Original Article
Protective effect of dihydromyricetin on renal damage in diabetic rats

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Abstract: Diabetic nephropathy (DN) is now the main cause of end-stage renal disease. As an important factor causing end-stage renal disease, diabetic nephropathy is correlated with oxidative stress and inflammation response. This study aimed to investigate the protective function of dihydromyricetin (DHM) on the kidneys of diabetic rats. Adult male SD rats were randomly divided into a control group (group A), a model group (group B), and a DHM treatment group with high (group C), moderate (group D), and low (group E) dosage. After streptozotocin induction, DHM was applied via intragastric administration for 16 consecutive weeks with dosages of 160, 320 and 480 mg/(kg·d) for C, D, and E groups, respectively. Fasting blood glucose (BG), serum creatinine (Scr), blood urea nitrogen (BUN), and total cholesterol (TC), triacylglycerol (TG) were measured, along with morphological observation of renal cells. Antioxidant enzymes and pro-inflammatory cytokine production were estimated to reflect the oxidative stress and inflammatory state in the damaged kidney. Finally, the main proteins in the nuclear factor kappa B (NF-κB) and nuclear factor erythroid 2 (Nrf2) signaling pathway were measured to investigate the effect of DHM on these two pathways via western blot. The results demonstrated that model rats had significantly elevated levels of BG, Scr, BUN, TC and TG compared to controls (P<0.05). All these increases were partially but significantly suppressed by DHM (P<0.05), which also caused marked improvement of histopathological damages. In addition, DN-induced oxidative stress and inflammatory responses in the kidney were also prevented by DHM at each dosage (P<0.05). Moreover, after treatment with DHM for 16 weeks, the NF-κB signaling pathway inhibition and Nrf2 pathway activation in the kidney of DN rats (P<0.05). These results indicated that the renoprotective effect of DHM might be through its NF-κB and Nrf2 signaling pathway regulations.

Keywords: Diabetic, DHM, oxidative stress, inflammatory, NF-κB, Nrf2

Introduction

Diabetic nephropathy is one of the most serious complications in diabetes mellitus and the leading cause of end-stage renal disease worldwide, which remains a major cause of mortality and morbidity in the diabetic population [1, 2]. Studies have shown that various factors contribute to the renal damage and dysfunction, including long duration of stimulation from hyperglycaemia and hyperlipidemia, and the altered glomerular hemodynamics [3, 4]. Dia-"betic nephropathy is characterized by morphological and ultrastructural changes in the kidney including expansion of the molecular matrix and loss of the charge barrier on the glomerular basement membrane [5, 6]. The progression from normal albuminuria to microalbuminuria is considered the initial step in diabetic nephropathy which further progresses to macroalbuminuria as renal function continues to deteriorate and glomerular filtration rate (GFR) starts to decline [5, 6]. Due to the inherent complexity of metabolic disorders, treatment of end-stage DN is more difficult compared to other renal diseases. Therefore, it is urgent to develop new therapeutic strategies to manage the patients with diabetes and chronic kidney disease.

Studies showed that oxidative stress can interact with inflammation in diabetes, cooperatively promoting the development of DN [7-10]. Oxidative stress promotes pathological progression of DN by up-regulating production of pro-inflammatory cytokines such as tumor

necrosis factor (TNF)-α and interleukin (IL)-6 by activation of the nuclear factor kappa B (NF-κB). In turn, activated leucocytes in the inflammation also provoke oxidative stress through production of reactive oxygen species (ROS). Numerous reports suggest that nuclear factor erythroid 2-related factor 2 (Nrf2) signal pathway is a major pathway that is involved in the regulation of the natural antioxidant defense system, by activating this pathway, numerous genes that encode antioxidative proteins are transcribed and the antioxidative mechanisms are initiated [11, 12]. Nuclear factor kappa B (NF-κB), which defined as a hinge involved in regulating the express of inflammatory genes. Many studies have proved that targeting either NF-κB or Nrf2 signaling pathway could contribute to the recovery of animal models of kidney disease [13-16]. Therefore, the prevention and treatment that modulate either Nrf2 or NF-κB signaling pathway could contribute to the recovery of animal models of kidney disease.

