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Original Article

Improvement of ginkgolide B on diabetic nephropathy in streptozotocin (STZ)-induced rats

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Abstract: Aim: The goal of this study was to explore ginkgolide B as a therapy for amelioration of diabetic nephropathy. Methods: Healthy male Sprague-Dawley rats were randomly assigned to four groups: the control group, control treatment group, diabetes group, and the diabetes treatment group. Rats from the diabetes group and the diabetes treatment group were intraperitoneally injected with STZ (70 mg/kg) to induce diabetes. After 3 days, rats from the control and diabetes treatment groups received ginkgolide B, and animals of the control and diabetes groups received control diet. After administration of ginkgolide B for 8 weeks, the kidneys were collected for histological examination and analysis of Western blot. Blood samples were used to determine renal function. Results: Compared with the control group, levels of blood urea nitrogen (BUN), creatinine, and malondialdehyde (MDA) in the serum were significantly increased in the diabetes group, and activity of superoxide dismutase (SOD) was decreased. Expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 was increased in the diabetes group. Treatment with ginkgolide B significantly reduced the levels of blood urea nitrogen (BUN), creatinine, and MDA in serum, and 24 hour urea protein. Ginkgolide B significantly decreased expression of TNF-α, IL-6, transforming growth factor (TGF)-β, and cyclooxygenase (COX)-2 in the kidney from rats in the diabetes treatment group compared with the diabetes group. Levels of heme oxygenase (HO)-1 and Nrf-2 were increased in kidney in the diabetes treatment group compared with the diabetes group. Conclusion: The results suggest that ginkgolide B ameliorates diabetic nephropathy via improving antioxidant and anti-inflammatory functions, and easing kidney fibrosis.

Keywords: Ginkgolide B, diabetic nephropathy, fibrosis

Introduction

Diabetic nephropathy is one of the most common and complex vascular complications of diabetes, and regarded as a primary cause of chronic kidney lesions [1]. Less knowledge for the pathogenesis of diabetic complications including diabetic nephropathy results in therapeutic limitations. Various studies have suggested that multiple mechanisms are involved in the initiation and deterioration of diabetic nephropathy, such as metabolic disorders, hemodynamic failure, and genetic factors [2-4]. Increasing evidence shows that oxidative stress and inflammation contribute to vascular injures, and increase the risk of diabetic complications including diabetic nephropathy [5-7]. Sustained hyperglycemia leads to excessive production of reactive oxygen species (ROS) via several mechanisms such as glucose auto-oxidation, advanced glycosylation end products, xanthine oxidase activity, and activation of the polyol and hexosamine pathways [8, 9], while pro-inflammatory cytokines such as tumor necrosis factor-(TNF-) α, interleukin-(IL-) 6 are associated with hyperglycemia [10-12]. Further studies indicate that oxidative stress and inflammation are involved in the initiation and progression of diabetic nephropathy and remodeling of the extracellular matrix [12-14]. Therefore, antioxidative and anti-inflammatory agents may be beneficial to diabetic nephropathy.

Ginkgolide B is a natural terpene lactone with bioactivity from ginkgo biloba leaves. Previous studies indicated that ginkgolide B can bind to platelet-activating factor (PAF) receptor, and inhibit activation of platelet [15, 16]. Therefore,

ginkgolide B is considered as the PAF antagonist, and decreases release of PAF-induced inflammatory mediators [17, 18]. Further, ginkgolide B has been reported to possess various pharmacological properties including reduction of Toll-like receptors (TLR) 4-mediated inflammatory response [19], protection of neurons against apoptosis induced by ischemia [20], antioxidant function [21], and anti-tumor activity [22]. Our previous and other studies showed the ability of ginkgolide B to improve vascular disorders in diabetes and suppress atherosclerosis [23, 24].

In the present study, we hypothesized that ginkgolide B could ameliorated diabetic nephropathy. Further, we investigated factors that affected the progression of diabetic nephropathy including oxidative stress and inflammation, and the mechanisms.

