Original Paper

Renal Resistive Index as a Novel Indicator for Renal Complications in High-Fat Diet-Fed Mice

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Key Words

Renal injury • Resistive index • Renal fibrosis

Abstract

Background/Aims: The renal resistive index (RI) is a novel candidate as a renal injury prognostic indicator, but it remains unclear how renal RI levels correspond to renal injury in diabetic nephropathy. **Methods:** To examine this issue, we compared 8-week-old male C57BL/6 mice fed with high-fat diet (HFD) versus chow diet (CHD) for 16 weeks. At 8 and 12 weeks, the glomerular filtration rate (GFR), urinary albumin-to-creatinine ratio (UACR), and inflammatory factors (IL-1 β , IL-6, TNF α , and MCP-1) were measured, along with the increase in renal RI. **Results:** Our study suggests RI values positively correlate with GFR for the first 12 weeks of HFD feeding. In contrast, the GFR of 16-week HFD feeding is lower than that of 12-week HFD feeding, whereas RI levels are significantly increased. Additionally, our study suggests RI values accurately indicate the renal fibrosis and renal injury in HFD-fed mice treated with *lovastatin*. **Conclusion:** This study seems to confirm the utility of a noninvasive and repeatable ultrasound parameter to rapidly evaluate renal fibrosis in a HFD-induced type 2 diabetic mouse model *in vivo*. This highly sensitive and comparable renal RI measurement could monitor the whole procedure of disease development in real-time. RI measurement of the renal artery is capable of differentiating responses to standard therapy with *lovastatin* in HFD-fed mice from the CHD group.

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Published by S. Karger AG, Basel

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Introduction

As reported, type 2 diabetes is the leading cause of end-stage renal disease (ESRD) in developed countries [1, 2]. Among the patients who develop renal injury, 10% to 40% of those with type 2 (adult onset) diabetes eventually suffer kidney failure. In the U.K. Prospective Diabetes Study (UKPDS), 24.9% of patients developed microalbuminuria within 10 years after type 2 diabetes diagnosis and 0.8% developed ESRD and needed renal replacement [3].

Progressive renal structural and functional changes have been noted in diabetic patients [4-6]. The renal effects of diabetes include hemodynamic, morphological, and functional changes. These changes will lead to renal injury and renal failure [7].

Currently, glomerular filtration rate (GFR) is used in daily practice for evaluation of renal function in clinical and experimental research [7, 8]. However, it was reported that the use of filtered serum creatinine concentration may not accurately reflect the kidney function of a particular subject. This finding is due to multiple factors affecting the concentration of serum creatinine, such as aging, race, and lifestyle. Currently, the National Kidney Disease Education Program (NKDEP) of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), American Society of Nephrology (ASN), and National Kidney Foundation (NKF) recommend estimating GFR (eGFR) for serum creatinine in combination with age, sex, weight, or race to improve the accuracy of renal injury evaluation [6].

It is commonly agreed that diabetic nephropathy (DN) is a major cause of ESRD, and the incidence of diabetes mellitus increases rapidly [1]. There is a growing number of research studies focused on identifying the causes, progression, and effective therapeutic strategies for DN [2]. Indeed, diabetic mouse models with different strain backgrounds have been developed. Noticeably, the results from different labs around the world are not comparable due to the lack of a standard in evaluation of renal injury in diabetic mouse research. Since GFR stops increasing, or even slightly declines, after HFD feeding for a while the value of GFR cannot accurately indicate the degree of renal injury [9, 10].

In clinical and research applications, histological staining is another way to evaluate the degree of renal injury. However, for research purposes, the kidney tissues are only collected at the end points of the experiments such that the renal injury cannot be examined during experiments.

In the past two decades, Doppler waveforms of renal arterial blood flow have been extensively applied to study the pathologic and physiologic changes in the kidney [9, 10]. Among the parameters involved in Doppler waveforms, the renal resistive index (RI) is a reflection of renal parenchymal resistance, and has been widely accepted as one of the best diagnostic and therapeutic indexes [11]. In clinics, RI was first used by Rigsby et al. in studies on transplanted kidneys [12]. Subsequently, RI has been used for years in a variety of clinical settings such as the assessment of chronic renal allograft rejection [13], detection and management of renal artery stenosis [14], evaluation of progression risk in chronic kidney disease (CKD) [15], and differential diagnosis in acute and chronic obstructive renal disease. More recently, RI has been considered a predictor of renal and overall outcomes in critically ill patients [16]. Additionally, accumulating evidence indicates that renal RI was associated with changes in renal perfusion, and reflected the systemic hemodynamics and atherosclerosis. This finding was considered to be valuable prognostic information with therapeutic implications [17].