Dihydromyricetin (DHM), a bioactive flavonoid compound extracted from the stems and leaves of Ampelopsis grosse dentata, has been reported to exert a number of biological and pharmacological actions, including anti-oxidative, anti-inflammatory, hepatoprotective, lipid and blood glucose regulatory, and anti-cancer effects [17, 18]. Animal experimental showed that the administration of DHM can reduce the blood glucose levels, and perform antioxidation and anti-inflammation in diabetic nephropathy rats. However, few reports have been issued on the molecular mechanisms involved of DHM. In view of the high in view of the high level of oxidative stress and inflammation associated with DN and the crucial role of Nrf2 and NF-κB signaling pathways in oxidative stress and inflammatory response, we evaluated the possible mechanism of DHM in protecting kidneys via the measurement of expression of Nrf2 and NF-κB in diabetic rat kidneys.

Materials and methods

Animals

Adult male Sprague-Dawley (SD) rats, weighing 180 to 200 g, were obtained from Experimental Animal Center of Shandong Luye Pharmaceutical Co., Ltd (Yantai, China). The animals were kept in a controlled light room with a photoperiod of 12 h dark and 12 h light at a temperature of (22±2)°C and 55±5% relative humidity. All experimental procedures conducted in this study were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Qi Lu Hospital of Shandong University.

Experimental protocol

The rats were randomly assigned into a control group (group A) with standard rodent chow diet and a diabetic group with long-term high-fat diet (HFD). After 8 weeks, diabetes of the HFD-fed rats was induced by an intraperitoneal single injection of 35 mg kg^{-1} of streptozotocin (Sigma-Aldrich Trading Co., Ltd, Shanghai, China) dissolved in citrate buffer solution (0.1 M, pH 4.5), and the rats of the control group were treated with equal volumes of citrate buffer. Three days after streptozotocin injection, the diabetic condition was confirmed by measuring fast blood glucose (FBG) level. Rats with FBG levels >14 mmol/L [19] were diagnosed as diabetic condition and divided randomly again into groups as model (group B), DHM (Zelang Medical Technological Co., Ltd, Nanjing, China) 160 mg kg^{-1} (group C), DHM 320 mg kg^{-1} (group D) and DHM 480 mg kg^{-1} (group E). The rats were administrated intragastrically with the corresponding medicine for 16 weeks, and the control and model groups were treated with normal saline (0.9%) in equivalent volumes.

Fasting blood glucose

The fasting blood glucose was determined at weeks 0, 8, 12, 16, 20 and 24 in overnight-fasted rats. The blood samples taken from caudal vein were used to assay the blood glucose levels via a One Touch Basic Blood Glucose Monitoring System (AccuChek, Basel, Switzerland).

Blood lipid profile and kidney function parameters

At the end of the experiment (16 weeks after the injection), the rats were anaesthetized with intraperitoneal injection of sodium pentobarbital (30 mg per kg body weight), and blood samples were collected from peritoneal veins, centrifuged at 3500 rpm and extracted for serum. The levels of blood total cholesterol (TC), triacylglycerol (TG), blood urea nitrogen (BUN) and serum creatinine (Scr) were assayed using

Table 1. Effect of DHM on fasting blood glucose of diabetic rats (mmol^{-1})

	Control	Model	DHM (160 mg kg^{-1})	DHM (360 mg kg^{-1})	DHM (480 mg kg^{-1})
0	6.20±0.60	6.15±0.78	6.21±0.81	6.16±0.68	6.19±0.74
8	6.24±0.13	7.89±1.62	7.88±1.56	7.89±1.50	7.91±1.72
12	6.18±0.16	24.85±1.70	22.56±9.08	19.35±5.10*	18.15±7.08*
16	6.25±0.20	22.94±2.10	16.27±4.46*	15.26±4.23*	14.87±1.43**
20	6.21±0.17	20.59±2.54	18.66±1.95	16.79±1.63**	15.98±1.95**
24	6.30±0.24	20.47±2.12	19.74±6.15	16.88±3.94**	15.55±2.37**

*$P<0.05$, **$P<0.01$ vs. model group.

