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Original Article

Rutin ameliorates renal fibrosis and proteinuria in 5/6-nephrectomized rats by anti-oxidation and inhibiting activation of TGFβ1-smad signaling

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Abstract: Objectives: Rutin, a polyphenolic flavonoid, was reported to have beneficial effect on drug induced nephropathy. The present study aimed to introduce 5/6 nephrectomized rat model to further evaluate its renal protective effect. Methods: Adult Wistar rats were induced to develop chronic renal failure through 5/6 nephrectomy (5/6 Nx). After that, animals were treated orally with saline, rutin at 15 and 45 mg/kg, and losartan (10 mg/kg) daily for 20 weeks; sham-operated animals were also involved as control. After treatment for 8 and 20 weeks, blood and urine samples were collected for biochemical examination; all the kidney remnants were collected for histological examination. The protein levels of TGF-β1, smad2 and phosphorylated-smad2 (p-smad2) in kidney were measured. Immunohistochemistry was used to analyze the expression of TGF-β1, fibronectin and collagen IV in kidney tissues. Results: Results suggested that rutin could reduce the proteinurea, blood urine nitrogen and blood creatinine in 5/6 Nx animals significantly, as well as oxidation stress in the kidney. By histological examination, rutin administration alleviated glomerular sclerosis scores and tubulointerstitial injuries in a dose-dependent manner ($P<0.01$). Immunohistochemistry also suggested rutin could reduce the expression of TGF-β1, fibronectin and collagen IV in kidney tissues. By western blot, we found the rutin could reduce the TGF-β1, p-smad2 expression in the kidney tissues of rats. Conclusions: This study suggests that the rutin can improve renal function in 5/6 Nx rats effectively. Its effect may be due to its anti-oxidation and inhibiting TGFβ1-Smad signaling.

Keywords: Rutin, 5/6 nephrectomy, TGF-β1, chronic renal failure

Introduction

Chronic kidney disease (CKD) is emerging as a major global health threat [1, 2], however, there is lack of long-term effective medicine against CKD currently; Searching for new potency compounds against CKD is still a major challenge.

Rutin works as a scavenger of reactive oxidative species (ROS) by donating hydrogen atoms to peroxy radicals, superoxide anions, and singlet oxygen and hydroxyl radicals; it also functions as a terminator and chelator of metal ions that are capable of oxidizing lipid peroxidation [3, 4]. Rutin has been shown to function as an anti-cancer, anti-viral, anti-bacterial and anti-inflammatory agent. It is also used to treat cardiovascular and neurodegenerative disorders because of its appreciable free radical-scavenging and anti-oxidant capacities [5-8]. Additionally, studies suggest that rutin alters signal transduction, causes activation of transcription factors and gene expression, and may also protect DNA by interacting with carcinogens that have escaped detoxification processes [9, 10]. Several studies also demonstrated that rutin has significant beneficial kidney protection effect in ischemia/reperfusion renal injury [11, 12], drug-induced nephropathy [3] and diabetic nephropathy [13].

To further study its renal protective effect on chronic kidney dysfunction besides diabetic nephropathy, the remnant kidney model of 5/6 nephrectomy (5/6 Nx) in rats was introduced in the current investigation. This model is often used to study the mechanisms of and potential therapeutic approaches to progression of chronic kidney disease (CKD) with renal mass reduction [14, 15]. In this model, systemic hypertension and proteinuria contribute to kidney injury and to the expression of pro-inflam-

matory and pro-fibrotic molecules by kidney cells. The nephrectomy is widely used as an animal model for glomerular hyperfiltration followed by glomerulosclerosis [16], it can mimic the advanced-stage nephropathy which is caused by various factors [17]. TGFβ-smad signal pathway was considered to play important roles in renal end-stage fibrosis in this model. In the current study, besides analysis of renal function and histopathological changes, we also measure the blood pressure and expression of TGFβ1, smad2 and p-samd2 in the kidneys of 5/6 Nx rats, aimed to understand the underlying mechanisms of rutin on renal beneficial effect.

Materials and methods

Chemicals

Rutin was purchased from sigma Aldrich (Germany), enzymic antioxidants (superoxide dismutase [SOD], catalase, glutathione peroxidase [GPx] and glutathione reductase [GRx]) and nonenzymic antioxidants (reduced glutathione [GSH] were measured by commercial kits (Nanjing Jiancheng Ltd, Nanjing, China).

