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Effects of *Ginkgo biloba* on prevention of development of experimental diabetic nephropathy in rats[1]

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Key words

Ginkgo biloba extract; diabetic nephropathy; transforming growth factor-β1; matrix metalloproteinases-2; connective tissue growth factor

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Abstract

Aim: To observe the preventive and therapeutic effects of *Ginkgo biloba* extract (*Gb*E) on early experimental diabetic nephropathy (DN) in rats. **Methods:** After an early DN model was induced by streptozotocin, rats were administered *Gb*E at 3 doses for 12 weeks. Fasting blood glucose, creatinine (Cr), blood urea nitrogen (BUN), urine protein, kidney index, anti-oxidase, advanced glycosylation end products (AGE), collagen IV and laminin, matrix metalloproteinases-2 (MMP-2) and the tissue inhibitor of metalloproteinase-2 (TIMP-2), connective tissue growth factor (CTGF), and transforming growth factor-β1 (TGF-β1) mRNA were measured by different methods. The ultrastructural morphology and the thickness of glomerular base membrane (GBM) were observed by a transmission electron microscope. **Results:** For the *Gb*E-treated DN rats, when compared with the vehicle-treated DN rats, the fasting blood glucose level, Cr, BUN, urine protein level, and the intensity of oxidative stress were significantly decreased. The expression of MMP-2 greatly increased, and TIMP-2 decreased. Also, AGE, either in serum or in renal, the collagen IV, laminin, CTGF levels, and TGF-β_1 mRNA were reduced. Furthermore, both relative grades of mesangium hyperplasia by microscopical observation and the thickness of GBM by electron microscope measurement decreased significantly. **Conclusion:** *Gb*E has protective effects on several pharmacological targets in the progress of DN and is a potential drug for the prevention of early DN.

Introduction

In many countries diabetic nephropathy (DN) is a major complication of diabetes. At present, it affects about 15%–25% of type 1 and 30%–40% of type 2 diabetic patients, causing disabilities and a high mortality rate. It is characterized by the thickening of the basement membranes, mesangial expansion and proliferation, and excessive accumulation of extracellular matrix (ECM), and ultimately leads to nodular glomerulosclerosis and chronic renal failure.

However, the mechanisms underlying the pathogenesis of DN are not completely understood. Many scholars consider the progression of DN a result of the interaction of multiple factors, such as high glucose, the polyol pathway, oxidative stress, protein non-enzymatic glycation, and cytokines[1]. Studies have found that high glucose is presumed to be an initiating agent which increases the formation of advanced glycosylation end products (AGE) and induces oxidative stress. It also increases transforming growth factor-β1 (TGF-β1) expressions. TGF-β1 is thought to be the key cytokine involved in the progression of DN[2]. It increases the synthesis of ECM components, including collagens, fibronectin, and laminin, and results in hypertrophy of the mesangial cell and glomerulosclerosis. Connective tissue growth factor (CTGF) has been described as a growth factor that acts downstream of TGF-β1 and is a potent inducer of ECM in the fibrotic process[3,4]. Matrix metalloproteinases (MMP) are the major physiological regulator of ECM degradation in the glomerulus. A balance between ECM synthesis and degradation is a prerequisite

for maintaining the structural and functional integrity of the glomerulus. MMP and tissue inhibitors of metalloproteinase (TIMP) keep this balance together[5]. For the complexity of mechanisms, there are no definitive drugs to delay the development of DN. Therefore, it is necessary to develop new drugs for DN that can deal with more than 1 pharmacological target in this intricate mechanism.

The tree *Ginkgo biloba* has long been believed to have medicinal properties, and its extracts are among the most widely-sold herbal supplements in the world. *Ginkgo biloba* extract (*Gb*E), extracted from *Ginkgo biloba* leaves, is a defined, complex mixture containing 24% *Ginkgo* flavone glycoside (quercetin, kaempferol, and isorhamne) and 6% terpene lactones (ginkgolides and bilobalide). It has been used as a therapeutic agent in some cardiovascular and neurological disorders[6,7]. Although the exact mechanism is unknown, evidence accumulated *in vitro* and *in vivo* shows that *Gb*E has a number of benefits, including ameliorating hemodynamics, suppressing the platelet-activating factor, scavenging reactive oxygen species (ROS), and relaxing vascular smooth muscles[8]. All of these offer us a pharmacological foundation of *Gb*E for DN therapy. However, there are still few published reports that focus on the protective mechanisms of *Gb*E on DN. Therefore, it is worthwhile for us to explore its potential effects in preventing the progression of DN.