Table 2. Effect of DHM on kidney function and blood lipid profiles of diabetic rats

Groups	TG (mmol/l)	TC (mmol/l)	BUN (mmol/l)	SCr (µmol/l)
Control	2.43±0.62	2.56±0.14	6.45±0.82	61.46±11.53
Model	4.27±0.41	5.23±0.52	17.71±2.49	158.83±14.71
DHM (160 mg kg^{-1})	3.98±0.63	3.36±0.45**	15.23±1.85*	147.56±12.19
DHM (360 mg kg^{-1})	2.86±0.38**	3.04±0.27**	14.58±1.59*	122.57±30.46*
DHM (480 mg kg^{-1})	2.63±0.59**	3.24±0.31**	11.25±1.71**	99.36±25.22**

*$P<0.05$, **$P<0.01$ vs. model group.

commercially available kits (JianCheng Biological Engineering Institute, Nanjing, China).

Histopathological analysis

The kidneys taken from sacrificed rats were fixed in 10% neutral formalin solution, embedded in paraffin, and cut into 5 µm sections. The sections were stained with hematoxylin-eosin and then examined by light microscopy.

Detection of antioxidant enzymes and inflammatory cytokines

Each kidney tissue sample was weighed using an analytical balance, and 100 mg tissue of each sample was homogenized at 0.01 M PBS buffer (pH 7.2). After the homogenate was centrifuged at 12,000×g for 30 min at 4°C, the supernatant was collected and quantitatively assayed for superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), interleukin (IL)-1β, tumor necrosis factor α (TNF-α) and IL-6, using ELISA kits (JianCheng Biological Engineering Institute, Nanjing, China) according to the manufacturers' instructions.

Western blot analysis

Total protein lysates were extracted from kidney tissues in NP-40 lysis buffer (Beyotime) containing 1% Triton X-100 with 1 mM PMSF. Protein extracts were centrifuged at 12,000×g for 10 min. And the supernatants were collected. Nuclear and cytosolic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) following the manufacturer's instruction. Total protein content was determined using BCA protein assay kit (Beyotime). Equal amounts of protein (40 µg) were electrophoresed and subsequently transferred onto PVDF membranes (Millipore, Boston, MA, USA). The blotted membranes were blocked with 5% skim dry milk for 1 hr at room temperature then incubated with the corresponding primary antibodies (Nrf2, HO-1, NF-κB, IκBα, β-actin; Histone, Wanleibio, Shenyang, China) in a blocking buffer overnight at 4°C. Thereafter, the membrane was incubated with appropriate secondary antibodies for 1 h at room temperature, and the blotted proteins were detected using the enhanced chemiluminescence reagent (7 Sea Pharmtech, Shanghai, China).

Statistical analysis

The results were expressed as means ± standard deviation (SD). The effect of DHM on each parameter was examined using one-way analysis of variance. Individual difference among groups were analyzed by Dunnett's test, and significance was accepted at $P<0.05$.

Results

Effects of DHM on blood glucose of diabetic rats

Table 1 shows the effects of DHM treatment on the blood glucose. In comparison, the levels of fasting blood glucose in D and E groups were