Animals

Male Wistar rats with an average weight of 200-240 g (institute of laboratory animal science, Chinese Academy of Medical Sciences, Beijing, China) were used in this study. Animals were housed in a climate-controlled vivarium with 12-h day and night cycles and were fed a standard laboratory diet and water ad libitum. The animals were randomly assigned to the chronic renal failure (CRF) and sham-operated control groups. The CRF group underwent 5/6 nephrectomy by surgical resection of the upper and lower thirds of left kidney, followed by tight nephrectomy 7 days later. The control group underwent sham operation. The procedures were carried out under general anesthesia (sodium pentobarbital, 50 mg/kg, i.p.) using strict hemostasis and aseptic techniques. The 5/6 nephrectomized animals were randomly divided into untreated and rutin-treated (15 and 45 mg/kg, dissolved in 0.5% carboxymethycellulose-Na buffer, p.o.) subgroups. The whole administration lasted for 20 weeks. The untreated group received regular water instead. Losartan (10 mg/kg. p.o.) was also administrated as positive control. Ten animals were included in each group. At the time of 16 weeks and 20 weeks, blood pressure, including the systolic blood pressure (SAP) and diastolic blood pressure (DAP), were measured by tail-cuff plethysmography (BP-98A; softron, Tokyo, Japan) with prior training to minimize variability in the blood pressure measurement.

At the end of the experiment, animals were anesthetized (sodium pentobarbital, 50 mg/kg i.p.) and euthanized by exsanguinations using cardiac puncture. Kidneys were removed. A piece of the kidney was separated and fixed in 10% formalin for histological examination.

All the animal experiments were approved by the Ethics Committee of Laboratory Animals of Chinese PLA General Hospital (Beijing, China), the protocol was approved on 15th, June 2013.

Blood and urine chemistry

At the times when rutin was administered for 8 and 20 weeks, the blood of rats was sampled through the eyes after rats were anesthetized with diethyl ether. Twenty-four-hour urine collections were obtained in each animal after placement in metabolic cage the day before collecting blood samples. Urine protein concentration, blood urea nitrogen (BUN) and plasma creatinine (Scr) were measured by the standard biochemical kits (Nanjing Jiancheng LTD, Nanjing, China) respectively.

Assessment of antioxidant profile

Renal tissue was homogenized in 10 volume of 100 mmol KH_2PO_4 buffer containing 1 mmol EDTA (pH 7.4) and centrifuged at 12,000× g for 30 min at 4°C. The supernatant was collected and used for enzymatic studies. Catalase assay (CAT), superoxide dismutase assay (SOD), Estimation of lipid peroxidation assay (TBARS), Glutathione-S-transferase assay (GST), Glutathione reductase assay (GSR), Glutathione peroxidase assay (GSH-px), Reduced glutathione assay (GSH) was conducted respectively according to the instruction of kit manual.

Histological examination

The fixed renal tissue blocks were embedded in paraffin. Sections of 2 μm thickness were cut and stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The stained sections were examined under a light microscope at a magnification 200× and the severity of glomerulosclerosis was graded in a blind manner on a scale of 0-4 as previously [18]: Grade 0,

normal; Grade 1, sclerotic area up to 25% (minimal); Grade 2, sclerotic area 25-50% (moderate); Grade 3, sclerotic area 50-75% (moderate to severe) and Grade 4, sclerotic area 75-100% (severe). The scores from each individual glomerulus examined (100 glomeruli from each rat) were averaged for each rat.

As for tubulointerstitial damage, a scoring system was applied (from 0 to 4), in which tubular atrophy, dilation, casts, interstitial inflammation, and fibrosis were assessed in 10 kidney fields at a magnification of ×200: 0, normal; 1, lesions in <25% of the area; 2, lesions in 25% to 50% of the area; 3, lesions in >50% of the area; and 4, lesions involving the entire area [19, 20].

Immunohistochemistry

Expression of TGFβ1, fibronectin and collagen IV in kidney was detected by the streptavidin-peroxidase-biotin (SP) immunohistochemical method. The kidneys were cut into 4 μm thick sections, which were placed on immunohistochemical slides. After dehydration with xylene and alcohol, the antigen retrieval was accomplished. The sections were incubated in a blocking solution of 10% normal goat serum diluted in PBS, and incubated with polyclonal rabbit anti- TGFβ1, fibronectin and collagen IV antibodies (1:200, Abcam, USA) for 2 h, then incubated for 10 min at room temperature with biotinylated goat anti-rabbit secondary antibody (1:500). After that, sections were exposed to streptavidin-biotin complex conjugated to HRP. Sections were then incubated with diaminobenzene (DAB) for 5-10 min and observed under the microscope. Each sample was examined at 200× magnification under a light microscope (Leica, Germany). For each slide, the labeled surface area for three different fields, which came from three random, non-overlapping areas of one slice from each mouse, was evaluated by quantitative image analysis using Image-Pro Plus software (Bethesda, MD, USA), and the mean of the three different fields was calculated. Positive areas were expressed as percentage of positive area per glomerulus.