This study focused on a type 2 diabetic mouse model to evaluate renal injury. We hypothesize that renal RI measurement will be more sensitive and accurate than the current widely used analysis of GFR. Thus, RI measurement could become a novel method for evaluating renal injury under pathological conditions and during treatment in small animals.

Materials and Methods

Animals

All experimental procedures described here were approved by and carried out in accordance with the guidelines of the Institutional Animal Use and Care Committee of Tianjin Medical University. In brief, eight-week-old male C57BL/6 mice (n=80) weighing 22–24 g were randomly assigned to be fed with a high-fat diet (60 kcal% fat, 20 kcal% protein, 20 kcal% carbohydrate; Research Diets) or a chow diet (5 kcal% fat, 24.5 kcal% protein; Research Diets). Mice were housed individually in a climate-controlled environment with a 12:12-h light-dark cycle and free access to diets (unless food restricted) and water.

Mice were placed in metabolic cages for 24-hour urine collection after weeks 8, 12, and 16 with treatment, and blood samples were collected at different time points. The body weight was also measured. The mice were measured at room temperature. To assess test-retest variability, two echocardiograms were taken by two experienced physicians for all mice at the same time point with a gap of 24 h. Blinded repeat analyses of echocardiographic data were performed by another two experienced physicians.

Thirty male mice were fed HFD, and 30 male mice were fed with CHD. Ten mice from each group were randomly selected at weeks 8, 12, and 16 with HFD or CHD diets. After euthanasia, kidneys were weighted, and portions of both kidneys were snap-frozen in liquid nitrogen for RNA isolation. An additional portion was fixed in 4% PAF for Masson's trichrome and Picrosirius red staining.

Another set of experiments were conducted as follows. Twenty male mice that were fed with HFD were further randomized into two groups. One group (10 mice) was treated with oral lovastatin (5 mg/kg/day) in distilled water for 8 weeks after 12 weeks with HFD. Another group (10 mice) served as a placebo with water. Additionally, 10 male mice fed with CHD were randomized into two groups; one group with 10 mice was treated with lovastatin, and another group with 10 mice was given water as a control. The concentration of lovastatin was adjusted once every other day based on water consumption and body weight, and 24-hour urine and blood samples from each mouse were collected on the last day of the treatment period.

Glomerular filtration rate

FITC-inulin was injected in bolus to measure the glomerular filtration rate (GFR) based on previous methods [12, 47, 56, 62]. First, 5% inulin was dissolved in normal saline and dialyzed for 24 h to remove unbound inulin. This solution was sterilely filtered and then injected ($2 \mu\text{l} \cdot \text{body wt}^{-1}$) retro-orbitally to anesthetized mice. Twenty microliters of blood were collected from the tail vein at 3, 5, 7, 10, 15, 30, 60, and 75 min into a Na⁺-heparinized microcap. The samples were spun down, and 1 μl of plasma was reconstituted in HEPES buffer. The fluorescence was then determined using a Nano drop ND-3300 fluorospectrometer (Nanodrop Technologies). The GFR was calculated using a two-phase exponential decay.

Urine and plasma analysis

The urine albumin and creatinine were measured with a mouse Albuwell ELISA kit (Exocell) and a Creatinine Companion kit (Exocell), according to the manufacturer's instructions. The urinary albumin-to-creatinine ratio (UACR) was calculated as $\text{UACR} = \text{urine albumin} / \text{urine creatinine}$ ($\mu\text{g}/\text{mg}$). Blood glucose levels (after 12 h of fasting) were determined using an Accu-Chek III glucose analyzer.