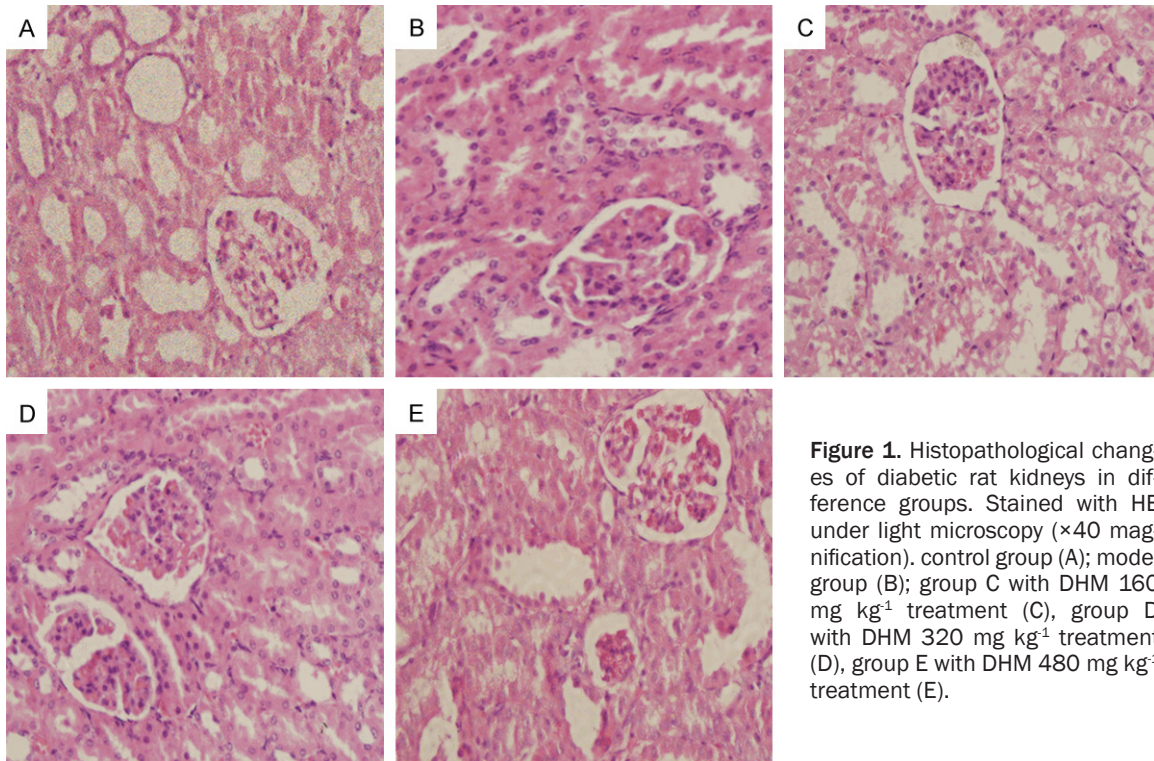**Figure 1.** Histopathological changes of diabetic rat kidneys in difference groups. Stained with HE under light microscopy (×40 magnification). control group (A); model group (B); group C with DHM 160 mg kg^{-1} treatment (C), group D with DHM 320 mg kg^{-1} treatment (D), group E with DHM 480 mg kg^{-1} treatment (E).

significantly lower than those of the group B at week 12, 16, 20 and 24 throughout the administration period. The significantly decreased fasting blood glucose was also observed at week 16 in the group C.

Effects of DHM on blood lipid profile and kidney function parameters

As shown in **Table 2**, Serum TC levels was significantly lower in the DHM treatment groups ($P<0.01$). A similar tendency was demonstrated on serum TG and a significant difference was observed between the D ($P<0.01$) and E ($P<0.01$) groups and the group B. The BUN, Scr levels were increased significantly by diabetic. After administration of DHM, BUN declined significantly at all doses ($P<0.05$). In addition, the markedly lower Scr, compared with group B, was shown in D ($P<0.05$) and E groups ($P<0.01$).

DHM treatment ameliorates renal injury in the kidney of diabetic rats

Renal histological findings with HE staining are shown in **Figure 1**. All parts of kidney showed normal appearance in group A (**Figure 1A**). After streptozotocin induction, a remarkable thickening of the glomerular basement mem-

brane and an obvious expansion of mesangium were observed in these diabetic rats (**Figure 1B**). But no significant alteration of such histopathology in kidney was found in diabetic rats with DHM dosed continually for 16 weeks (**Figure 1C-E**).

DHM treatment ameliorates oxidative stress in the kidney of diabetic rats

SOD (**Figure 2A**) and GSH-Px (**Figure 2B**) activities in the kidney of diabetic rats decreased as compared to the group A, DHM treatment significantly ameliorated the reduction of activities of these antioxidant as compared to the group B ($P<0.05$). Conversely, DHM treatment obviously decreased the level of MDA (**Figure 3C**) in the kidney of diabetic rats ($P<0.01$).