Materials and methods

Materials

Streptozotocin (STZ) and sodium pentobarbital were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). A horseradish peroxidase-conjugated secondary anti-rabbit antibody, rabbit polyclonal antibodies, β-actin, heme oxygenase (HO)-1, tumor necrosis factor (TNF)-α, interleukin (IL)-6, transforming growth factor (TGF)-β, nuclear factor-erythroid 2 (NF-E2)-related factor (Nrf)-2, and cyclooxygenase (COX)-2 were purchased from Abcam (USA). TNF-α and IL-6 specific ELISA kits were purchased from Hefei Bomei Biotechnology CO., LTD, (Hefei, China). Malondialdehyde (MDA) and superoxide dismutase (SOD) commercially available kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals and induction of diabetes

Healthy male Sprague Dawley (SD) rats were obtained from the Animal Experimental Center in Wannan Medical College. Animals were fed in a standard animal laboratory with a controlled temperature of 22°C and a twelve-hour day/night alternate. After 2 weeks of acclimatization, rats were fasted for 12 hours for induction of diabetes. Fasting animals were administrated STZ (70 mg/ml, dissolved in pH 4.5 citrate buffer) via intraperitoneal injection, and control rats with the same volume buffer. Diabetes was diagnosed at 72 hours after injection of STZ by determining the fasting blood glucose concentration. Rats were considered as diabetic while their blood glucose concentrations were above 16.7 mmol/L.

Experimental protocol and treatment

After diabetes was confirmed, control and diabetic animals were randomly assigned to four groups: the control group, control treatment group, diabetes group, and the diabetes treatment group. Rats from the control and diabetes groups received a standard food and water *ad libitum,* and animals from the control treatment group and the diabetes treatment group received a standard food and water *ad libitum* and were treated with ginkgolide B (5 mg/kg body weight per day). At the end of the experiment, 24-hour urine was collected for determination of urea protein. After 8 weeks of treatment with ginkgolide B, rats were anesthetized with sodium pentobarbital (45 mg/Kg body weight), and sacrificed. Fasting blood samples were collected for biochemical analyses. Kidneys were harvested, weighed, and divided into two parts, one part was fixed in 4% paraformaldehyde for histopathological observation and the other was preserved under -80°C for analysis of Western blot.

Biochemical analysis

Blood urea nitrogen (BUN), creatinine, and 24-hour urea protein were determined with an automatic biochemistry analyzer.

Determination of inflammatory cytokines

Serums were separated from fasting blood samples by centrifugation at 2300 g. Inflammatory cytokines including TNF-α and IL-6 in serum were determined by specific ELISA kits according to manufacturer's specifications. Concentrations of TNF-α and IL-6 are presented as pg/ml.

Analysis of antioxidant effects

MDA content and SOD activity in serum were measured with commercially available kits according to manufacturer's specifications. MDA content and SOD activity are expressed as nmol/ml and U/ml, respectively.

Table 1. Effects of ginkgolide B on body weight (BW), kidney weight (KW), BW/KW ratio (mean ± SD)

	Initial BW (g)	Final BW (g)	KW (g)	BW/KW (× 10^{-3})
Control	261.1±11.73	432.1±31.22	2.86±0.28	6.62±0.38
Control Treatment	259.5±11.02	424.3±32.65	2.82±0.23	6.64±0.39
Diabetes	264.9±10.07**	243.3±22.02**	2.50±0.30**	10.27±0.71**
Diabetes Treatment	263.4±9.42##	322.8±39.40##	2.56±0.27##	7.96±0.27##

**P<0.01 compared with the control group; ##P<0.01 compared with the diabetes group.

Table 2. Effects of ginkgolide B on BUN, creatinine, and 24-hour urea protein (mean ± SD)

	BUN (mmol/L)	Creatinine (mmol/L)	24-h urea protein (mg)
Control	4.90±0.61	48.91±7.48	3.97±0.95
Control Treatment	4.71±0.65	47.74±8.92	4.12±0.91
Diabetes	16.44±2.27**	94.20±9.43**	19.92±4.77**
Diabetes Treatment	10.85±1.27##	69.18±9.35**	11.55±2.36**

**P<0.01 compared with the control group; ##P<0.01 compared with the diabetes group.

Examination of histology

Fixed kidneys in 4% paraformaldehyde were imbedded in paraffin, and then cut 5-μm-thickness sections for Hematoxylin-Eosin (H-E) staining. Morphological changes were observed under a light microscope.