Whole blood was obtained by cardiac puncture under anesthesia at the end point of the experiment. The blood samples were left for 2 h, and then centrifuged at 3,500 rpm for 15 min to collect serum, followed by storage at -80°C until assays. TNF- α and IL-6 were measured by the mouse TNF α ELISA MAX Standard kit and the mouse IL-6 ELISA MAX Standard kit according to the manufacturer's instructions, respectively (BioLegend).

Ultrasound imaging

The high-resolution ultrasound imaging was performed with a Vevo2100 Imaging System by using an MS550D (22–55 MHz) transducer (Visualsonics, Toronto, Canada). After mice were anesthetized using 0.5% to 2% isoflurane inhalant anesthesia with oxygen at 1 L/minute, they were placed in the supine position on a heated pad at 37°C to minimize data variations caused by fluctuating body temperatures or hair was removed from the thorax to the abdomen with a depilatory cream. After the transducer was placed on the mouse abdomen, transverse images of the abdominal artery at the level of the suprarenal gland were taken.

Then, the transducer was switched into the parasternal position to obtain the longitudinal images of the abdominal artery to confirm the scanning position and to confirm the suprarenal artery with color Doppler. Subsequently, blood flow velocity and physiological signals in Pulse Wave Doppler Mode were acquired simultaneously, including ECG signals and respiratory signals [18].

Ultrasound imaging analysis

After acquisition, the obtained images were stored and analyzed on the Vevo Working-station, a built-in off-line analysis system. The blood flow in the renal artery was analyzed from Doppler pulse velocity images. The peak systolic velocity (Vs) and the minimal end diastolic velocity (Vd) were calculated over an average of three to four cardiac cycles. Then, the resistive index (RI) was calculated as $RI = (Vs - Vd) / Vs$.

Masson staining

Masson staining was performed on formalin-fixed, paraffin-embedded 4- μ m sections, which were dehydrated in graded alcohol and then embedded in paraffin. Sections were stained with Masson staining to assess the level of collagen deposition. Semi-quantitative evaluation was performed at ten non-overlapping vision fields that were randomly selected and photographed in each group.

Picrosirius red staining

Kidney tissues were immediately fixed in 4% paraformaldehyde and then embedded with paraffin. The kidney tissues were sectioned (5- μ m slices), and then Picrosirius red staining was performed. Slides were stained (2 h) in saturated picric acid with 0.1% Sirius Red F3BA (Fisher Chemical), followed by washing in 0.01 M of hydrochloric acid for 2 min, and then being dehydrated through graded alcohols. Image analysis was performed with a Nikon E600 microscope. Quantitative evaluation was performed at ten non-overlapping vision fields that were randomly selected and photographed in each mouse.

Quantitative real-time PCR

Real-time PCR was performed as previously described²⁶. In brief, total RNA samples were isolated from kidneys using TRIzol (Invitrogen) precipitated with isopropanol. The synthesis of the first-strand cDNA was performed using M-MLV Reverse Transcriptase (Promega). Real-time RT-PCR analysis was performed using the FastStart Universal SYBR Green Master (Roch) and a 7900 HT Fast Real-Time PCR System (ABI).

The sequences for RT-PCR primers were 5'-CTTCTGGGCTGCTGTTCA -3' (sense) and 5'-CTTCTGGGCTGCTGTTCA -3' (antisense) for mouse MCP-1, 5'-CAACCAACAAGTGATATTCTCCATG -3' (sense) and 5'-GATCCACACTCTCCAGCTGCA -3' (antisense) for mouse IL-1 β , 5'-GAGGATACCACTCCCAACAGACC -3' (sense) and 5'-AAGTGCATCATCGTTGTTTCATACA -3' (antisense) for mouse IL-6, 5'-CACGTCGTAGCAAACCAAGTGGA -3' (sense) and 5'-TGGGAGTAGACAAGGTACAACCC -3' (antisense) for mouse TNF α , and 5'-CAACCAACAAGTGATATTCTCCATG -3' (sense) and 5'-GATCCACACTCTCCAGCTGCA -3' (antisense) for mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH served as an internal standard. The data were analyzed using 2^{- $\Delta\Delta$ Ct} relative expression as described.