DHM treatment ameliorates inflammatory response in the kidney of diabetic rats

Inflammatory response was quantified through measuring levels of inflammatory cytokines in kidney tissue and the result exhibit in **Figure 3**. Compared with rats in the control group, rats with diabetic demonstrated increased TNF-α (**Figure 3A**, $P<0.05$), IL-6 (**Figure 3B**, $P<0.05$) and IL-1β (**Figure 3C**, $P<0.05$) levels. Treatment
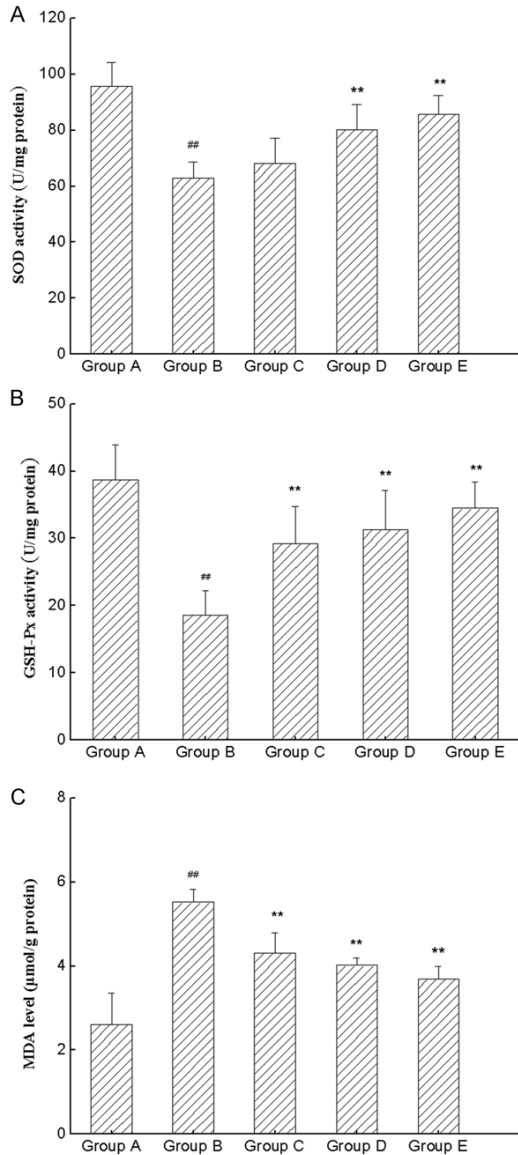
Figure 2. Effect of DHM on oxidative stress in the kidney of diabetic rats. SOD (A), GSH-Px (B) activity decreased and MDA (C) level increased in the kidney of diabetic rats, and DHM treatment reversed this changes. Data were shown as mean ± SD. ##$P<0.01$ vs. group A, *$P<0.05$, **$P<0.01$ vs. group B.
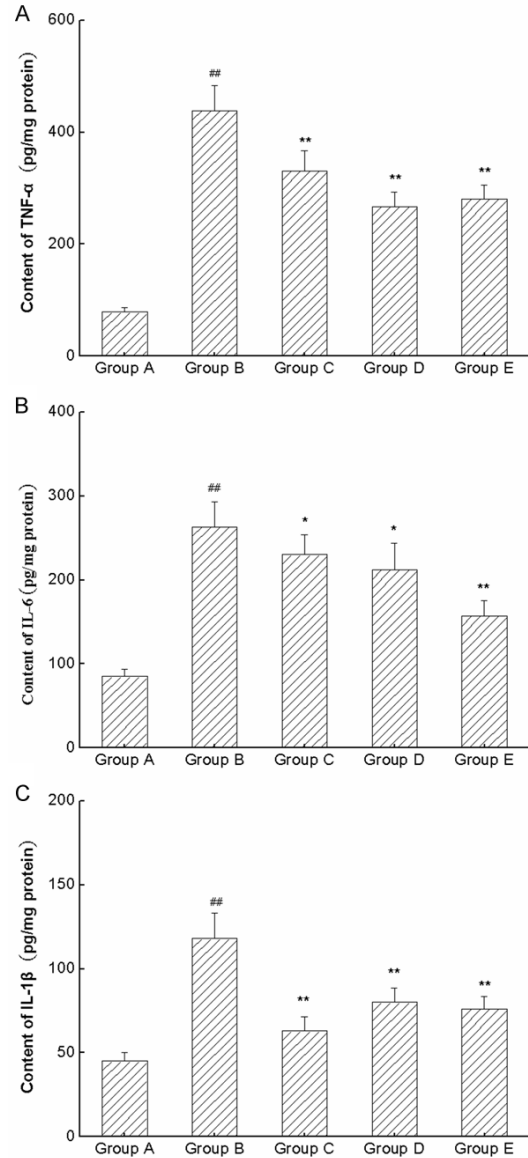
Figure 3. Effect of DHM on inflammatory response in the kidney of diabetic rats. (A) TNF-α (B) IL-6 (C) IL-1β levels in the kidneys of diabetic rats and DHM prevented inflammatory response. Data were shown as mean ± SD. ##$P<0.01$ vs. group A, *$P<0.05$, **$P<0.01$ vs. group B.