To evaluate the effects of *Gb*E on DN, in our present work we used captopril (CAP) as an antifibrotic control drug[9,10], and studied the possible influences of *Gb*E on the parameters that indicate protective effects against the progress of DN, such as blood glucose, AGE, TGF-β1, MMP-2, TIMP-2, CTGF, collagen IV and laminin in the kidney cortex, anti-oxidases in the serum, and the thickness of the glomerular base membrane (GBM) on early experimental DN rats, and observed the morphological changes on DN rats.

Materials and methods

Drugs *Gb*E (Lot No 040029) was provided by the Pizhou Fuwei Biochemical Company (Xuzhou, China), and was dissolved in 1% carboxymethyl cellulose (CMC) solution. Streptozotocin (STZ, Lot No P7993b) was purchased from Biomol Research Lab (Plymouth Meeting, PA, USA). Captopril (Lot No 050050), serving as a positive control drug, was kindly provided by Changzhou Pharmaceutical Factory (Changzhou, China) and was suspended in 1% CMC solution.

Animals Male Sprague-Dawley rats (Certificate No SYXK 2001-0050), weighing 165.7±10.2 g (150–190 g) were obtained from the Laboratory Animal Center of Xuzhou Medical College (Xuzhou, China), following the Guiding Principles for Care and Use of Laboratory Animals of Xuzhou Medical College.

Induction of DN model and study protocol Diabetes mellitus was induced in the male Sprague–Dawley rats, by ip injection of 60 mg/kg of the beta-cell toxin STZ (dissolved in pH 4.5 citrate buffer immediately before the injection), while controlled normal standard rats (NS group, n=13) received 6 mL/kg citrate buffer. The induction of the diabetic state was confirmed by the blood glucose level measurement on the third day after STZ administration. The rats with fasting blood glucose concentrations=13.88 mmol/L were randomly allotted into 5 groups: DN rats were treated with 1% CMC solution (DN group, n=13); DN rats with 50, 100, and 200 mg/kg *Gb*E for the GL group (low dose, n=12), the GM group (moderate dose, n=14), and the GH group (high dose, n=14), respectively; the DN rats were treated with 10 mg/kg of captopril (CAP group, n=11). The same volume of CMC solution was administered to the NS group (n=13). The animals housed in the barrier environment refer to breed specific pathogen-free grade animals, and they were allowed food and water *ad libitum*. After 12 weeks, the urine and blood samples were collected. After the animals were sacrificed, fresh kidney cortices were stored in formaldehyde solution for immunohistochemical measurements, and 1 mm×1 mm×1 mm cubes of kidney cortices were fixed in 2.5% glutaraldehyde for electron microscopic measurement. The rest of the kidneys were stored at –80 ºC for the later analysis.

Measurement of renal function and biochemical parameters Blood glucose was measured by the glucose oxidase method with kits purchased from Dong-Ou Bioengineering (No 2005110008, Wenzhou, China). The values of urine protein, creatinine (Cr), and blood urea nitrogen (BUN) were determined by the automatic biochemistry analyzer (Olympus-2000, Tokyo, Japan). The kidney index was 1000×kidney weight/body weight.

Collagen IV and laminin, the main component of ECM, were measured by radioimmunoassay kits from Shanghai High Biotech Center (Lot No 20060601, Shanghai, China).

Total antioxidative capability (T-AOC), catalase (CAT), total superoxidase dismutase (T-SOD) and glutathione-peroxidase (GSH-Px) activities in the serum were measured by spectrophotometry, using kits from Jiancheng Bioengineering Institute (Lot No 20050522, Nanjing, China).

AGE plays a critical role in diabetic nephropathy by stimulating ECM synthesis. AGE either in the renal cortex or in the serum was measured by fluorescence spectrophotometry

(fluorospectrophotometer F-4500, Hitachi, Tokyo, Japan), and the concentration of AGE was represented by the fluorescence optical density. The final value of AGE in the tissue was modulated by the total protein in the tissue which was measured by the Lowry method[11].

