Mechanisms of Protective Effect of Ramulus Mori Polysaccharides on Renal Injury in High-Fat Diet/Streptozotocin-Induced Diabetic Rats

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Key Words
Ramulus mori polysaccharides • Diabetic nephropathy • Antioxidant activities • Renoprotection

Abstract
Background: Diabetic nephropathy (DN) is the most important complication of diabetes and the most common cause of end-stage renal disease (ESRD). Aims: A recent study established that the Ramulus mori polysaccharides (RMP) exert antioxidant effects on DN in rats. Methods: The diabetic rats which induced by high-fat diet and streptozotocin injection were orally administered RMP by doses of 250, 500 and 1000 mg/kg daily for 8 weeks. The effects of RMP on hyperglycemia and other biochemical changes were examined in the sera and kidney tissues. Additionally, the pathological and ultrastructural changes and expressions of nuclear-factor kappa B (NF-κB) and transforming growth factor-β1 (TGF-β1) were assessed. Results: The results revealed that the serum levels of blood glucose, total cholesterol (TC) and triglycerides (TG) were significantly decreased by RMP. Furthermore, the blood urea nitrogen (BUN), serum creatinine (SCr) and 24-hour urine protein levels in the RMP-medicated rats were lower than those in untreated diabetic rats. Moreover, treatment of the DN rats with RMP normalized all biochemical changes, including the malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels in the serum and kidney tissues. In contrast, the protein expression levels of NF-κB and TGF-β1, which were enhanced in the kidneys of DN rats, were reduced by RMP. Conclusion: These results suggest that RMP improving the renal function of diabetic rats possibly via its ameliorating antioxidant activities.

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Introduction

DN is one of the important microvascular complications of diabetes mellitus and is also the leading cause of ESRD. The prevalence of DN is increasing yearly with the increasing in the numbers of diabetes mellitus patients [1]. The pathogenesis of DN is complicated and involves genetic susceptibility, lipid metabolism disorders, oxidative stress, hemodynamic changes and multiple cytokines and ultimately leads to chronic kidney injury [2, 3].

The excessive generation of reactive oxygen species (ROS) that are produced under conditions of oxidative stress might cause disorder in the balance between oxidation and antioxidation and led to subsequent cell damage in the tissues [4, 5]. Hyperglycemia promotes the generation of ROS in mesangial cells and tubular epithelial cells, and these processes accelerate the progression of diabetes and ultimately result in DN [6, 7]. It has been reported that the SOD activity of DN patients is much higher than those of diabetic patients without nephropathy or healthy people [8]. The up-regulation of SOD activity might protect diabetic rats against DN by improving oxidative stress. Moreover, the MDA content of DN rats is abnormally increased, and the GSH-Px activity is decreased [9-11]. In this context, increasing numbers of studies support the notion that an adverse lipid profile is a risk factor of DN [12, 13]. Hyperglycemia in patients with type 2 diabetes usually accompanies dyslipidemia. The increasing of TC and TG serum levels have been observed in the early stage of DN [14]. In addition to lipids, the relative levels of other renal metabolic factors, particularly advanced glycation end products (AGEs), have been reported in the development of diabetic kidney disease [15]. AGEs promote the accumulation of extracellular matrix (ECM) in the glomeruli and tubulo-interstitium. Moreover, the stimulatory capability of TGF-β1 on ECM accumulation is activated by AGEs [16, 17], and thus, TGF-β1 is considered to be one of the crucial factors that modulate renal cell proliferation, glomerulosclerosis and interstitial fibrosis in diabetes [18].

Morus alba is an folk medicine widely used for DN treatment. Previous studies have demonstrated that Morus alba exhibits comprehensive pharmacological effects that include, for example, hypoglycemic, immunomodulatory and antioxidant activities [19-21]. Our previous studies have found that RMP exerts hypoglycemic effect via regulating the intrapancreatic JNK/p38 pathway and blocking the IL-1/NF-κB pathway to protect against streptozotocin-induced apoptosis in pancreatic tissue and cytotoxicity in renal tissue [22-24]. Thus, we hypothesized that RMP might exert a beneficial effect on the development of DN. The aim of the present study was to investigate the underlying mechanisms of RMP on high-fat diet/streptozotocin-induced DN in rats.