Western blotting

Kidneys were homogenized and lysed in pre-cooled lysis buffer (50 mmol/L HEPES, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 1% Triton-X 100, 100 mmol/L sodium orthovanadate) with 2 mmol/LPMSF, 2 μg/L leupeptin and aprotinin. Protein in supernatant was quantified with a BCA kit (Bio-Rad). Equal amounts of protein were separated in 10% SDS-PAGE gel and then transferred to nitrocellulose membrane. Membranes were blocked with 5% nonfat milk dissolved in Tris-HCl buffer saline with 0.5% Tween 20 (TBS-T), then incubated with primary antibodies including HO-1, TNF-α, IL-6, TGF-β, Nrf-2, and COX-2 (1:500) dissolved in TBS-T containing 5% nonfat milk overnight at 4°C. After washing, the membranes were incubated with a secondary antirabbit antibody (1:10,000) dissolved in TBS-T for 2 hours. Immunoblot of protein was visualized by DAB.

Statistical analysis

All data are presented as mean ± SD. Comparisons between groups were analyzed with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), or unpaired Student t test by using SPSS 18.0. A value of P<0.05 was considered to be statistically significant.

Results

General characteristics

After injection with STZ, animals gradually presented many diabetic characteristics such as polyuria, increase in drinking water, and body weight (BW) loss. On day 3, rats injected with STZ showed hyperglycemia. As shown in **Table 1**, rats from diabetes group significantly lost BW in the final compared with the initial, and BW was significantly lower in diabetes group than in control group at the end of experiment (P<0.01). Change of kidney weight (KW) was not significant in diabetes group compared with control group, but KW/BW ratio was increased (P<0.01). Ginkgolide B treatment significantly reduced loss of BW, and prevented the increase of KW/BW ratio in diabetes treatment group compared with diabetes group.

Biochemical changes

BUN, creatinine, and urea protein levels were significantly increased in the diabetes group compared with the control group (P<0.01) (**Table 2**). Administration of ginkgolide B to rats from the diabetes treatment group significantly reduced levels of BUN, creatinine, and urea protein to near normal levels (P<0.01) (**Table 2**).

Morphological changes

Diabetic nephropathy is characterized by kidney hypertrophy, glomerulosclerosis, tubular edema, and thickened basement membrane. Sections stained with HE were observed under light microscopy, the results showed that rats