Western blot analysis of TGFβ1 and samd2/p-smad2

To detest the expression of TGF-β1, samd2 and p-smad2 in renal tissues, we performed the western blotting. Renal tissue lysate was prepared using a lysis buffer containing 25 mmol/l Tris-HCl, 150 mmol/l NaCl, 5 mmol/l ethyleneglycol bis (2-aminoethyl ether) tetraacetic acid (EGTA), 5 mmol/l ethylenediamine tetraacetic acid (EDTA), 10 mmol/l NaF, 1 mmol/l phenylmethyl sulfonylfluoride (PMSF), 1% TritonX-100, 0.5% Nonidet P40, 10 mg/l aprotinin, 10 mg/l leupeptin and quantified by Bradford dye-binding procedure. Equal amounts of protein were separated by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) (5% stacking gel and 10% separating gel for β-actin (mouse monoclonal, Santa Cruz, USA), smad2 (rabbit monoclonal, CST, USA) and p-smad2 (rabbit monoclonal, CST, USA) or 12% separating gel for TGF-β1 (rabbit monoclonal, CST, USA) and electroblotted onto nitrocellulose. After blocking with 3% bovine serum albumin (BSA), the membranes were incubated with appropriate antibodies overnight. Membranes were then incubated with a horseradish peroxidase-conjugated secondary anti-body against rabbit or mouse immunoglobulin G (IgG) at a 1:2000 dilution for 1 h at room temperature after being washed. Reactive proteins were viewed by ECL. The signals were detected with FujiFilm Las-3000 (Tokyo, Japan). The intensity of the detected bands was analyzed using Image J program.

Statistical analysis

All the values were represented as the means ± standard error (S.E.M.) and were analyzed by ANOVA and post hoc Bonferroni test. Difference was considered significant when $P<0.05$.

Results

Rutin decreased the SAP and DAP in 5/6 Nx rats

As shown in **Figure 1**, 20 weeks later, the 5/6 Nx rats established significant higher blood pressure compared to sham control after operation. After treatment for 20 weeks, rutin treatment reduced the SAP by almost 20% at 45 mg/kg compared to model control ($P<0.05$); meanwhile, 15 mg/kg rutin treatment also showed a certain effect. Rutin also could reduce DAP at dose dependent manner.

Rutin reduced the Scr, BUN and proteinurea in 5/6 Nx rats during the long-term treatment

As expected, all the 5/6 nephrectomized animals had significant elevation of Scr, BUN and