Statistical analysis

Results are shown as the mean $\pm$ SD. Differences between two groups were determined by one-way ANOVA followed by Tukey's post-hoc test or Student's *t*-test as appropriate. For correlation analysis, Pearson's correlations were used after passing the D'Agostino-Pearson omnibus normality test, and Spearman correlations were used in other cases. The level for statistical significance was defined as $p < 0.05$ or $p < 0.01$.

Results

Basic characterizations of a HFD-fed type 2 diabetic mouse model

The basic conditions of mice fed with HFD or CHD are presented in Fig. 1. There was an obvious body weight increase in HFD-fed mice after 8 weeks compared with those fed with CHD (Fig. 1A). Blood glucose levels were sharply increased after initiation of 8 weeks

of feeding with HFD, along with high levels of blood glucose in the following 8 weeks of HFD compared with CHD (Fig. 1B). All these results showed that HFD for 8 weeks can establish a type 2 diabetic mouse model. Meanwhile, the increase in mouse kidney weights was noted starting at 8 weeks of HFD compared with those of CHD (Fig. 1C). Fig. 1D shows that glomerular fibrosis appeared after 12 weeks of HFD and became worse after 16 weeks of HFD. Fibrosis was significantly increased throughout the period of HFD feeding ($p = 0.036$). To further confirm the renal fibrosis, Picrosirius red staining was applied to show the increase in renal fibrosis percent coincided with HFD feeding (Fig. 1E). As previously reported, HFD could induce kidney injury and renal fibrosis. Together, these results suggest that long-term HFD feeding aggravates renal fibrosis.

GFR cannot illustrate renal injury after HFD feeding in type 2 diabetic mice

With the HFD-induced kidney injury model, we further investigated renal function. As shown in Fig. 2A, the HFD induced a significant and sustained elevation in UACR after week 8. However, the levels of UACR were not obviously increased for the following 8 weeks of HFD-feeding ($p = 0.570$). The GFR was also progressively elevated in HFD-fed mice compared

Fig. 1. High fat diet caused renal injury. A. 8-week-old male C57BL/6 mice were fed with CHD or HFD. Changes in body weight in mice are presented at the indicated times. B. Temporal evolution of blood glucose in mice fed with CHD or HFD at the different time points. C. Temporal increase in kidney weight in mice fed with HFD compared with CHD at the same time. D. Renal fibrosis was increased after HFD feeding. Masson's staining was used to identify and quantitate glomerular fibrosis in mice fed with CHD or HFD for the indicated time. The left panel is the representative image of glomerular fibrosis, the middle is the quantification of the

area of the glomerular fibrosis, and the right is the quantification of the area of renal fibrosis. E. Renal fibrosis shown by Picrosirius red staining. The left panel is the representative image of Picrosirius red staining, the others are the quantification of Sirius positive and fibrotic positive areas. $n = 10$ each group, * $p < 0.05$ vs. CHD at the same time point.

Fig. 2. HFD led to renal injury and renal inflammation. A. 8-week-old male C57BL/6 mice were fed with CHD or HFD. Urine albumin/creatinine ratio (UACR) increased with HFD at 8, 12, and 16 weeks. B. Temporal evolution of glomerular filtration rate (GFR) in mice fed with CHD or HFD at the different time points. The quantitative glomerular filtration rate was determined by the renal FITC-inulin clearance expression in $\mu\text{l}/\text{min}$. C. Renal $\text{TNF}\alpha$ mRNA levels were elevated with an HFD at 8, 12, and 16 weeks. D. IL-6 mRNA levels were increased in renal tissues from HFD-fed mice for 8, 12, and 16 weeks compared with CHD-fed mice over the same time. E. Temporal evaluation of renal MCP-1 mRNA levels in renal tissues were shown at the indicated time. F. Renal IL-1 β mRNA levels were increased with an HFD at 8, 12, and 16 weeks. G and H, the protein levels of $\text{TNF}\alpha$ and IL-6 in serum are shown after 16 weeks HFD feeding. $n=10$ each group, * $p<0.05$ vs. CHD at the same time point.