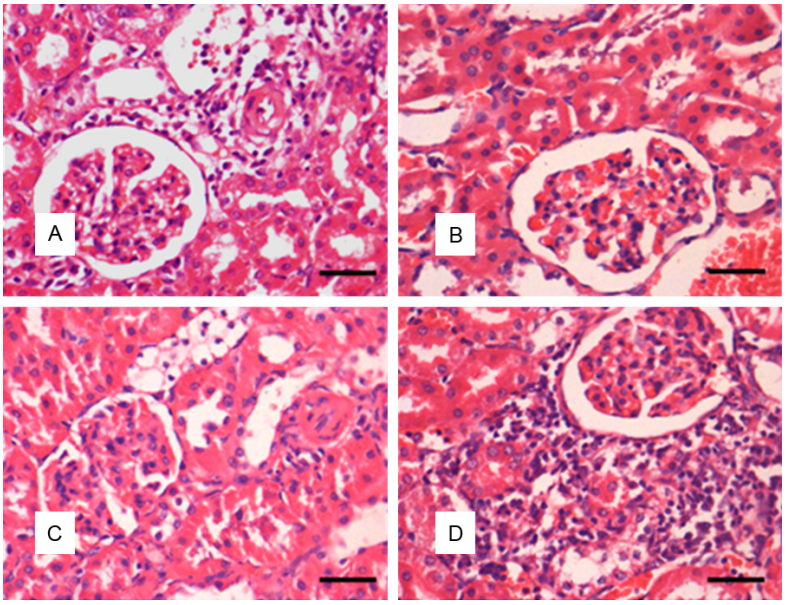

Figure 1. Histological changes of the kidney of each group. A. Control group. B. Control rat treatment. C. Diabetes group. D. Diabetes treatment. C. Kidney from the diabetic rats showed markedly severe lesion characterized by glomerular injury, interstitial expansion, and interstitial cellular infiltration. D. Morphology of glomerulus and tubulointerstitial impairments was ameliorated by treatment with ginkgolide B.

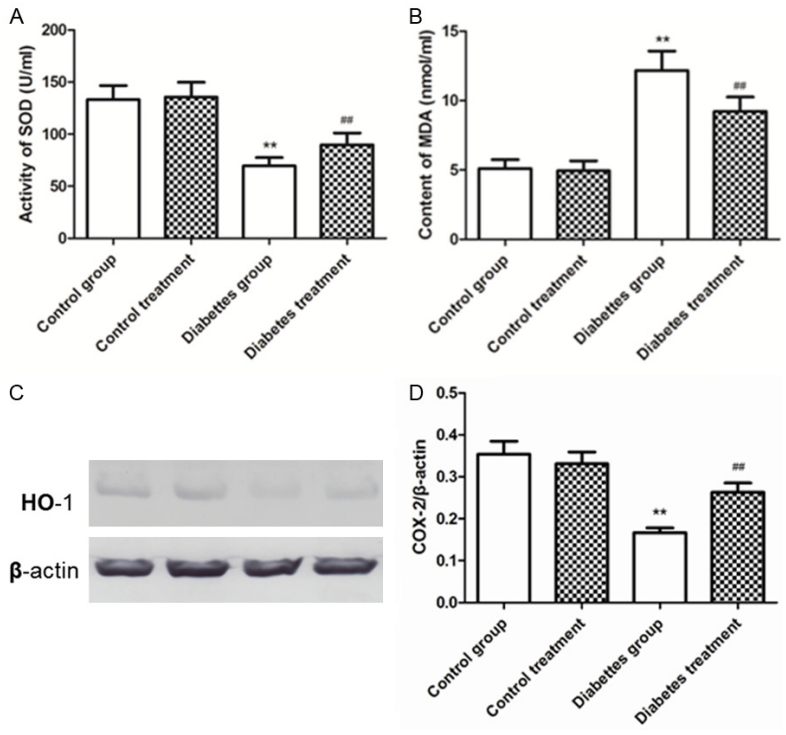

Figure 2. Changes of anti-oxidative effects. A. SOD activity in serum was significantly elevated by treatment with ginkgolide B in diabetic rats (**P<0.01 versus control group; ##P<0.01 versus diabetes group). B. Treatment with ginkgolide B reduced MDA content in serum (**P<0.01 versus control group; ##P<0.01 versus diabetes group). C. Expression of HO-1 was detected. D. Relative level of HO-1 was enhanced in diabetic rats treated ginkgolide B (**P<0.01 versus control group; ##P<0.01 versus diabetes group).

from the diabetes group presented marked increase in the glomerular volume compared with control group. Ginkgolide B treatment markedly improved the pathological alterations of the kidney such as decrease of the glomerular volume, and attenuation of renal swelling in diabetes treatment group compared to the diabetes group (**Figure 1**).

Effects of ginkgolide B on inflammation

It is well known that ginkgolide B is a natural antagonist of PAF, and reduces release of inflammatory cytokines [15, 16]. Our results showed that levels of inflammatory cytokines such as TNF-α and IL-6 in serum significantly were increased in diabetes group compared with the control group (P< 0.01) (**Figure 3**). Treatment with ginkgolide B significantly decreased serum levels of TNF-α and IL-6 in the diabetes treatment group compared to the diabetes group. Further, levels of TNF-α and IL-6 in renal tissues were significantly decreased in the diabetes treatment group (P<0.01) (**Figure 3**).