with DHM markedly decreased levels of these cytokines in the kidney at all doses ($P<0.05$).

DHM treatment activates Nrf2 in the kidney of diabetic rats

We performed Western blot analyses to examine the expression of Nrf2 and down-stream molecules HO-1 in the kidney. The results in **Figure 4** demonstrated that rats in group B showed decrease in the levels of Nrf2 ($P<0.01$)

and the expression of HO-1 ($P<0.01$) compared with normal rats, but when treated with DHM, the expression of Nrf2 ($P<0.01$) and HO-1 ($P<0.05$) were obviously upregulated in the kidney of the DHM treatment groups.

DHM treatment inhibits NF-κB activation in the kidney of diabetic rats

As shown in **Figure 5**, NF-κB was up-regulated with the stimulation of high blood lipid and glu-
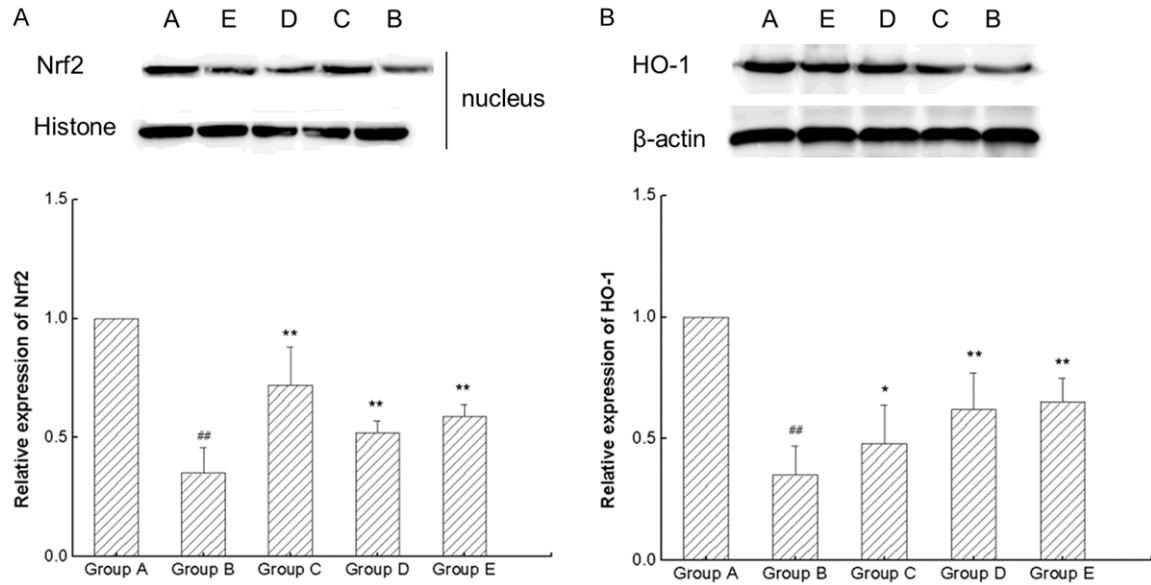
Figure 4. Effect of DHM on Nrf2 signaling pathway in the kidney of diabetic rats. DHM contributes to Nrf2 nuclear accumulation (A), and promotes HO-1 expression (B). ##$P<0.01$ vs. group A, *$P<0.05$, **$P<0.01$ vs. group B.
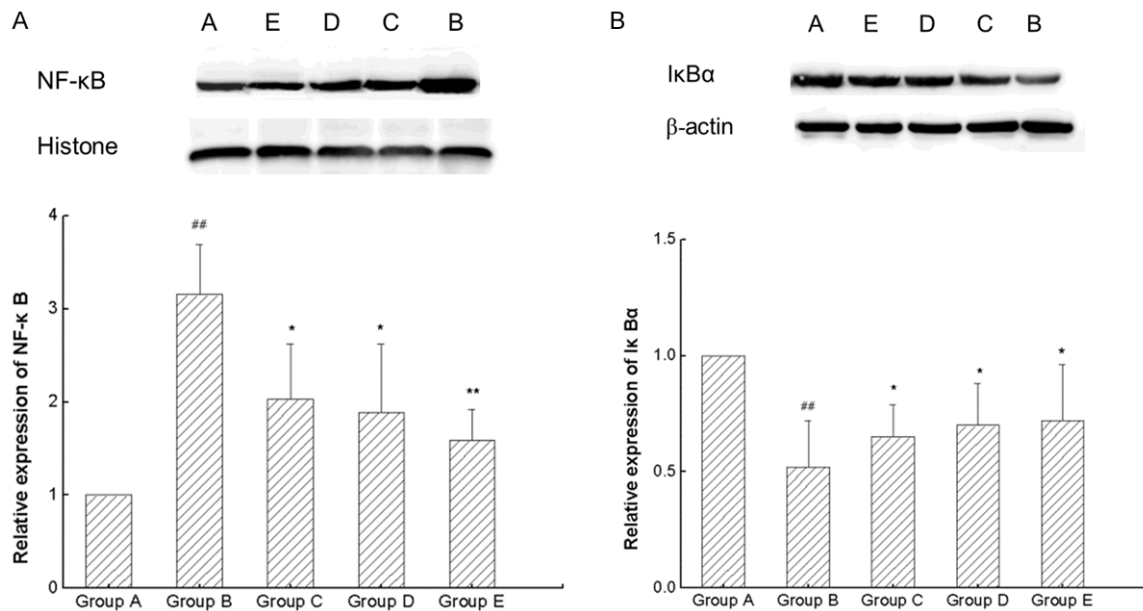

Figure 5. Effect of DHM on NF-κB signaling pathway in the kidney of diabetic rats. Protein expression of inhibitors of IκBα (A), and nuclear protein expression of NF-κB (B) in the kidney of rats. ##$P<0.01$ vs. group A, *$P<0.05$, **$P<0.01$ vs. group B.