with that in CHD-fed mice after week 8, and then it reached its highest level at week 12 (Fig. 2B). The high levels of GFR in HFD-fed mice were still noted after week 16 compared with those of CHD feeding. However, the values of GFR declined after 16 weeks of HFD-feeding compared with 12 weeks of HFD-feeding (HFD for 12 w, $211\pm 20 \mu\text{l}/\text{min}$, 95% CI, 197 to 225; HFD for 16 w, $187\pm 26 \mu\text{l}/\text{min}$, 95% CI, 168 to 206, $p=0.032$). Additionally, the mRNA levels of $\text{TNF}\alpha$, IL-6, monocyte chemotactic protein-1 (MCP-1), and IL-1 β were obviously increased in the kidney tissues from HFD-fed mice for 8 weeks compared with those from CHD-fed mice (Fig. 2C-F). The differences in $\text{TNF}\alpha$, IL-6, MCP-1, and IL-1 β were maintained following 8 weeks of HFD feeding ($p < 0.001$, $= 0.002$, < 0.001 , $= 0.001$, respectively). Meanwhile, we found an increase in $\text{TNF}\alpha$ and IL-6 in sera in the mouse model with HFD feeding compared with that of CHD feeding (Fig. 2G and 2H). Thus, this finding illustrated that GFR may not be a reliable marker for renal function in HFD-fed mice, as it could not represent renal injury or renal fibrosis.

Changes in renal blood flow in HFD-fed mice

Blood flow analysis was performed in pulsed-wave Doppler images to obtain peak systolic velocity (Vs) and minimal end diastolic velocity (Vd) in renal arteries (Fig. 3A). HFD-feeding could suppress the velocity of Vs compared with CHD-feeding, but the differences among Vs were not significant at the different time points with HFD feeding (Fig. 3A, 3B). The values of Vd were greater in CHD-fed mice than those of HFD-fed mice (Fig. 3A, 3C). Decreases in Vd after 12 weeks of HFD-feeding were significant compared with those after 8 weeks of HFD-feeding. However, there was no significant difference in Vd levels between 12 weeks and 16 weeks of HFD-feeding (Fig. 3A, 3C). Surprisingly, it was found that RI was significantly greater in HFD-fed mice than in CHD-fed mice at each time point (Fig. 3D). Additionally, RI values increased successively from 8 weeks to 16 weeks in the HFD-treated group (12 w, 0.73 ± 0.04 , 95% CI, 0.70 to 0.76; 16 w, 0.76 ± 0.01 , 95% CI, 0.75 to 0.77, $p = 0.031$) (Fig. 3D). There were significantly different RI values across the process of HFD feeding ($p = 0.0002$). Together, the results showed that HFD can increase the levels of RI over time.

Correlation between RI and renal fibrosis

To evaluate whether RI can exactly represent renal injury, we first analyzed the correlation between metabolic parameters and RI. The results reflect that RI was correlated with body weight ($r = 0.577$, 95% CI 0.521 to 0.612, $p = 0.03$). Otherwise, there was no association between RI and blood glucose ($r = 0.327$, $p = 0.62$). Additionally, we analyzed the

Fig. 3. HFD affected blood flow in the renal arteries. A. Representative ultrasound images in pulse-wave Doppler mode showed the typical peak systolic velocity (Vs) and the minimal end diastolic velocity (Vd) in renal arteries from HFD or CHD mice. B. Temporal evolution of Vs in mice fed with CHD or HFD at the different time points. C. Renal Vd levels were elevated with an HFD at 8, 12, and 16 weeks. D. Resistive index (RI) levels were increased in renal arteries from HFD-fed mice for 8, 12, and 16 weeks compared with CHD-fed mice over the same time. $n = 10$ each group, * $p < 0.05$ vs. CHD at the same time point.

Table 1. Correlation between renal fibrosis and GFR or RI after HFD feeding. GFR indicates glomerular filtration rate; RI indicates resistive index; CHD indicates chow diet; HFD indicates high fat diet. * indicates p value <0.05; ** indicates p value < 0.01

	Feeding time		R	p-value
GFR	8w	CHD	0.991	<0.0001**
		HFD	0.997	<0.0001**
	12w	CHD	0.997	<0.0001**
		HFD	0.982	<0.0001**
	16w	CHD	0.977	<0.0001**
		HFD	0.024	0.947
RI	8w	CHD	0.756	0.011*
		HFD	0.825	0.003**
	12w	CHD	0.786	0.007**
		HFD	0.736	0.015*
	16w	CHD	0.747	0.013*
		HFD	0.763	0.01*

