln[S(n, b)/S_0] = b \sum_{j=1}^{3} \sum_{l=1}^{3} n_{ij} + \frac{1}{6} b^2 D^2 \sum_{j=1}^{3} \sum_{l=1}^{3} \sum_{k=1}^{3} \sum_{l=1}^{3} n_{ijk} W_{ijkl}
\]

(1)

where S(n,b) is the diffusion signal intensity for diffusion weighting b and diffusion-encoding direction n, S0 is the signal intensity for b0, Dj is the diffusion tensor and Wijkl is the kurtosis tensor.

The DKI parameters were independently measured by two readers (with 6 years and 8 years of diagnostic imaging experience, respectively) using a workstation (GE, ADW 4.5) equipped with Functool software. The method that we used to draw regions of interests (ROIs) was done in reference to the study by Hueper et al. (15). The ROIs were manually placed within both the renal cortex (CO) and the medulla (ME) (Fig 1), avoiding the renal sinus and blood vessels on the b=0mm/s² image and then mapping to various DKI functional parameters (Fig 2). The sizes of the ROIs in the
renal cortex and medulla were approximately 40–50 mm² and 20–30 mm², respectively. In order to optimize data accuracy, the three continuous coronal images were chosen to draw ROIs (at or adjacent to the level of the renal hilum). The average DKI parametric values of the renal cortex and medulla were then calculated.

Pathological Analysis

The right kidneys of rat were weighed and then fixed in 10% neutral formalin solution for histological examination. Tissue was embedded by paraffin wax, cut into 3 μm sections and stained with hematoxylin and eosin (H&E). The sections were analyzed by a renal pathologist (with 20 years of pathological diagnostic experience).

Statistical Analysis

SPSS v.20.0 software for Windows (Chicago, IL) was used to perform the statistical analysis. Data were expressed as the mean ± standard deviation with < 0.05 considered significant. The intraclass correlation coefficient (ICC) was calculated to evaluate the correlation between the two observers for the current study. ICC > 0.80 represented good agreement. The basic information and measurement of the DKI data between the control and the DM groups were compared by a paired sample t-test at each time point. A Pearson correlation analysis was adopted to assess the relationship between the DKI derived parameters of diabetic rats and some biochemical values, including UV, BUN and Scr. Correlation coefficients were classified as follows: 0.0–0.2, very weak to negligible correlation; 0.2–0.4, weak correlation; 0.4–0.6, moderate correlation; 0.6–0.8, strong correlation; and 0.8–1.0, very strong correlation.

RESULTS

Basic Information

Eight rats of the DN group were excluded from the experiment, on account of blood glucose levels lower than 16.7 mmol/L. A total of 25 rats were successfully induced by STZ-injection. Unfortunately, one of them was sacrificed due to an anesthesia accident. The remaining 24 diabetic rats and 24 control rats were then included in our experiment (Supplementary Fig.). As shown in Table 1, the diabetic rats weighed significantly less than controls since the 4th week, but a completely opposite trend was shown in the ratios of kidney weight/body weight (p < 0.05). Cataracts were observed in diabetic rats at the 7th week.

Biological Results

Compared to the controls, there was a significant increase in the UV of diabetic rats at the 4th, 8th, and 12th week, but a precipitous drop was noted at the 12th week (p < 0.05). During the period of our study, an increased trend was noted in the BUN and Scr values of diabetic rats. The difference was significant between the two groups in BUN from the 4th week (p < 0.05); However, for Scr, a nonsignificant difference was found at each time point.
TABLE 1. Changes in Basic Index Between the Two Rats Groups at Each Points and Comparison Results