Immunohistochemical measurements of MMP-2, TIMP-2, and CTGF The glass slides were sealed with 10% polylysine. The 4 μm renal tissue sections were used to perform immunohistochemical staining for MMP-2, TIMP-2, and CTGF. The renal tissue sections were incubated with rabbit polyclonal anti-MMP-2 (Lot No 200604, Boster Biological Technology Company, Wuhan, China) at a dilution of 1:50 at 37 ºC for 2 h. After washing, goat anti-rabbit IgG-horseradish peroxidase (Lot No 015090, Zhongshan Golden Bridge Biotechnology Company, Beijing, China) was added. To visualize MMP-2, the renal tissue sections were stained with 3,3'-diaminobenzidine (DAB) for 10 min and then examined by light microscopy (×400). All steps were performed at room temperature. The TIMP-2 and CTGF measurements were identical to MMP-2. The stained MMP-2, TIMP-2, and CTGF were quantified by gray scale analysis (Leica Qwin Standard V2.6, Leica Microsystems, Welzlar, Germany).

RT-PCR for the relative quantities of TGF-β1 mRNA in the kidney cortex[12] A RT-PCR procedure was performed to determine the relative quantities of TGF-β1 mRNA in the kidney cortex, while β-actin mRNA, a housekeeping gene, was used as an internal control. The total RNA was extracted from the kidney cortex with the Promega Total RNA Isolation System (Lot No 182207, Promega, Madison, USA). The upstream and downstream primers for rat TGF-β1 mRNA were: 5'-CCCGCATCCCAGGACCTCTCT-3' and 5'-CGGGGGACTGGCGAGCCTTAG-3', yielding a 519 bp product; whereas those for β-actin were: 5'-GCTGCGTGTGGCCCCTGAG-3'and5'-ACGCAGGATGGCATGAGGGA-3', yielding a 25 -bp product. Equal amounts (3 μL) of each total RNA sample were added in a 50 μL reaction mixture exerting one-step amplification with the Promega RT-PCR System (Lot No 199676, Promega, USA). The reaction mixture was incubated at 48 ºC for 45 min to reverse transcript, then went into cycles. The cycle conditions were set to initial denaturation for 5 min at 94 ºC, 40 cycles at 94 ºC for 1 min, 57 ºC for 50 s, 72 ºC for 1 min, with a final elongation at 72 ºC for 7 min. The RT-PCR products were separated by 1% agarose electrophoresis, and the band densities were analyzed using laser densitometry. The relative quantities of TGF-β1 mRNA in the kidney cortex were represented by the ratio of band density of TGF-β1 versus that of β-actin.

Morphological observation and measurement of the thickness of GBM The kidney cortex samples stored in formaldehyde solution were embedded with paraffin and stained with periodic acid-Schiff (PAS). Every PAS-stained sample in each group was observed under light microscope. Three kidney samples from each experimental group were randomly chosen for electron microscopic observation. The specimens were embedded in epoxy resin and cut into ultra-thin sections and then stained with plumbum citrate for ultrastructural observation under a transmission electron microscope (H600A-2, Hitachi, Japan). Five photos were taken at different views for each kidney sample. The images were amplified 6 K and the photos were scanned into a computer so that the thickness of GBM was measured by an image analysis system (Leica Qwin Standard V2.6, Leica Microsystems, Germany).

Statistical analysis Statistical analysis was performed to compare the effects of *Gb*E on early DN rats using ANOVA and Dunnett's *t*-test (2-side) for different groups using SPSS 10.0 (Chicago, USA). Data were expressed as mean±SD. Differences were considered to be significant at $P<0.05$.

Results

Effects of *Gb*E on physical behaviors, blood glucose, urinary protein, Cr, BUN, and the kidney index In our experiment, the rats in the DN group had hypopraxia, cachexia, polyuria/polydipsia, yellowish and damp fur, kyphosis, and tardy weight gain; the rats in the NS and GH groups were vibrant and vigorous, had white and tidy fur, and weight gain.

Table 1 shows that the fasting blood glucose level, urine protein, Cr, BUN, and the kidney index of the DN group were significantly higher than those of the NS group ($P<0.01$), suggesting that our early DN model was successful. Low doses of *Gb*E markedly reduced blood glucose and Cr in the DN rats ($P<0.05$), but was of no significant difference in reducing BUN, urine protein, and the kidney index. Moderate and high doses of *Gb*E and captopril significantly reduced blood glucose, urine protein, Cr, BUN, and kidney index levels in the DN rats ($P<0.01$).