Materials and Methods

Chemicals

Streptozotocin was purchased from Sigma Co., Ltd. (Missouri, USA), dissolved in fresh sodium citrate buffer and stored under cold condition until use. More details about RMP (including preparation, purity analysis, structure characterization and medicinal safety) were identified in our previous achievements [25]. Other required materials were labelled as described below.

Animals

Seven-week-old male Wistar rats (180-200 g) were obtained from the Experimental Animal Center of Guangxi Medical University (registration number SCXK 2009-0002). The animals were housed at a temperature of 24-26°C on a 12-h light/dark cycle and fed with standard rodent chow and water. The experimental procedures and protocols involved in this study were approved by the Ethical Committee for the Experimental Use of Animals at Guangxi Medical University (Guangxi, China).

Experimental induction of diabetes

The rats were fed with high-fat diet (consisting of 100 g lard, 25 g cholesterol, 10 g sodium deoxycholate and 5 g sucrose combined with 45 g ordinary fodder). After 4 weeks, the rats were injected streptozotocin (40 mg/kg). After 72 h, blood samples were collected through the tail veins and used for blood glucose
measurements. Rats with fasting blood glucose (FBG) levels higher than 16.7 mmol/L were considered diabetic.

**Experimental procedures**

The diabetic rats were randomly assigned into following groups with 10 animals per group: Group I: healthy rats that were administered distilled water, i.e., the normal control group. Group II: diabetic rats that were administered distilled water, i.e., the model control group. Group III: diabetic rats that were administered 500 mg/kg metformin, i.e., the positive control group. Group IV: diabetic rats that were administered 250 mg/kg RMP. Group V: diabetic rats that were administered 500 mg/kg RMP. Group VI: diabetic rats that were administered 1000 mg/kg RMP.

The RMP and metformin groups were received oral administrations once daily for 8 weeks. The normal control and model control groups were given equal volumes of distilled water. FBG levels were tested during the RMP treatment at weeks 0, 4 and 8 using accurate blood glucose meter (Accu-check Performa, Roche, Germany). At the end of the experiment, the rats were placed in individual metabolic cages for 24 h to collect urine samples. The blood and kidney tissue samples were collected after the overnight fasted rats were sacrificed and then stored at −80°C for further determinations. The left kidney samples were cut into small pieces and fixed in 2.5% pre-cooling glutaraldehyde immediately for 2 h at 0°C for transmission electron microscope examination.

**Biochemical analysis**

TC, TG, BUN, SCR and 24-h urinary protein excretion were measured by the laboratory of the First Affiliated Hospital of Guangxi Medical University (Guangxi, China).

**Assessment of SOD and GSH-Px activities and MDA contents in the sera and kidney tissues**

The kidney tissues were homogenized with ice-cold saline and then were centrifuged at 3500 rpm for 10 min at 4°C. The activities of SOD, GSH-Px and the contents of MDA in the homogenates and serum samples were determined using commercial kits (Nanjing Jiancheng, Institute of Biotechnology, Nanjing, China) to investigate the antioxidant function of the kidney tissues. The operating procedures were conducted according to the manufacturer’s instructions.

**Histopathological examination**

The kidney samples were fixed with 10% formaldehyde for 24 hours and then embedded in paraffin. The samples were stained with hematoxylin and eosin after being cut into sections and were then examined under a light microscope.

**Electron microscopy examination**

For the electron microscopy examinations, the fresh kidney tissues were cut into small pieces and fixed in 2.5% pre-cooling glutaraldehyde immediately at the temperature of 0°C. Ultrathin sections were cut to perform uranyl acetate and lead citrate staining. Finally, the samples were observed under a transmission electron microscopy (Hitachi H-7650).

**Western blot analysis**

Briefly, the kidney tissues were homogenized in lysis buffer. The renal proteins were obtained, and the protein concentrations were determined using protein assay reagent (Bio-Rad). The renal proteins were fractionated by SDS-PAGE and transferred to polyvinyl difluoride membranes. The membranes were blocked with PBST buffer (20% Tween-20, PBS) and then incubated with primary antibodies against NF-κBp65 and TGF-β1 at 4°C overnight. Following extensive washing, the membranes were incubated with a goat anti-rabbit IgG (Boster Biotechnology) for 2 h at 20°C. The signals were visualized with a gel image analysis system (UVP) after washing. The protein bands were analyzed with Scion Image software (Scion Corp., Frederick, MD, USA).