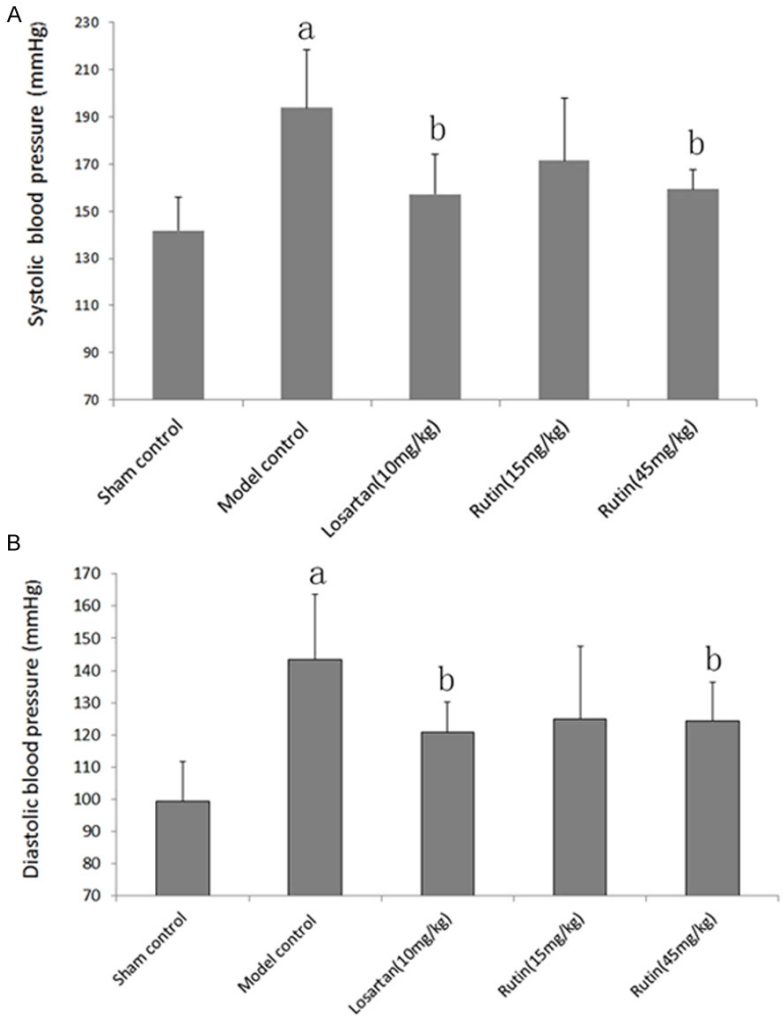

Figure 1. Rutin decreased blood pressure and heart rate on 5/6 nephrectomized rats: A. Systolic blood pressure; B. Diastolic blood pressure. Data are means ± S.D. (n=10). [a]*P*<0.01, versus sham control; [b]*P*<0.05, versus model control.

proteinuria (**Tables 1** and **2**). Eight-week administration of rutin already significantly improved these biochemical parameters, which shared the similar effect as losartan (10 mg/kg) (**Table 1**, *P*<0.05). As shown in **Table 2**, after administration for 20 weeks, rutin showed significant beneficial effect on reducing Scr, BUN and proteinuria, increase the creatinine clearance (*P*<0.01). Proteinuria decreased by 63.70% and 69.40% respectively at dose of 15 and 45 mg/kg, compared with model control, *P*<0.01. Rutin also reduced BUN by 34.30% (45 mg/kg, vs. model control) and Scr by 37.14% (45 mg/kg, vs. model control), compared with model control, both *P*<0.05, meanwhile increased the creatinine clearance by 125% and 158% respectively at 15 mg/kg and 45 mg/kg.

Effects of rutin on antioxidant profile

The results regarding the protective effects of rutin against the oxidative stress in rat on kidney protein and activities of antioxidant enzymes such as CAT, SOD, GSH-Px, GSR and GST are shown in **Table 3**. Activities of antioxidant enzymes such as CAT, SOD, GSH-Px, GSR and GST were reduced (*P*<0.01) in the 5/6 Nx as compared to control group. This reduction in enzymes activity was reversed significantly (*P*<0.01), in a concentration dependent way, by the treatment of rutin as compared to the 5/6 Nx group. Losartan at 10 mg/kg also showed a certain beneficial effect in antioxidant profile.

Benefits of rutin on renal histopathological injuries

The model control group displayed glomerular hypertrophy, mesangial cell proliferation, mesangial matrix accumulation, telangiectasia or occlusions of the capillaries, thickening of the glomerular capsule wall, and focal or global sclerosis of some glomeruli. Furthermore, the renal tubules in this group showed dilation or atrophy, a large number of protein casts, interstitial widening, substantial infiltration of inflammatory cells, and focal distribution of renal interstitial microangiopathy, with narrowing and distortion of capillary cavities.

Compared with the model control group, the pathological changes in both rutin-treated groups were alleviated to different extents: lower glomerular sclerosis scores and tubulointerstitial scores were observed in these groups in a dose dependent manner (**Figure 2**, *P*<0.05). The losartan-treated group also showed similar amelioration, while its effect was less significant than rutin (**Figure 2**).

Table 1. General data in the 5/6 nephrectomized rats after treatment with the rutin for 8 weeks

Index	Sham control	Model control	Losartan (10 mg/kg)	Rutin (15 mg/kg)	Rutin (45 mg/kg)
N	10	10	10	10	10
Body weight (g)	428.0±21.9	412.0±11.7	399.0±18.2	410.6±31.2	393.0±26.5
24-h urine protein (mg/day)	14.66±4.91	91.22±44.5[a]	31.48±17.66[c]	44.77±22.04[c]	35.01±5.62[c]
Blood urea nitrogen (mg/dL)	20.7±1.8	52.9±17.2[a]	38.1±4.8[b]	41.9±8.9	42.2±5.6
Plasma creatinine (mg/dL)	1.74±0.24	2.72±0.60[a]	2.01±0.33[c]	1.70±0.40[c]	1.66±0.43[c]
Creatinine clearance (ml/min)	3.5±1.22	2.4±0.76[a]	3.2±1.14[b]	3.1±1.21[b]	3.3±1.40[b]

[a]$P<0.01$, versus sham control; [b]$P<0.05$, [c]$P<0.01$, versus model control.