Effects of *Gb*E on oxidative stress The activities of the CAT, GSH-Px, T-AOC, and T-SOD of the DN group were all lower than those of the NS group ($P<0.01$), suggesting that the DN group exhibited oxidative stress. We also found that low, moderate, and high doses of *Gb*E increased these 4 anti-oxidase activities ($P<0.05$ or $P<0.01$). These results indicated that *Gb*E could ameliorate the oxidative stress state of DN rats. Captopril also significantly increased CAT and GSH-Px activities ($P<0.05$), but it had no evident effect on T-AOC and T-SOD ($P>0.05$; Figure 1).

Table 1. Effects of *Gb*E on blood glucose, urine protein, and kidney index of rats. Mean±SD. $^{c}P<0.01$ *vs* NS group; $^{e}P<0.05$, $^{f}P<0.01$ *vs* DN group. NS: normal saline. DN: diabetes. GL, GM, GH: *Gb*E 50, 100, 200 mg/kg. CAP: captopril 10 mg/kg.

Group	*n*	Fasting blood glucose/mg	24 h urinary protein/mmol·L^{-1}	Kidney index (×1000)	BUN /mmol·L^{-1}	Cr /μmol·L^{-1}
NS	13	5.28±1.11	15.06±6.78	6.47±0.33	7.25±1.57	40.17±4.15
DN	13	16.83±4.01^{c}	103.58±18.12^{c}	13.18±2.35^{c}	23.08±4.04^{c}	65.38±11.38^{c}
GL	12	13.44±3.86^{e}	88.86±17.81	11.99±2.64	20.09±6.49	56.08±11.73
GM	14	11.56±3.18^{f}	67.07±19.21^{f}	9.95±2.15^{f}	16.44±4.85^{f}	49.79±8.68^{f}
GH	14	10.24±4.15^{f}	55.60±19.32^{f}	9.33±1.67^{f}	15.40±4.37^{f}	45.50±12.58^{f}
CAP	11	9.96±3.15^{f}	56.37±20.24^{f}	10.70±3.37^{e}	17.02±7.16^{e}	47.73±7.90^{f}

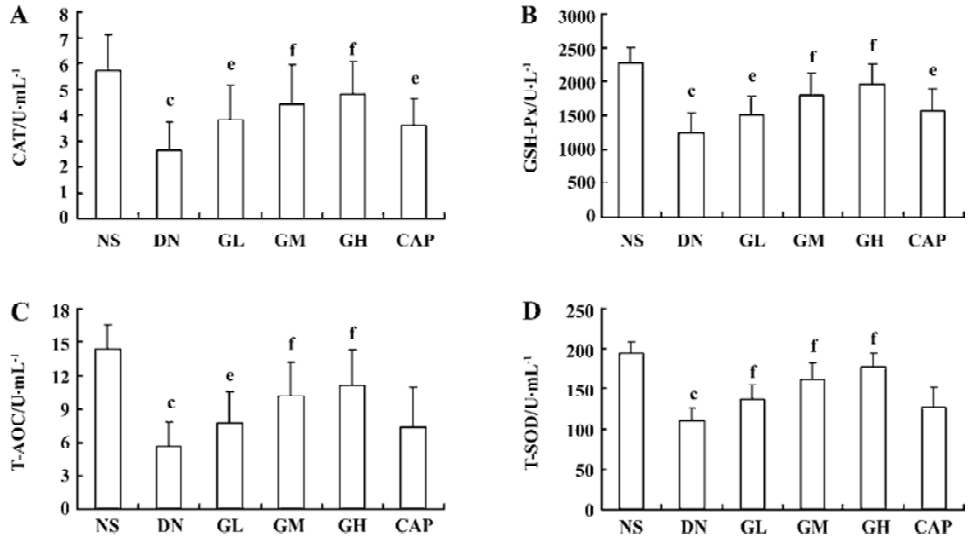

Figure 1. Effects of *Gb*E on the activities of the CAT, GSH-Px, T-AOC, and T-SOD in serum of rats. NS group received 6 mL/kg citrate buffer (n=13). DN group were treated with 1% CMC solution (n=13). GL group: DN rats treated with *Gb*E 50 mg/kg (n=12); GM group: with *Gb*E 100 mg/kg (n=14); GL group: with *Gb*E 200 mg/kg (n=14); CAP group: the DN rats were treated with 10 mg/kg of captopril (n=11). Mean±SD. $^{c}P<0.01$ *vs* NS. $^{e}P<0.05$, $^{f}P<0.01$ *vs* DN.