**Statistical analyses**

The data are expressed as means ± S.E. The statistical differences between groups were analyzed with one-way ANOVAs followed by Tukey’s tests for comparisons between groups using SPSS16.0. P-values < 0.05 were considered statistically significant.
Results

Effects of RMP on FBG, TC and TG

FBG levels were determined after the rats were fasted for 12 h at weeks 0, 4 and 8 during the RMP treatment. The results revealed that, at week 0, the FBG levels of the high-fat diet/streptozotocin-induced diabetic rats were significantly higher than those of the normal control. After 4 and 8 weeks of treatments with metformin and RMP, the FBG levels were obviously decreased, whereas these levels were enhanced in the model control group (Fig. 1). The serum TC and TG contents of the RMP-treated groups were significantly lower than those of the model control (Fig. 2).

Fig. 1. Effect of Ramulus mori polysaccharides (RMP) on fasting blood glucose (FBG). I: normal control group; II: model control group; III: 500 mg·kg⁻¹·d⁻¹ metformin; IV: 250 mg·kg⁻¹·d⁻¹ RMP; V: 500 mg·kg⁻¹·d⁻¹ RMP; VI: 1000 mg·kg⁻¹·d⁻¹ RMP. The results are presented as the mean ± the S.E.* P < 0.05 compared with the normal control group; # P < 0.05 compared with the model control group.

Fig. 2. Effect of Ramulus mori polysaccharides (RMP) on the serum contents of total cholesterol (TC) and triglycerides (TG). I: normal control group; II: model control group; III: 500 mg·kg⁻¹·d⁻¹ metformin; IV: 250 mg·kg⁻¹·d⁻¹ RMP; V: 500 mg·kg⁻¹·d⁻¹ RMP; VI: 1000 mg·kg⁻¹·d⁻¹ RMP. The results are presented as the mean ± the S.E. * P < 0.05 compared with the normal control group; # P < 0.05 compared with the model control group.
Effects of RMP on renal function
Compared with the normal control group, the serum levels of BUN and SCr and the 24-h urinary protein excretion were markedly increased in the model control group. However, these indices of renal function were reduced following the treatments of the diabetic rats with metformin and 500 and 1000 mg/kg RMP. In addition, the SCr and the 24-h urinary protein levels of 250 mg/kg RMP group were also lowered. But the BUN in 250 mg/kg RMP treated rats was reduced without significance when compared to model group (Table 1).

Effects of RMP on oxidative parameters in the sera and kidney tissues
Oxidative stress was assessed by detecting the activities of SOD and GSH-Px and the MDA levels in the sera and kidney tissues. The diabetic rats exhibited significant decreases in the SOD and GSH-Px activities, while the MDA levels were enhanced compared with the normal control group both in the sera and kidney tissues of the rats. Following the administration of RMP for 8 weeks, elevations in SOD and GSH-Px activities were observed and were greater in the 1000 mg/kg RMP group (Table 2, 3).

Histological and ultrastructural results
H&E staining of the kidneys of the model control group revealed tubular vacuolar degeneration, glomerular collapse and dilatation of the renal tubules compared with the

| Table 1. Effect of Ramulus mori polysaccharides (RMP) treatment on renal function parameters. I: normal control group; II: model control group; III: 500 mg.kg⁻¹.d⁻¹ of metformin; IV: 250 mg.kg⁻¹.d⁻¹ of RMP; V: 500 mg.kg⁻¹.d⁻¹ of RMP; VI: 1000 mg.kg⁻¹.d⁻¹ of RMP. The results were presented as the means ± S.E. * P < 0.05 compared with the normal control group; # P < 0.05 compared with the model control group |
|---|---|---|---|---|---|
| Items | I | II | III | IV | V | VI |
| BUN (mg/dl) | 17.9±1.6 | 31.5±2.6* | 18.1±2.7* | 28.6±2.2* | 21.0±1.9** | 19.3±1.7* |
| SCr (mg/dl) | 0.361±0.051 | 0.506±0.069* | 0.410±0.056* | 0.462±0.074** | 0.451±0.055** | 0.428±0.054* |
| Urine protein (mg/dl) | 2.31±0.65 | 8.15±0.88* | 3.58±0.91* | 6.10±0.53* | 3.16±0.32* | 3.44±0.51* |