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Group</th>
<th>Weight of Rat (g)</th>
<th>Weight of Kidney/Body (%)</th>
<th>UV (ml)</th>
<th>BUN (mmol/L)</th>
<th>Scr (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0W</td>
<td>CO(n=6)</td>
<td>338.64±15.82</td>
<td>7.71±0.65</td>
<td>39.87±8.65</td>
<td>5.79±0.59</td>
<td>23.17±4.45</td>
</tr>
<tr>
<td></td>
<td>DM(n=6)</td>
<td>334.72±16.53</td>
<td>8.29±0.63</td>
<td>42.15±8.70</td>
<td>6.25±0.45</td>
<td>24.33±3.78</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>0.741</td>
<td>0.146</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4W</td>
<td>CO(n=6)</td>
<td>427.78±19.26</td>
<td>6.67±0.83</td>
<td>43.98±22.54</td>
<td>5.89±0.98</td>
<td>24.00±2.00</td>
</tr>
<tr>
<td></td>
<td>DM(n=6)</td>
<td>339.42±41.72*</td>
<td>10.28±0.64*</td>
<td>188.78±44.32*</td>
<td>13.51±2.40*</td>
<td>27.33±4.50</td>
</tr>
<tr>
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<td></td>
<td>0.014</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.081</td>
</tr>
<tr>
<td>8W</td>
<td>CO(n=6)</td>
<td>489.59±25.15</td>
<td>5.81±0.35</td>
<td>48.63±30.14</td>
<td>5.96±1.03</td>
<td>24.22±3.25</td>
</tr>
<tr>
<td></td>
<td>DM(n=6)</td>
<td>327.60±32.00*</td>
<td>10.88±1.05*</td>
<td>271.07±109.05*</td>
<td>13.22±2.37*</td>
<td>27.33±3.72</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.002</td>
<td>0.189</td>
</tr>
<tr>
<td>12W</td>
<td>CO(n=6)</td>
<td>522.40±10.94</td>
<td>5.89±0.30</td>
<td>39.57±25.15</td>
<td>6.01±1.02</td>
<td>25.36±4.27</td>
</tr>
<tr>
<td></td>
<td>DM(n=6)</td>
<td>301.39±47.57*</td>
<td>13.13±2.53*</td>
<td>126.22±29.72*</td>
<td>14.19±3.30*</td>
<td>30.17±7.51</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.002</td>
<td>0.001</td>
<td>0.225</td>
</tr>
</tbody>
</table>

Note: UV, urine volume; BUN, blood urea nitrogen; Scr, serum creatinine; * the results of t test for two groups of two samples in each time point (p < 0.05)

ICC

The ICCs of two observers were identified as good, ranging from 0.924 to 0.997 (Supplementary Table). Then, the data which performed for this study was acquired by the first observer.

Comparisons of DKI Parameters

For the diabetic rats, FA and MK values in the cortex and medulla continued to fall over time. The cortical and medullary MD values rose at the 4th week and then gradually declined. However, The MRI quantitative parameters fluctuated slightly in the control group, and no similar trends were found as DM group.

At the 4th week after injection, the FA<sub>cortex</sub>, FA<sub>medulla</sub>, MD<sub>cortex</sub>, and MK<sub>cortex</sub> values of diabetic rats were significantly lower than those of the controls (p < 0.05). At the 8th week, compared to the control group, significantly lower FA<sub>cortex</sub> and MK<sub>cortex</sub> values were discovered in the diabetic rats (p < 0.05). At the 12th week following injection, there were significantly lower FA<sub>medulla</sub>, MD<sub>medulla</sub> and MK<sub>medulla</sub> values in diabetic rats compared to those in the controls (Fig. 3 and Table 2).

Correlation Analysis of DKI Parameters and UV, BUN, and Scr

As the statistical shows in Figure 4 and Table 3, the FA values in the cortex and medulla negatively correlated with the BUN (r = -0.43, p = 0.03, and r = -0.49, p = 0.01, respectively). A moderate correlation was observed between the cortical MK and UV or BUN values (r = -0.46, p = 0.03, and r = -0.55, p = 0.01, respectively). Furthermore, no DKI parameters were related to Scr.

The Pathological Findings

At the 0th week, there was no visible pathological change in the glomeruli and tubule interstitium (Fig 5).

At the 4th week, the lumen of the renal tubule dilated slightly, but the glomeruli still had no pathological changes visible to the naked eye (Fig 5).

At the 8th week, we found tubulointerstitial hyperplasia of the renal tubules, tubular epithelial edema, and loose cytoplasm, in addition to glomerular hyperplasia, cytoplasm of the renal tubular epithelial cells adjacent to the glomeruli that were loose and lightly stained, and epithelial cells that had shed (Fig 5).

At the 12th week, the tubular lumen was significantly dilated and the vascular hyperplasia was more obvious, while the cytoplasm of the renal tubular epithelial cells was more improved than before. The glomeruli further proliferated, and the lumen of the renal tubule adjacent to the glomerulus expanded significantly (Fig 5).

All of DM rats showed the same pathological changes at the same timing as the Figure 5 displayed. The control rats did not experience the previous changes.

DISCUSSION

In the present study, the DKI technique was used to monitor the early alternations of kidneys in STZ-induced diabetic rats, and the biochemical and histopathological evidence were adopted to estimate the feasibility of this method. Our results revealed lower MK in the cortex and FA in the cortex and medulla in diabetic rats starting in the 4th week after DM induction. Moderate correlations were discovered between the cortical MK and UV or BUN and between the FA in the cortex or medulla and BUN. In addition, changes in the DKI-derived parameters were also supported by the pathological findings. Therefore, these results indicate that DKI may be a supplementary method for monitoring early renal function in diabetic rats.