Effects of *Gb*E on collagen IV and laminin level in the kidney cortex The levels of collagen IV and laminin in the kidney cortex of early DN rats significantly increased when compared with those of the normal rats ($P<0.01$). The collagen IV levels of the GM, GH, and CAP groups were strikingly lower than those of the DN group ($P<0.05$ or $P<0.01$). The laminin levels of the GL, GM, GH, and all the CAP groups decreased ($P<0.01$). These results suggested that *Gb*E could decrease the expressions of collagen IV and laminin of DN rats, and that captopril's capability of decreasing expressions of collagen IV was between that of *Gb*E's moderate and low dose (Figure 2).

Effects of *Gb*E on AGE and the thickness of GBM The AGE levels in the kidney cortex and in the serum of the DN rats were greatly higher than those of normal rats ($P<0.01$). In the kidney cortex and in the serum, there was significant decrease in the AGE levels of the GL, GM, and GH groups when compared with those of the DN group ($P<0.05$ or $P<0.01$), whereas captopril significantly decreased the AGE levels ($P<0.01$).

There was a significant difference in the thickness of the GBM between the NS group and DN group ($P<0.01$). The thickness of GBM decreased as the doses of *Gb*E increased. There was significant decrease in the thickness of the GBM of the GL, GM, GH, and CAP groups, compared with that of the DN group ($P<0.05$ or $P<0.01$; Table 2).

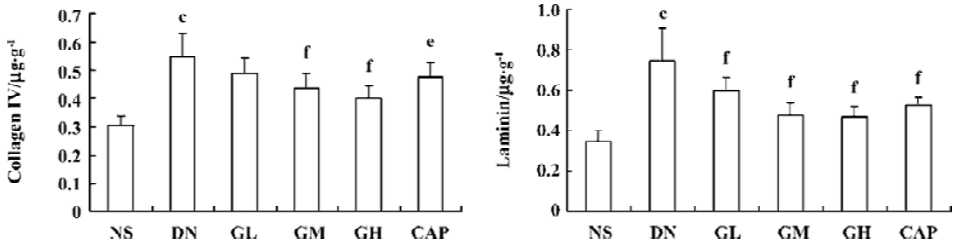

Figure 2. Effects of *Gb*E on the collagen IV and laminin in kidney cortex. NS group received 6 mL/kg citrate buffer (n=13). DN group were treated with 1% CMC solution (n=13). GL group: DN rats treated with *Gb*E 50 mg/kg (n=12); GM group: with *Gb*E 100 mg/kg (n=14); GL group: with *Gb*E 200 mg/kg (n=14); CAP group: the DN rats were treated with 10 mg/kg of captopril (n=11). Mean±SD. ^{c}P<0.01 *vs* NS. ^{e}P<0.05, ^{f}P<0.01 *vs* DN.

Table 2. Effects of *Gb*E on AGE and GBM of rats. Mean±SD. ^{c}P<0.01 *vs* NS group; ^{e}P<0.05, ^{f}P<0.01 *vs* DN group.

Group	n	AGE in kidney cortex /AUF·mg^{-1} cortex	AGE in serum /AUF·mL^{-1} plasma	Thickness of GBM /nm
NS	13	4.47±0.97	2.81±0.93	125.2±15.8
DN	13	9.78±1.87^{c}	7.64±1.54^{c}	267.5±38.4^{c}
GL	12	8.10±1.24^{e}	6.12±1.21^{e}	212.2±27.5^{e}
GM	14	6.77±1.07^{f}	5.05±1.15^{f}	171.7±18.8^{f}
GH	14	6.06±1.19^{f}	4.52±1.18^{f}	158.4±14.9^{f}
CAP	11	6.23±1.15^{f}	5.57±0.94^{f}	162.3±12.2^{f}