Table 2. Correlations between RI and GFR. GFR indicates glomerular filtration rate; RI indicates resistive index; CHD indicates chow diet; HFD indicates high fat diet. * indicates p value <0.05; ** indicates p value < 0.01

			R	p-value
GFR	8w	CHD	0.794	0.006**
		HFD	0.763	0.01*
	12w	CHD	0.795	0.006**
		HFD	0.711	0.021*
	16w	CHD	0.748	0.013*
		HFD	0.117	0.747

correlation between GFR and renal fibrosis. In the CHD-fed mice, GFR was high-positively correlated with the percent of glomerular fibrosis (Table 1). Until 12 weeks of HFD-feeding, the values of GFR were related to the glomerular fibrosis. However, the relationship between GFR and glomerular fibrosis disappeared after 16 weeks of HFD feeding ($r = 0.024$, $p = 0.947$) (Table 1). Nevertheless, RI was significantly correlated with renal fibrosis, even at the late stage of HFD feeding ($r = 0.763$, 95% CI 0.760 to 0.779, $p = 0.01$) (Table 1).

RI was significantly correlated with GFR at an early stage of HFD-feeding for the first 12 weeks (8 w, $r = 0.763$, 95% CI 0.760 to 0.769, $p = 0.01$; 12 w, $r = 0.711$, 95% CI 0.709 to 0.714, $p = 0.021$, respectively). After that, RI was not correlated with GFR after 16 weeks ($r = 0.117$, $p = 0.747$) (Table 2). These data indicate that RI is better than GFR at reflecting the condition of renal fibrosis and renal injury in HFD-fed mice.

Additionally, the significant correlation between IR and mRNA levels of MCP-1 and IL-1 β in kidney tissue (MCP-1, $r = 0.512$, 95% CI 0.473 to 0.564, $p = 0.01$; IL-1 β , $r = 0.346$, 95% CI 0.294 to 0.389, $p = 0.02$) further supports our choice of RI.

Ability of RI analysis to evaluate the effects of lovastatin treatment

To determine RI as a sensitive indicator in assessing potential therapy, a subset of the HFD-induced type 2 diabetic mice underwent treatment with lovastatin for renal injury. It was noted that lovastatin significantly suppressed the HFD-induced body weight gain (HFD, 50.03 ± 2.18 g, 95% CI, 48.47 to 51.59; HFD+Lovastatin, 39.58 ± 2.71 g, 95% CI, 37.65 to 41.51, $p < 0.001$) (Fig. 4A). Blood glucose was maintained after lovastatin treatment (Fig. 4B), while kidney weight and blood glucose in HFD-fed mice were reduced with lovastatin treatment group (HFD, 0.45 ± 0.05 g, 95% CI, 0.41 to 0.48; HFD+Lovastatin, 0.40 ± 0.04 g, 95% CI, 0.37 to 0.43, $p = 0.038$) (Fig. 4C). As illustrated in Masson's staining, glomerular fibrosis caused by HFD-feeding was prevented by lovastatin (Fig. 4D). Furthermore, Picrosirius red staining also showed that lovastatin can suppress the renal fibrosis caused by HFD-feeding (Fig. 4E).

Additionally, the increase in GFR in mice fed an HFD was significantly suppressed by lovastatin (HFD, 211 ± 27 μ g/ml, 95% CI, 192 to 231; HFD+Lovastatin, 131 ± 21 μ g/ml, 95% CI, 115 to 146, $p < 0.001$) (Fig. 5A). Furthermore, lovastatin reduced the levels of UACR in HFD-fed mice compared with CHD (HFD, 103 ± 25 μ g/ml, 95% CI, 85 to 121; HFD+Lovastatin, 70 ± 14 μ g/ml, 95% CI, 60 to 81, $p = 0.002$) (Fig. 5B). HFD-induced increases in TNF- α , IL-6,