Change of antioxidant effects

SOD activity was significantly reduced, and MDA content increased in serum from the diabetes group compared with the control group. Treatment with ginkgolide B significantly enhanced SOD activity, and decreased content in serum in the diabetes treatment group compared to the diabetes group (P<0.01) (**Figure 2**). Ginkgolide B significantly increased expres-

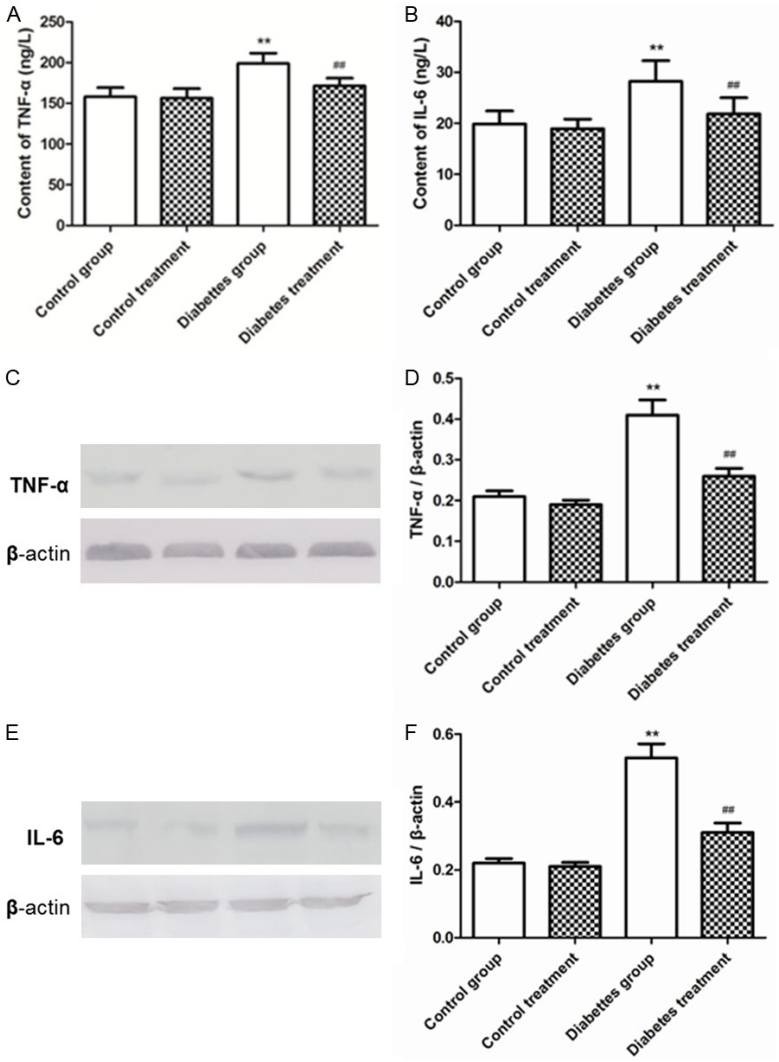

Figure 3. Changes of inflammatory response. A. TNF-α level in serum was significantly decreased in diabetic rats treated with ginkgolide B in diabetic rats (**$^{**}P$<0.01 versus control group; $^{\#\#}P$<0.01 versus diabetes group). B. Treatment with ginkgolide B decreased IL-6 level in serum ($^{**}P$<0.01 versus control group; $^{\#\#}P$<0.01 versus diabetes group). C. Expression of TNF-α was detected. D. Relative level of TNF-α was decreased in diabetic rats treated ginkgolide B. E. Expression of IL-6 was detected. F. Relative level of IL-6 was decreased in diabetic rats treated ginkgolide B. ($^{**}P$<0.01 versus control group; $^{\#\#}P$<0.01 versus diabetes group).

sion of HO-1 protein in kidney from the diabetes treatment group while compared to the diabetes group (P<0.01) (**Figure 2**).