cose in group B ($P<0.01$). Elevated NF-κB further induced decreased IκBα protein levels ($P<0.01$). DHM of 16 week administration significantly blocked the elevation of NF-κB ($P<0.05$) and reversed the change of IκBα protein levels ($P<0.05$).

Discussion

Dyslipidemia, hyperglycemia, insulin resistance and hypertension, which are the major driving factors of diabetes, exacerbated kidney dysfunction to proteinuria and a decline in the glo-

merular filtration rate leading to end-stage renal disease [20, 21]. Additionally, hyperglycemia induced states of low-grade inflammation and oxidation stress resulting in kidney damage and dysfunction. Therefore, therapeutic strategies targeting the management and prevention of diabetic nephropathy include agents for controlling hyperglycemia, glucose-derived oxidative stress and inflammatory response.

In this study, the effect of DHM on kidney of diabetic rats was evaluated. We found that the glucose level and blood lipid profile of diabetic rats showed significant increase. However, DHM inhibited this increase and DHM administration significantly improved the renal function. In addition, increased activities of antioxidant enzymes and levels of pro-inflammatory cytokines were normalized by DHM treatment. Furthermore, the nuclear accumulation of NF-κB was inhibited, while the nuclear accumulation of Nrf2 was promoted by DHM. The effect of DHM on these two signaling pathway might contribute to the antioxidative and anti-inflammatory effects of DHM in the kidney.