Fig. 4. Lovastatin suppressed the kidney injury caused by HFD feeding. A. 8-week-old male C57BL/6 mice were fed with CHD or HFD for 12 weeks. Then, these mice were randomly separated into two groups. One was treated with vehicle as a control, and another was treated with lovastatin for 8 weeks. Body weights of mice were presented after 8 weeks of lovastatin treatment. B. Blood glucose levels in mice fed with CHD or HFD with/without lovastatin treatment are shown. C. The increase in kidney weight in mice fed with HFD was prevented by lovastatin compared with control. D. Lovastatin prevented HFD-induced renal fibrosis. Masson's staining was used to identify and quantitate glomerular fibrosis in CHD- or HFD-fed mice with/without lovastatin. The left panel is the representative image of glomerular fibrosis, the middle panel is the quantification of the area of glomerular fibrosis, and the right panel is the quantification of the area of renal fibrosis. E. Renal fibrosis is shown with Picrosirius red staining. The left panel is the representative image for Picrosirius red staining, the others are the quantification of Sirius positive and fibrotic positive areas. n =10 each group, * p<0.05 vs. CHD; # p<0.05 vs. Veh.

MCP-1, and IL-1 β mRNA levels were suppressed by lovastatin, indicating that lovastatin also had an anti-inflammatory role in type 2 diabetes (Fig. 5C-F). The results of the protein levels of TNF- α and IL-6 in sera were consistent with the mRNA level results (Fig. 5G, H).

As shown in Fig. 6A, there was no significant change in Vs in HFD-fed mice between lovastatin and control groups. However, lovastatin could increase Vd in HFD-fed mice compared with control treatment in HFD-fed mice (Fig. 6B). The increase in RI in HFD-fed mice was significantly suppressed by lovastatin treatment (HFD, 0.80 ± 0.49 , 95% CI, 0.76 to 0.83; HFD+Lovastatin, 0.60 ± 0.06 , 95% CI, 0.56 to 0.65, $p < 0.001$) (Fig. 6C).

Fig. 5. Lovastatin can recover HFD-caused renal injury and inflammation.

A. Lovastatin could affect the GFR in mice with HFD feeding. B. Lovastatin can sharply prevent the increase in UACR caused by HFD after 20 weeks. C. Renal TNF α mRNA levels were decreased by lovastatin in HFD-fed mice. D. The increase in IL-6 mRNA levels caused by HFD feeding was prevented by lovastatin treatment. E. Lovastatin could obviously inhibit the increase in MCP-1 mRNA levels in renal tissues induced by

HFD feeding. F. HFD-induced increases in renal IL-1 β mRNA levels were blocked by lovastatin treatment. G and H. The increases in TNF α and IL-6 protein levels in serum caused by HFD feeding were prevented by lovastatin treatment. n = 10 each group, * p < 0.05 vs. CHD; # p < 0.05 vs. Veh.

Fig. 6. RI can correctly reflect the improvement in renal injury by lovastatin in HFD-fed mice. A. No changes were noted in Vs after lovastatin treatment in HFD-fed mice. B. Vd levels were increased a small amount after lovastatin treatment in HFD-induced diabetic mice. C. Lovastatin can sharply suppress the increase in RI levels induced by HFD feeding. n = 10 each group, * p < 0.05 vs. CHD; # p < 0.05 vs. Veh.

Discussion

In this study, we suggested the utility of a noninvasive, repeatable, and highly sensitive parameter for the evaluation of renal injury in an HFD-induced type 2 diabetic mouse model *in vivo*. Renal RI measurements performed with *real-time* monitoring showed high sensitivity and comparability during the whole disease development and therapeutic treatment. Furthermore, RI measurement is sensitive and accurate for monitoring the changes in renal injury to standard therapy with lovastatin for HFD-induced type 2 diabetic mice.

Here, we found that GFR is recognized as a marker of renal function but does not accurately reflect renal injury. Currently, considering the difference in gender, race, weight,

and life style, estimated glomerular filtration rate (eGFR) is widely used in daily practice for evaluation of renal function. However, due to the lack of a standard formula for mouse eGFR evaluation, it is impossible to evaluate eGFR for mice based on strain background, food and age in research. Therefore, the results of GFR from different labs around the world are not comparable and are inconclusive. Additionally, our results are consistent with previous reports that GFR values in the early stage of renal injury increase, yet these values decreased to less than those of the normal condition (Fig. 2B). GFR levels increase during the decompensated renal injury and then decline from mild chronic kidney disease to renal failure. From our study, we noticed that GFR levels stop increasing any more after long-term HFD-feeding (Fig. 2B). It was demonstrated that GFR may be maximized in certain hyperfiltration states, such as diabetic nephropathy, and thus cannot increase further in response to some stimuli. Therefore, GFR is not suitable for evaluating renal injury in research.