The cortical MK value significantly decreased in diabetic rats at the 4th week in our study, which suggests a reduction in overall diffusional heterogeneity. As we know, MK value
is the specific parameter of the DKI sequence, which reflects the water molecules' diffusion deviation degree from Gaussian diffusion and can be used to assess the complexity of the microstructural environment of tissue (17–19). The more complicated the microstructure of biological tissue, the greater the MK value will be (17, 18) and vice versa. In addition, the prominently increased urinary volume, weight ratio of kidney/body and hypertrophic glomeruli and proximal tubules were also noted at the same time, indicating glomerular hyperfiltration. Thus, we speculate that the reduction of cortical MK values is attributed to glomerular hyperfiltration. In hyperfiltration states, there is an increase in glomerular basement membrane (GBM) length, while the ability of the podocyte to grow is limited. Thus, a mismatch between the GBM area and the GBM area covered by foot processes leads to podocyte injury and the detachment of viable podocytes (20,21). Also, Fu et al. (22) found apparently reduced glomerular podocytes in STZ-induced diabetic rats. Additionally, the injury and apoptosis of glomerular endotheliocyte were noted by Li et al. (23). Third, hyperfiltration is associated

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>FAco</th>
<th>FAme</th>
<th>MDco</th>
<th>MDme</th>
<th>MKco</th>
<th>MKme</th>
</tr>
</thead>
<tbody>
<tr>
<td>0w</td>
<td>Con</td>
<td>0.24±0.02</td>
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<td>3.20±0.36</td>
<td>3.12±0.06</td>
<td>0.76±0.02</td>
<td>0.64±0.01</td>
</tr>
<tr>
<td></td>
<td>DM</td>
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<td>0.69±0.04</td>
<td>2.86±0.11</td>
<td>2.79±0.48</td>
<td>0.78±0.14</td>
<td>0.65±0.22</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.460</td>
<td>0.454</td>
<td>0.074</td>
<td>0.152</td>
<td>0.765</td>
<td>0.914</td>
</tr>
<tr>
<td>4w</td>
<td>Con</td>
<td>0.27±0.02</td>
<td>0.68±0.02</td>
<td>3.12±0.25</td>
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<td>0.70±0.01</td>
<td>0.59±0.03</td>
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<tr>
<td></td>
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<td>0.60±0.04</td>
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<td>0.62±0.04</td>
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<tr>
<td></td>
<td>p</td>
<td>&lt;0.001</td>
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<td>0.665</td>
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<tr>
<td>8w</td>
<td>Con</td>
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<td>3.11±0.26</td>
<td>3.08±0.43</td>
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<td>0.63±0.05</td>
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<tr>
<td></td>
<td>DM</td>
<td>0.20±0.02</td>
<td>0.55±0.12</td>
<td>2.91±0.24</td>
<td>2.55±0.41</td>
<td>0.62±0.03</td>
<td>0.61±0.09</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.002</td>
<td>0.147</td>
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<td>0.054</td>
<td>0.001</td>
<td>0.665</td>
</tr>
<tr>
<td>12w</td>
<td>Con</td>
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<tr>
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<tr>
<td></td>
<td>p</td>
<td>0.165</td>
<td>0.001</td>
<td>0.243</td>
<td>0.030</td>
<td>0.019</td>
<td>0.326</td>
</tr>
</tbody>
</table>

Note: FA, fractional anisotropy; MD, mean diffusivity; MK, mean kurtosis; MD is given in $10^{-3}$ mm²/s, while FA and MK is dimensionless.
with an increased Bowman’s space volume and increased proximal tubular cells and lumen volume (24). Finally, the proximal tubular sodium reabsorption is increased due to hyperfiltration. The enhanced proximal sodium transport load and consequent elevated oxygen consumption will result in hypoxia. All the aforementioned elements collaboratively reduce the limitation of the renal cortex on water molecules, and thus decrease the MK value. In this study, the urinary volume of diabetic rats peaked at the 8th week and then dropped at the 12th week, but this volume was still significantly higher than that of controls at the 12th week. The weight ratio of kidney/body continuously increased until the 12th week. Also, the severe damage of renal glomerulus, including the uneven expansion of the glomerular balloon, adhesion of the balloon wall and highly dilated capillary lumen, were observed in the 12th week. These findings suggest sustained glomerular hyperfiltration until the 12th week. Although tubulointerstitial inflammation and fibrosis occurred from the 8th week, hyperfiltration outweighs them, and the MK value of the renal cortex remained lower during this period.