Hyperglycemia is the principle factor responsible for structural alterations at the renal level, and The Diabetes Control and Complications Trial Research Group has elucidated that hyperglycemia is directly linked to diabetic microvascular complications, particularly in the kidney; therefore, glycemic control remains the main target of therapy. In this study, the glucose level of diabetic rats showed a significant increase; however, DHM inhibited this increase dose-dependently. In addition, the typical pattern of serum constituents, that is, a decrease in total protein and albumin due to their excessive excretion via urine, and also an increase in lipids, e.g., total cholesterol and triglyceride, whose abnormal metabolism has been proven to play a role in the pathogenesis of diabetic nephropathy [22] and to enhance lipid peroxidation, were all improved by administration of the DHM. Therefore, we feel that DHM had a positive effect on serum glucose and lipid metabolic abnormalities.

A progressive decline in the glomerular filtration rate, reflecting Scr and BUN levels, is the most common characteristic in the development of diabetic nephropathy, which causes proteinuria, leading to histological damage in the kidney [23]. The results of the study presented here demonstrate that diabetic nephropathy rats showed significant increases in the serum urea nitrogen and Cr, representing a decline in renal function. However, the DHM treatment positively affected these parameters of renal function, indicating the renoprotective effect of DHM in diabetic nephropathy rats.

TNF-α, IL-1β and IL-6 are the major pro-inflammatory cytokines, involved in the inflammatory process, playing a significant role as regulators as well as final effectors, including DN [24]. These pro-inflammatory cytokines have been found to be up-regulated in the diabetic kidney [25, 26]. Oxidative stress has also been implicated in the pathogenesis of diabetic nephropathy [27]. Levels of oxidative stress markers are observed to have increased, while levels of antioxidative enzymes are found to be decreased in the kidney. In this study, both inflammation and oxidative stress were observed in the kidney, which indicated a vicious circle between inflammation and oxidative stress. However, DHM administration inhibited inflammatory response by decreasing the production of cytokines and diminished oxidative stress through enhancing the activities of antioxidative enzymes. These results suggest that the renoprotective effect of DHM may source from its anti-inflammatory and antioxidative effects.

The nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is one of the most critical endogenous defense systems which regulate antioxidant and anti-inflammatory cellular responses in the body. When oxidative stress or other covalent modification of thiols occur in some of these cysteine residues, Nrf2 dissociates from Keap1 and translocates to the nucleus and activated gene expression of downstream antioxidantenzymes, such as SOD and HO-1, enhancing cell response to oxidative stress resistance [28, 29]. In contrast with Nrf2 signaling pathway that contributes to the antioxidant responses, NF-κB signaling pathway is believed to be another important pro-inflammatory signaling pathway in human and experimental kidney disease. Activation of the transcription factor NF-κB by cytokines is rapid, mediated through the activation of the IKK complex with subsequent phosphorylation and degradation of the inhibitory IkB proteins [30]. The expression of NF-κB in kidney of diabetes animal model of kidney enhanced markedly,

and its activation promoted the glomerular mesangial cells proliferation [31]. In this study, we found a down-regulation of Nrf2 protein and a decreased HO-1 protein in the kidney of diabetes rats. Nuclear accumulation of NF-κB was increased, accompanied with down-regulating the expression of IκBα. As expected, after treatment with DHM for 16 weeks, the Nrf2 signaling pathway was more activated and the activity of NF-κB signaling was inhibited by DHM treatment. These results indicate that and Nrf2 pathway activation and the NF-κB signaling pathway inhibition may contribute to the renoprotective effect of DHM.

This study shows that DHM could significantly improve early-stage renal damage in diabetic rats. This renoprotection may be due to its anti-inflammatory and antioxidative effects. The underlying mechanisms may include its NF-κB signaling pathway inhibition and Nrf2 pathway activation effects. DHM therefore has potential to become a candidate drug in treating early-stage DN, although its detailed mechanism needs further elucidation.

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Disclosure of conflict of interest

None.

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