In addition, UACR is not a good biomarker for renal function evaluation in research, although it is widely considered valuable for renal function evaluation. Our results showed that renal injury by HFD-feeding can be identified by UACR compared with CHD feeding (Fig. 2A). However, UACR is not sensitive to the worsening progression of renal injury in the process of HFD feeding ($p = 0.570$). Furthermore, renal fibrosis is an ideal parameter for evaluating only the end point of the renal injury. It is not suitable for monitoring the process of disease development or therapeutic progression *in real-time*.

Prior investigations have used velocity of renal blood flow to reflect renal injury. It was demonstrated that HFD can induce arterial stiffness, so the minimal end diastolic velocity can be used as marker of arterial stiffness. Our experiments show Vd levels declined in HFD-fed mice compared with those of CHD-fed mice. In addition, the Vd levels were decreased along with HFD feeding time. Noticeably, the velocity of blood flow is subjectively affected by measurement angles, body temperature, and some other factors. With these disadvantages, it is not suitable and comparable to take Vd as a good parameter for renal injury.

In clinical studies, subtle abnormalities in renal RI are associated with the presence of renal injury and the long-term outcomes in chronic nephropathies [19]. Renal risk factors result in elevated renal RI during the development of renal injury. Currently, clinical studies have shown that detection of subtle changes in RI were associated with renal vascular tone during sepsis, acute kidney injury and stage of chronic kidney disease in patients with renal allograft [9, 11]. Additionally, RI could be used for the management of hypertensive patients [20]. In experimental studies, the adaptation of renal RI to facilitate detailed renal injury has been limited, primarily because of technical difficulties in imaging mice versus humans, including the small size of mice, the high heart rates of small animals, and the high speed for data acquisition and storage. However, recently developed imaging techniques allow us to obtain high-frequency and high-resolution images with ultrasound to monitor blood flow and analyze RI values in the tiny vessel [21]. Accordingly, we observed that RI levels were increased in the early stage of HFD-feeding, and they continued to increase with long-term HFD-feeding.

Our results also suggest that the use of RI measurement is sensitive and reliable for determining the effectiveness of a pharmacological therapy for type 2 diabetes. Compared to conventional renal injury measurements, measures of RI value exhibited the benefit of standard therapy during the entire therapeutic procedure. Prior studies have shown that statin can suppress renal injury caused by HFD feeding. The values of GFR and tissue staining at the final time point are missing detailed information during the process of treatment. It has been shown from our data that it is possible that the values of RI present the changes in renal injury during the entire process of statin treatment in HFD-fed mice.

Conclusion

Our study suggested that noninvasive assessment of renal injury using RI evaluation in a small animal model provided a highly sensitive *in vivo* characterization of distinct trajectories of renal fibrosis and renal injury caused by HFD feeding. Thus, the use of RI assessment to serially track the changes in renal blood flow could provide insight into patterns of renal structure, renal injury, and recovery in response to therapy. This finding will potentially benefit future clinical diagnosis and monitoring of treatment effectiveness.

Disclosure Statement

None.

Acknowledgements

This study was supported by a grant from the National Natural Science Foundation of China (No. 81500720, 81303109), a grant from the Health Bureau Technology Fund of Tianjin (No. 2014KZ092), the Research Fund for the Doctoral Program of Higher Education of China (20113250120006), and the Natural Science Fund of Yangzhou City (YZ2014047). We gratefully acknowledge Dr. Pei Sun for her contribution in discussing some parts of the current study, and Dr. Fang Wang for data analysis.

Zejun Ma and Qiulun Lu conceived and designed the study. Zejun Ma, Shan Lu, Rui Li, and Liangkun Lyu performed the experiments. Hairong Xu, Zejun Ma, Rui Li, Lexi Ding and Qiulun Lu analyzed data and interpreted the results of the experiments. Zejun Ma, Shan Lu, and Lexi Ding drafted the manuscript.

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