Table 2. General data in the 5/6 nephrectomized rats after treatment with the rutin for 20 weeks

Index	Sham control	Model control	Losartan (10 mg/kg)	Rutin (15 mg/kg)	Rutin (45 mg/kg)
N	10	10	10	10	10
Body weight (g)	493.7±34.1	456.7±32.1	461.3±34.7	440.0±44.7	446.3±23.3
24-h urine protein (mg/day)	18.39±6.37	209.8±106.3[a]	105.73±63.12[b]	76.10±37.75[c]	64.21±15.48[c]
Blood urea nitrogen (mg/l)	20.0±3.5	52.8±20.4[a]	45.0±11.5[b]	43.5±11.4	34.7±8.9[b]
Plasma creatinine (mg/l)	1.00±0.17	2.10±1.06[a]	1.51±0.22[b]	1.39±0.24	1.32±0.16[b]
Creatinine clearance (ml/mim)	3.5±1.12	1.2±0.34	2.6±0.85	2.7±0.68	3.1±0.97

[a]$P<0.01$, versus sham control; [b]$P<0.05$, [c]$P<0.01$, versus model control.

Table 3. Effects of rutin on renal antioxidant profile

Treatment	CAT (U/min)	SOD (U/mg protein)	GSH-Px (nmol/mg protein)	GSR (nmol/min/mg protein)	GST (nmol/min/mg protein)
Sham control	17.7±2.3	19.45±2.11	44.77±1.77	201.5±11.5	129.9±15.6
Model control	7.2±0.78[a]	8.13±1.78[a]	22.14±2.11[a]	101.4±9.8[a]	60.34±13.5[a]
Losartan (10 mg/kg)	9.4±1.21[b]	10.87±1.89[b]	32.58±3.20[b]	145.2±13.4[b]	99.5±15.2[b]
Rutin (15 mg/kg)	12.3±1.32[b]	12.55±1.84[b]	33.17±2.57[b]	156.4±12.8[b]	103.4±13.5[b]
Rutin (45 mg/kg)	15.34±1.83[c]	16.49±1.73[c]	41.25±3.12[c]	184.5±13.9[c]	110.3±12.7[c]

[a]$P<0.01$, versus sham control; [b]$P<0.05$, [c]$P<0.01$, versus model control.

Expression of TGFβ1, fibronectin and collagen IV in kidneys by immunohistochemistry

As shown in **Figures 3** and **4**, after raising for 20 weeks, animals with 5/6 Nx showed higher expression of TGFβ1, fibronectin and collagen IV by immunohistochemistry in glomeruli compared with that of sham controls ($P<0.05$). Treatment with 15 or 45 mg/kg rutin dose-dependently decreased expression of these fibrosis-related biomolecules in glomeruli and renal interstitium.

Expression of TGFβ1 and samd2 in kidney tissues

Western blotting analyses showed that the expression of TGF-β1 in kidney tissues was reduced by the skimmin treatment, in a dose dependent manner (**Figure 5**), same trend as well as smad2 and p-smad2. These findings suggested that rutin might inhibit the renal fibrosis via modulation of TGF-β1 signaling pathway in the end stage of renal dysfunction.

Discussion

The 5/6 nephrectomy rat is a classic model of progressive renal scarring characterized by both glomerulosclerosis and interstitial fibrosis, in which both glomerular and peritubular capillary endothelial injuries have been reported [21-23]. In this study, the levels of creatinine, BUN and proteinuria in the 5/6 Nx group were progressively elevated, which were also accompanied by typical pathological changes. Oral administration of rutin was shown to ame-