Effects of ginkgolide B on expression of TGF-β, COX-2, and Nrf-2

Rats from diabetes group showed an increase in expression of TGF-β and COX-2, and a decrease in expression of Nrf-2 compared with the control group (P<0.01) (**Figure 4**). Treatment with ginkgolide B reduced expression TGF-β and COX-2, and increased in expression of Nrf-2 in kidney (P<0.01) (**Figure 4**).

Discussion

The results from the present study show that treatment with ginkgolide B significantly decreased levels of BUN and creatinine, and volume of 24-hour urea protein in STZ-induced diabetic rats. Further, ginkgolide B protected against development of diabetic nephropathy by reducing inflammatory response, and decreasing oxidation. Ginkgolide B treatment increased expression of Nrf-2 and HO-1 protein, and reduced expression of COX-2 and TGF-β protein.

Diabetic nephropathy is one of the most common microvascular complications of diabetes mellitus, and regarded as an important contributor for the development of cardiovascular disease [25, 26]. Pathogenesis of diabetic nephropathy is poorly elucidated. Therefore, the effective therapies of diabetic nephropathy are few, even though when blood glucose was strictly controlled, diabetic nephropathy still developed [27]. In this study, we explored the effect of ginkgolide B on diabetic nephropathy and its possible mechanisms such as antioxidant and anti-inflammatory activities. Our results show that administration of ginkgolide B to diabetic rats reduced levels of creatinine and BUN, and volume of 24-urea protein. Creatinine and BUN are considered as markers of kidney lesions, and are increased in rats with diabetic nephropathy [28, 29]. Further, ginkgolide B attenuated edema of tubular and glomerular lesion. These results suggest that ginkgolide B ameliorated diabetic nephropathy.