| Table 2. Effect of Ramulus mori polysaccharides (RMP) treatment on oxidative parameters in kidney tissue. I: normal control group; II: model control group; III: 500 mg.kg⁻¹.d⁻¹ of metformin; IV: 250 mg.kg⁻¹.d⁻¹ of RMP; V: 500 mg.kg⁻¹.d⁻¹ of RMP; VI: 1000 mg.kg⁻¹.d⁻¹ of RMP. The results were presented as the means ± S.E. * P < 0.05 compared with the normal control group; # P < 0.05 compared with the model control group |
|---|---|---|---|---|---|
| Items | I | II | III | IV | V | VI |
| MDA (nmol/mg) | 2.36±0.82 | 4.51±0.53* | 2.89±0.28* | 4.18±0.42* | 3.16±0.26** | 2.95±0.35* |
| SOD (U/mg) | 14.06±1.40 | 8.42±0.65* | 12.51±0.83* | 10.36±0.16* | 9.58±1.81** | 11.09±0.59** |
| GSH-Px(U/mg) | 2.8±0.16 | 1.2±0.36* | 2.5±0.23** | 1.94±0.42** | 2.6±0.50** | 2.4±0.36** |

| Table 3. Effect of Ramulus mori polysaccharides (RMP) treatment on oxidative parameters in serum. I: normal control group; II: model control group; III: 500 mg.kg⁻¹.d⁻¹ of metformin; IV: 250 mg.kg⁻¹.d⁻¹ of RMP; V: 500 mg.kg⁻¹.d⁻¹ of RMP; VI: 1000 mg.kg⁻¹.d⁻¹ of RMP. The results were presented as the means ± S.E. * P < 0.05 compared with the normal control group; # P < 0.05 compared with the model control group |
|---|---|---|---|---|---|
| Items | I | II | III | IV | V | VI |
| MDA (nmol/mL) | 5.42±0.25 | 13.64±0.82* | 7.46±0.34* | 12.05±0.54* | 9.67±0.34** | 8.66±0.30* |
| SOD (U/mL) | 270.46±24.13 | 213.65±23.05* | 255.20±21.35* | 223.30±33.05* | 246.26±29.54* | 261.07±30.51* |
| GSH-Px (U/mL) | 3016.15±124.14 | 1862.34±169.24* | 2875.14±206.23* | 2163.05±195.42* | 2451.36±145.50* # | 2693.45±258.36* |
normal appearance of the normal control group. After the 8-week treatment with metformin and RMP, the tubular vacuolar degeneration and glomerular collapse were obviously mitigated. Additionally, glomerular basement membrane (GBM) thickening was present in
the 1000 mg/kg RMP group. However, the thickness of GBM was increased in model rats and rats treated with 250 mg/kg RMP (Fig. 3).

As shown in Fig. 4, significant mesangial matrix expansion, GBM fusion and mitochondrial damage were observed in the electron photomicrographs from the model control group, whereas normal kidney ultrastructure was observed in the normal control group. However, varying degrees of amelioration of the ultrastructural alterations were observed in the diabetic rats that were treated with metformin and RMP, particularly in the rats that were treated with 1000 mg/kg RMP.

**Effects of RMP on the protein expressions of NF-κB and TGF-β1**

The western blot data suggested that the protein expressions of NF-κB and TGF-β1 were both remarkably increased in the untreated diabetic rats compared with the normal control group, which exhibited no significant increase in the expressions of these proteins. Compared with the model control group, the RMP medication groups exhibited dose-dependently normalized NF-κB and TGF-β1 levels (Fig. 5).