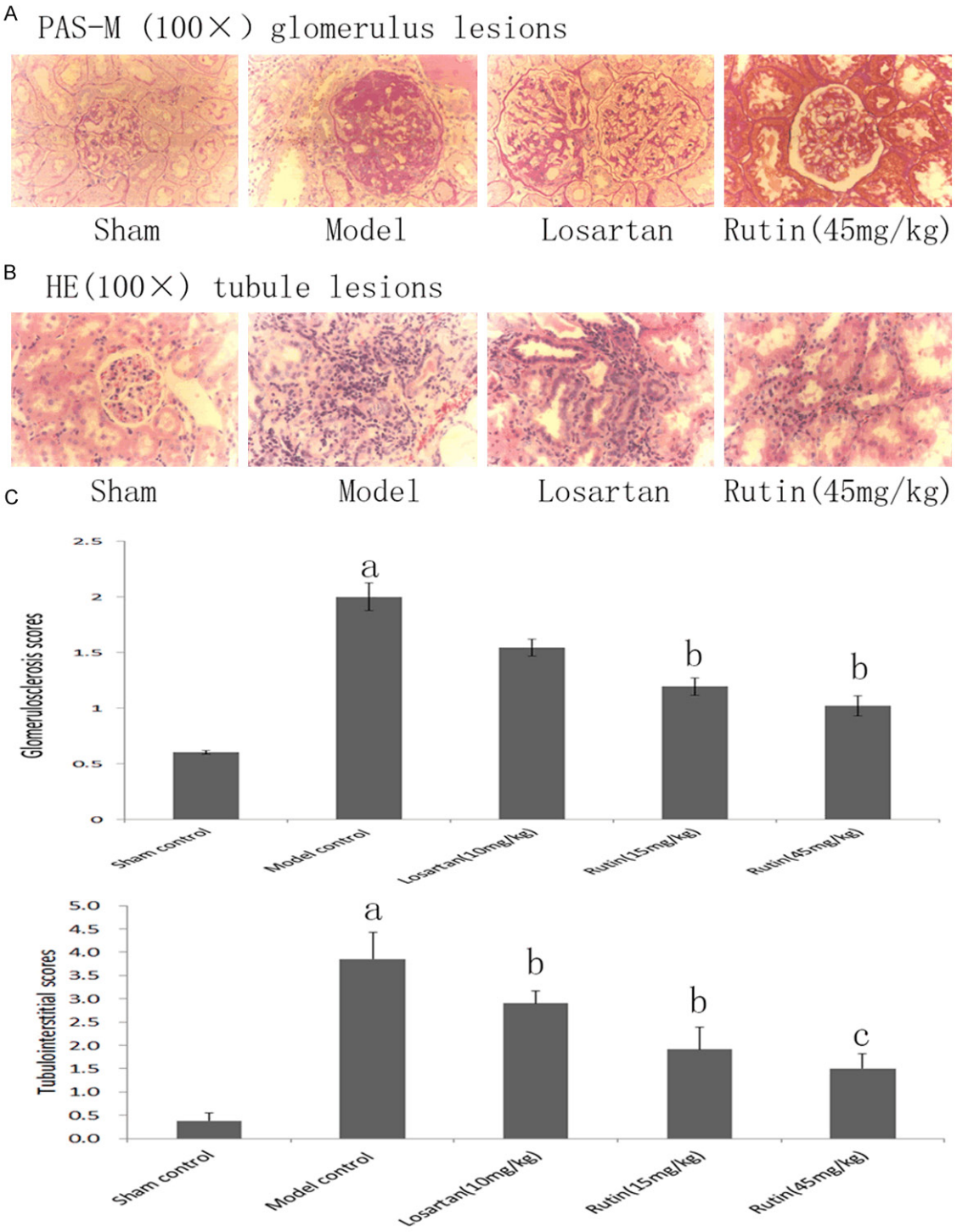

Figure 2. Representations of PAS-stained (A) or HE-stained (B) kidneys of 5/6 (Nx) sham-operated rats, model control, losartan-treated and rutin-treated animals; (C) is quantitative analysis of glomerulosclerosis scores and tubulointerstitial damage index Data are means ± S.D. (n=10). [a]*P*<0.01, versus sham control; [b]*P*<0.05, [c]*P*<0.01, versus model control.

liorate glomerulosclerosis and renal interstitial fibrosis in this study, which indicated that rutin has the same renoprotective effects in the 5/6 Nx model as it was reported to ameliorate diabetic nephropathy and other drug-induced nephropathy [24].