### TABLE 3. Correlation Analysis Between DKI Parameters and UV, BUN, and Scr

<table>
<thead>
<tr>
<th></th>
<th>UV</th>
<th>BUN</th>
<th>Scr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>FAc0</td>
<td>-0.38</td>
<td>0.62</td>
<td>-0.43</td>
</tr>
<tr>
<td>FAm0</td>
<td>-0.35</td>
<td>0.10</td>
<td>-0.49</td>
</tr>
<tr>
<td>MdCO</td>
<td>-0.08</td>
<td>0.73</td>
<td>0.25</td>
</tr>
<tr>
<td>Mdme</td>
<td>-0.32</td>
<td>0.12</td>
<td>-0.11</td>
</tr>
<tr>
<td>MkCO</td>
<td>-0.46</td>
<td>0.03</td>
<td>-0.55</td>
</tr>
<tr>
<td>Mkme</td>
<td>0.03</td>
<td>0.90</td>
<td>-0.12</td>
</tr>
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</table>

Note: UV, urine volume; BUN, blood urea nitrogen; Scr, serum creatinine; FA, fractional anisotropy; MD, mean diffusivity; MK, mean kurtosis; The p values in italics are of statistical significance.
The declined FA values of the cortex and medulla were also found in diabetic rats in comparison with those of controls from the 4th week in our study. FA is a dimensional parameter which is a mirror that displays the amount of local diffusion anisotropy (25,26). A diminution in the FA value indicates the damaged microstructure of tissue (27). The reductions in the directionality (FA) of water movement in the renal cortex and medulla have been verified previously in DN rat models (12,28). Hueper et al. (12) considered that glomerulosclerosis, tubulointerstitial fibrosis and tubular damage were the potential mechanisms for decreasing FA values. Our preclinical study provided the evidence to support this hypothesis. The histopathological changes occurred at the 4th week in DN rats compared to the control group and progressively worsened in the following weeks, including interstitial inflammatory cells infiltration, dilated tubules, glomerulosclerosis, tubular atrophy, etc. In addition, the level of BUN in the DM group was remarkably greater than in the controls, which suggested renal damage in the diabetic rats. Notably, we found that the descending magnitude of the medullary FA value was greater than that of the cortical FA value at each point. This is in line with the pathological results showing that more prominent abnormalities were observed in the renal medulla.

Previous literature has shown that the diffusion coefficient (e.g. ADC and MD) decreases with the development of renal disease (12). In this current study, a downward trend was also displayed in the MD values of renal parenchyma from the 4th week, suggesting that the diffusion of water molecules in the renal parenchyma became more restricted. However, the MD did not exhibit statistical differences between the two groups at most of the time-points. These findings indicate that MD value is insensitive in detecting the functional changes in DN, since the diffusion coefficient would be influenced by perfusion and flow effects. Many previous studies of various renal diseases have demonstrated similar results (12,29).

**Limitations**

Our research has some limitations. First, this diabetic SD rat model may not accurately simulate the pathological changes in the kidneys of human diabetes, as diabetes in humans may be the result of multiple factors, not just single induction by insulin cell damage. Secondly, the region of interest which the DKI value measurement selects cannot match perfectly with the biopsy site, which may render our analysis slightly inaccurate. Finally, only early changes of the kidneys in diabetic rats were evaluated in this study, and we did not assess the results of long-term kidney damage.

**CONCLUSION**

Our study demonstrates that DKI could be a feasible and sensitive technique to assess renal microstructure changes in a diabetic rat model. FA and MK values may serve as potential biomarkers for early diabetic nephropathy.

**ETHICS APPROVAL**

This study was approved by the ethics committee of the Institutional Research Ethics Committee of Jinan University, China.

**AUTHOR CONTRIBUTIONS**

Guarantors of integrity of entire study, X.R.C. and Z.Y.C; manuscript drafting or manuscript revision for important
intellectual content, all authors; approval of final version of submitted manuscript, all authors; literature research, Y.Z.F., X.Q.C and Z.Y.C; clinical studies, Y.Z.F., Z.Y.C., P.K.C.; Acquisition of data: Y.Z.F., P.K.C., Z.Y.C. and L.Q. Sequence debugging and data processing Z.Y.C and L.Q.

AVAILABILITY OF DATA AND MATERIALS
All data generated or analysed during this study are included in this published article.

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PRIOR PRESENTATION
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