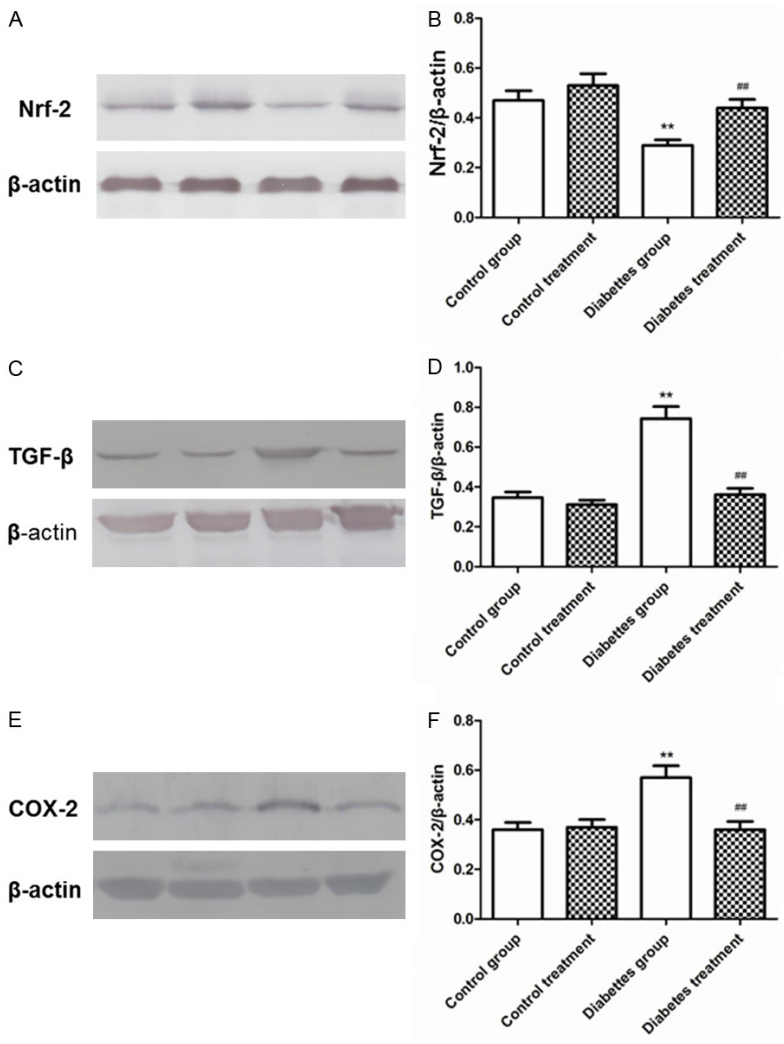

Figure 4. Effects of ginkgolide B on expression of Nrf-2 and TGF-β. A. Expression of Nrf-2 was detected. B. Relative level of Nrf-2 was increased in diabetic rats treated ginkgolide B. C. Expression of TGF-β was detected. D. Relative level of TGF-β was decreased in diabetic rats treated ginkgolide B. E. Expression of COX-2 was detected. F. Relative level of COX-2 was decreased in diabetic rats treated ginkgolide B. (**P<0.01 versus control group; ##P<0.01 versus diabetes group).

Ginkgolide B, a natural active terpene lactone from *ginkgo biloba*, is known as PAFR antagonist [30]. Increasing studies have shown that ginkgolide B exerts various pharmacological functions. Ginkgolide B has been reported to be able to enhance anti-oxidative activity by scavenging free radicals [31-33], and suppress inflammatory response by antagonizing PAFR and regulating TLR4 [17, 19]. In the present study, ginkgolide B reduced MDA content and levels of TNF-α and IL-6, and increased activity of SOD in serum. In addition, ginkgolide B increased expression of HO-1 and Nrf-1 protein, and decreased expression of TNF-α, IL-6, and COX-2 in kidney. These data supported anti-oxidant and anti-inflammatory activities of ginkgolide B. It is well known that oxidative stress and inflammation are closely associated with diabetic complications including diabetic nephropathy [5, 7, 34]. Many studies have confirmed that sustained hyperglycemia produces excessive reactive oxygen species (ROS) production via multiple mechanisms such as auto-oxidation of glucose and the polyol and hexosamine pathways [8, 35]. ROS causes vascular injury and results in diabetic complications including diabetic nephropathy [36]. A previous study also demonstrated that ROS stimulates inflammatory response [37]. Nrf2 is an important y transcription factor which mediates redox balance, and is regarded as a sensor of oxidative stress. Nrf2 has been reported to upregulate expression of HO-1 and glutathione S-transferase to mediate oxidative stress [38-40]. HO-1 and the by-products of heme catabolism by HO-1 such as heme into bilirubin and carbon monoxide (CO) have been reported to show anti-inflammatory, and anti-oxidant effects [41], and HO/CO pathway is implicated in regulation of anti-inflammatory and cytoprotection by reducing COX-2 signaling [42, 43]. Our study showed that ginkgolide B upregulates expression of Nrf-2 in kidney from diabetic rats. Therefore, ginkgolide B protected against diabetic renal lesions via enhancing anti-oxidant and anti-inflammatory function.

Increasing evidence confirmed that ROS and inflammatory cytokines elevate TGF-β level in the diabetic kidney cortex [44-46]. TGF-β has been reported to be implicated in fibrosis of

diabetic kidney, therefore plays a vital role in development of diabetic nephropathy [47, 48]. The study showed that TGF-β leads to accumulation of extracellular matrix (ECM) via increasing synthesis of ECM and reducing degradation of ECM [49]. Activation of TGF-β1 decreased expression of type I and III collagen, and PAI-1, which suggested that TGF-β1 is critical in the progression of renal fibrosis [50]. This study indicated that treatment with ginkgolide B reduced expression. This suggested that ginkgolide B attenuated diabetic nephropathy by inhibition of TGF-β signaling.

In summary, the present study shows that ginkgolide B ameliorates diabetic nephropathy through anti-inflammatory and anti-oxidant functions. Further, ginkgolide B reduced expression of TGF-β, a profibrogenic cytokine, which attenuated kidney fibrosis. Therefore, it is significant to further investigate the mechanism of the protective effects of ginkgolide B on diabetic nephropathy.

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Disclosure of conflict of interest

None.

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