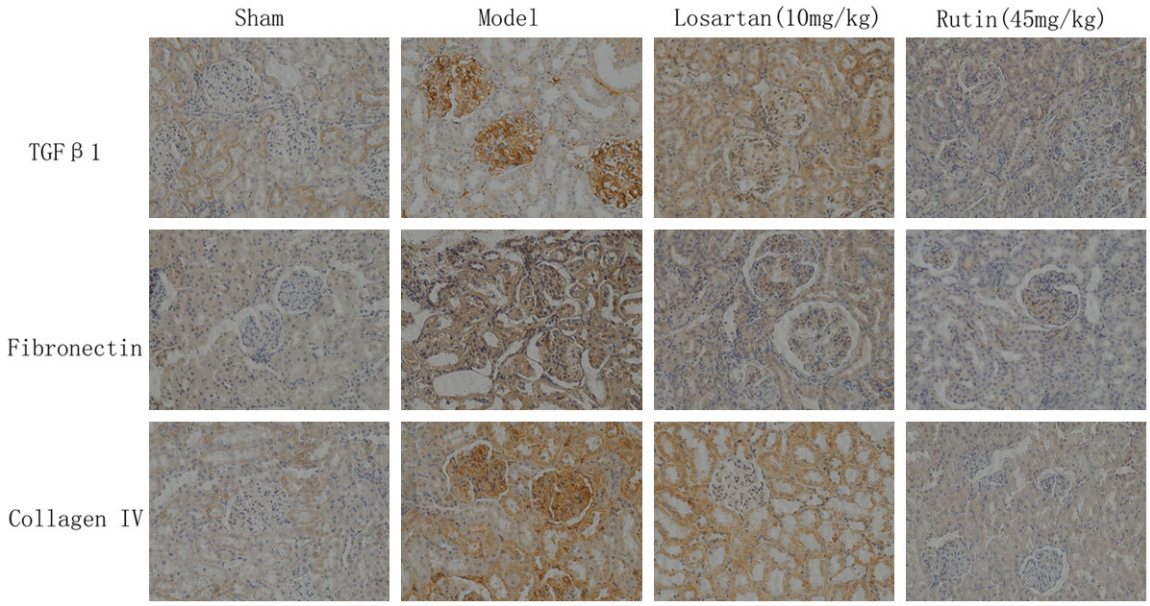

Figure 3. Expression of TGFβ1, fibronectin and Collagen IV in the cortex of kidney (200×).

Previous studies suggest that anti-oxidation characteristic of rutin plays an important role in its renal beneficial effect [12, 13, 25, 26]. In the current study, we introduce 5/6 Nx animal model to mimic the progression of renal damage resulting from reduced nephron mass. This model has been used to investigate the effects of drugs on pathophysiological events in the progression of glomerulosclerosis and chronic renal failure, which is characteristic of advanced-stage or end-stage renal dysfunction [27].

Currently, we successfully established 5/6 Nx rat model. The urinary excretion of protein in nephrectomised rats increased with time more markedly than in the sham-operated rats, as well as BUN and Scr; the blood pressure in the 5/6 Nx rats gradually rose during the experiment; in the histopathological examination, obvious glomerulosclerosis and interstitial fibrosis was found in the remnant kidneys of the model control animals. These findings were consistent with those in the previous study of rodents [18, 28].

After treatment for 20 weeks, rutin improved the blood urea nitrogen and creatinine clearance significantly, also it reduced the albumin excretion and urine protein level in the urine of 5/6 Nx rats (**Tables 1** and **2**), compared to model control. Urine protein level increases progressively with CRF progression, and urinary protein level was a key determinant for renal dysfunction via worsening tubulointerstitial fibrosis [29, 30]. By reducing the urine protein, rutin treatment improved the kidney filtration and slowed down the progress of chronic renal failure.

By histopathological analysis, we further confirmed that rutin has satisfactory beneficial effect on 5/6 Nx nephropathy in Wistar rats, rutin significantly suppressed the glomerulosclerosis and tubulointerstitial damage in a dose-dependent manner, and its effect is better than losartan at 10 mg/kg dosage.

Cytokines-driven glomerular mesangial cell proliferation and overproduction of extracellular matrix (ECM) play important roles in the response of the kidneys to injury and in the development of glomerulosclerosis. Fibronectin and collagen IV are important factors for overproduction of ECM. By immunohistochemistry, we could prove that rutin could inhibit the content of fibronectin and collagen IV in the renal interstitium.

TGFβ1 plays a key role in the renal fibrosis at the end stage of renal dysfunction. In the current study, we found lower TGFβ1 level in the rutin-treated groups in all three doses. TGFβ1 overexpression contributes to progressive renal fibrosis [31], TGF-β1 is widely expressed in all cells of the kidney, where it exerts proin-