**Discussion**

In the present study, protective effects of RMP against DN in type 2 diabetes model which was induced by high-fat diet and streptozotocin were clearly demonstrated and were mediated by the amelioration of oxidative stress and improvement of renal function via the inhibition of the expression of the NF-κB/TGF-β1 pathway. Obesity was induced by feeding rats with high-fat diet for 4 weeks and streptozotocin injection, which selectively acts on islet β-cells and results in insulin resistance, increases in blood glucose and reductions in insulin. The high-fat diet- and streptozotocin-treated rats were characterized by hyperglycemia and insulin resistance, which closely mimic the symptoms of human diabetes [26, 27].

The serum levels of BUN and SCr and the 24-h urinary protein excretion were examined in this study to identify changes in the renal function of the diabetic rats. In the model control group, the above parameters were markedly increased compared to the normal group. These changes were ameliorated by RMP. All these findings demonstrated that RMP might potentially be an agent that could exert beneficial effects on kidney tissue. This speculation was confirmed by the HE and ultrastructural results. The glomerular structures of the kidney were observed in the HE histology, and the pathological changes, including tubular vacuolar degeneration, glomerular collapse and dilatation of the renal tubules, were reversed by the 8-week RMP treatment. Furthermore, GBM fusion and mitochondrial damage were also inhibited by RMP as demonstrated by the electron photomicrographs.

Chronic hyperglycemia causes significant changes in oxidative stress markers. Diabetic patients exhibit much more severe oxidative stress than healthy people. Meanwhile, endoplasmic reticulum stress-induced podocyte apoptosis played a critical role in the development of DN [28]. Ouassila Aouacheri explored the differences in the oxidative stages of 59 diabetes patients and 48 healthy volunteers. The results revealed that the MDA levels of the diabetic patients were significant higher than those of the healthy people, while the GSH and SOD activities were decreased; moreover, antioxidant enzyme activities (i.e., glucose-6-phosphate dehydrogenase, glutathione peroxidase and glutathione reductase) were decreased in the type 2 diabetes patients [29]. MDA content is an important indicator of oxidative stress, and its formation is promoted by ROS in the kidney [30]. The results of our study revealed that the RMP-treated rats exhibited an amelioration of this oxidative stress, which might be an additional mechanism of anti-oxidant activity.

TGF-β1 is a multifunctional cytokine with a variety of regulatory effects on immune reactions that also mediates ECM production [31]. TGF-β1 gene polymorphisms are involved in diabetes complications, particularly DN disease in patients with hyperglycemia [32]. Hyperglycemia mediated by ROS activates transcription factors that ultimately lead to the activation of the profibrotic gene TGF-β1, which plays crucial role in the promotion of fibrosis.
One study demonstrated that low-dose TGF-β1 treatment in early DN attenuates the kidney hypertrophy and oxidative stress [35]. It is believed that activated NF-κB triggers the expression of the profibrotic gene TGF-β1, which further causes ECM accumulation via a mechanism in which activated NF-κB translocates into the nucleus, binds to the TGF-β1 DNA sequence, and triggers the transcription of TGF-β1, which in turn, ultimately promotes the occurrence of renal fibrosis [36]. In the present study, the NF-κB and TGF-β1 protein levels were significantly increased in the model control group according to the western blot results. However, the hyperglycemia-induced elevations of the NF-κB and TGF-β1 protein levels were effectively prevented by the 8-week treatment with RMP. This finding indicates that the renoprotective effect of RMP might be associated with the inhibition of the activation of the NF-κB-TGF-β1 pathway in DN.

In conclusion, our present study demonstrated that RMP exerted hypoglycemic effects and can attenuated oxidative stress in diabetic rats. Most importantly, we have shed new light on therapeutic targets for DN in the NF-κB-TGF-β1 pathway. Additional attention will be focused on RMP, which is a potential agent for the prevention of diabetic nephropathy.

Abbreviations

ESRD (end-stage renal disease); RMP (ramulus mori polysaccharides); NF-κB (nuclear-factor kappa B); TGF-β1 (transforming growth factor-β1); TC (total cholesterol); TG (triglycerides); MDA (malondialdehyde); SOD (superoxide dismutase); GSH-Px (glutathione peroxidase); DN (Diabetic nephropathy); ROS (reactive oxygen species); AGEs (advanced glycation end products); ECM (extracellular matrix); FBG (fasting blood glucose); BUN (blood urea nitrogen); SCr (serum creatinine).

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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