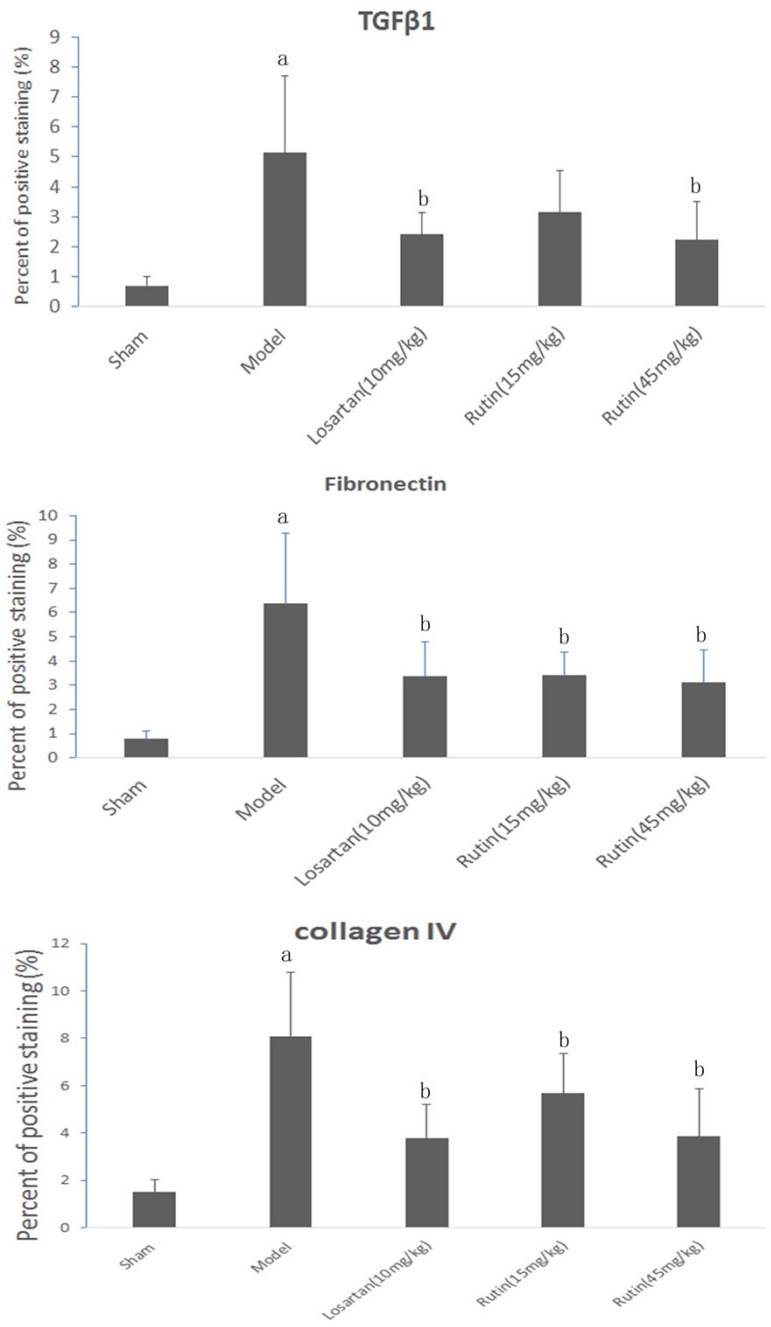

Figure 4. Expression and quantitation analysis of TGFβ1, fibronectin and Collagen IV in the glomeruli, (designated by percentage of positive areas in glomerulus). [a]*P*<0.01, versus sham control; [b]*P*<0.05, [c]*P*<0.01, versus model control.

flammatory and profibrotic effects, mediating extracellular matrix deposition, increasing the synthesis of matrix components and reducing their degradation [32]. Putative factors inducing TGF-β1 expression include overload of renal cells with excessive filtered plasma proteins, renin-angiotensin system activation and hyperglycemia [33, 34]. Experimental studies have suggested inhibiting TGF-β1 can prevent renal insufficiency [35, 36]. By present study, we further confirm one of underlying mechanisms of rutin's renal protective effect is due to down-regulation of TGF-β1. More interestingly, current study showed that after 13-week treatment, the concentration of TGFβ1 in 5/6 Nx animals was much lower than sham control, we assumed that the remnant kidney could not produce higher TGFβ1 because of less nephrons left.

Increased oxidation also enhanced the progression of CRF in 5/6 Nx rats. In the present study, the mean activity of the antioxidant enzymes CAT, SOD, GSH-Px, GST and GSR were found to be significantly lowered in the model group compared with that of the sham control group. However, the treatment of rutin modified the biochemical changes caused by nephron loss in rat. In the present study, the mean activities of antioxidant enzymes were significantly higher compared with those of the model group and thus had a potential protective effect.

In a conclusion, long-term rutin administration improves hypertension and proteinuria and attenuates glomerulosclerosis and tubular-intertinum injuries in the remnant kidneys of animals with CRF induced by subtotal nephrectomy; it may be due to the anti-oxidation effect and blocking of TGF-Smad signal pathway.

Disclosure of conflict of interest

None.

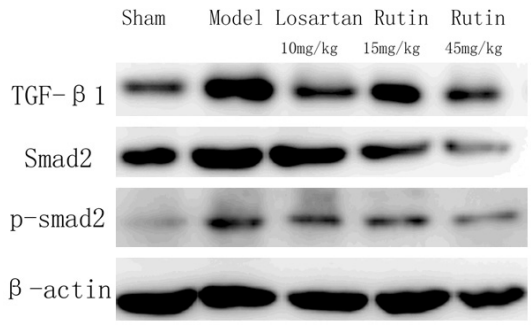

Figure 5. Western blot of TGFβ1, smad2 and p-smad2 in the kidney tissues.

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