Immunocytochemical analysis of MMP-2, TIMP-2, and CTGF MMP-2 and TIMP-2 are mainly expressed in the cytoplasm of mesangial cells and renal tubular epithelial cells. The color of the stained MMP-2 and TIMP-2 protein was brown. The staining intensity of TIMP-2 of the DN group highly increased. On the contrary, MMP-2 markedly decreased when compared with those of the NS group. We used gray scale analysis to quantify MMP-2 and TIMP-2 proteins and found that the levels of MMP-2/TIMP-2 of the DN group was significantly different from that of the NS group (P<0.01). With the increased concentration of *Gb*E, the expressions of MMP-2/TIMP-2 in the GM group and the GH group significantly increased/decreased (P<0.05 or P<0.01), respectively. The expressions in the CAP group had the same changes (P<0.01), and the levels of MMP-2 and TIMP-2 in the CAP group was between those of the GH group and GM group (Figures 3,4). Like TIMP-2, the levels of CTGF in the GL, GM, GH, CAP groups significantly decreased (P<0.01 or P<0.05; Figure 5). All of these results suggested that *Gb*E had a potent influence on the expressions of MMP-2, TIMP-2, and CTGF (Figure 6).

Effect of *Gb*E on the relative quantity of TGF-β1 mRNA in the kidney cortex The RT-PCR products of TGF-β1 were separated by 1% agarose electrophoresis, after which we could see distinct bands (Figure 7A). The relative quantity of TGF-β1 mRNA in the kidney cortex of the DN group was greatly higher than that of the NS group (P<0.01). The TGF-β1 mRNA level of the GM, GH, and CAP groups strikingly decreased when compared with that of the DN group (P<0.05 or P<0.01). The level of the GH group was similar to that of the CAP group. The results suggested that a high dose of *Gb*E had the same effect as captopril in decreasing the expression of TGF-β1 mRNA (Figure 7B).

Effects of *Gb*E on morphological change in kidneys The light microphotograph showed the existence of glomerular mesangial hyperplasia (Figure 8). In the transmission electron micrographs, the ultrastructure of glomerulus of the DN rat was changed. The GBM was wrinkled and partly thickened, but in the GH group, the thickness of the GBM appeared to be almost normal (Figure 9).

Discussion

DN is the leading cause of end-stage renal disease and the characteristics of this diabetic complication include macrovascular and microvascular damage, ECM accumu-

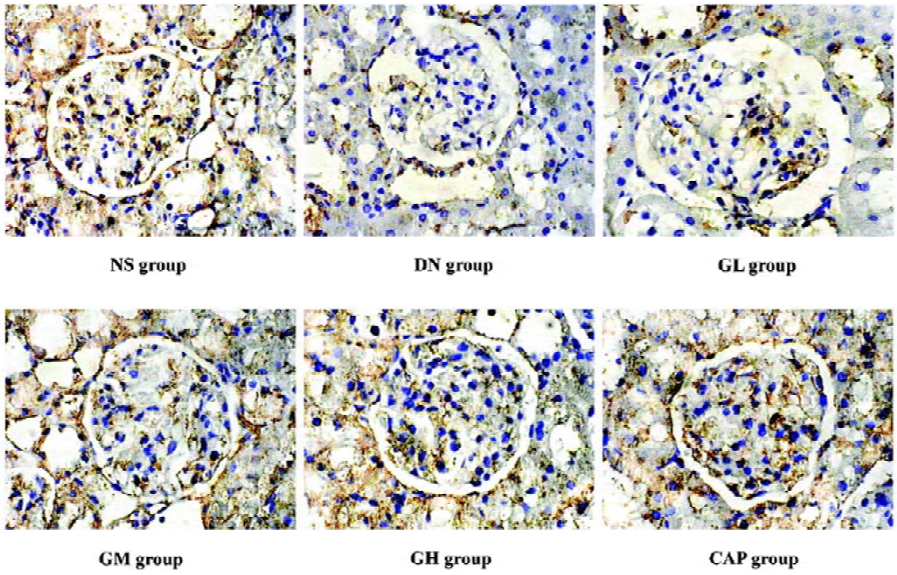

Figure 3. Immunocytochemical micrographs of MMP-2 in kidney cortex. MMP-2 was stained brown. GL, GM, and GH: *Gb*E 50, 100, and 200 mg/kg. CAP: captopril 10 mg/kg. ×400.

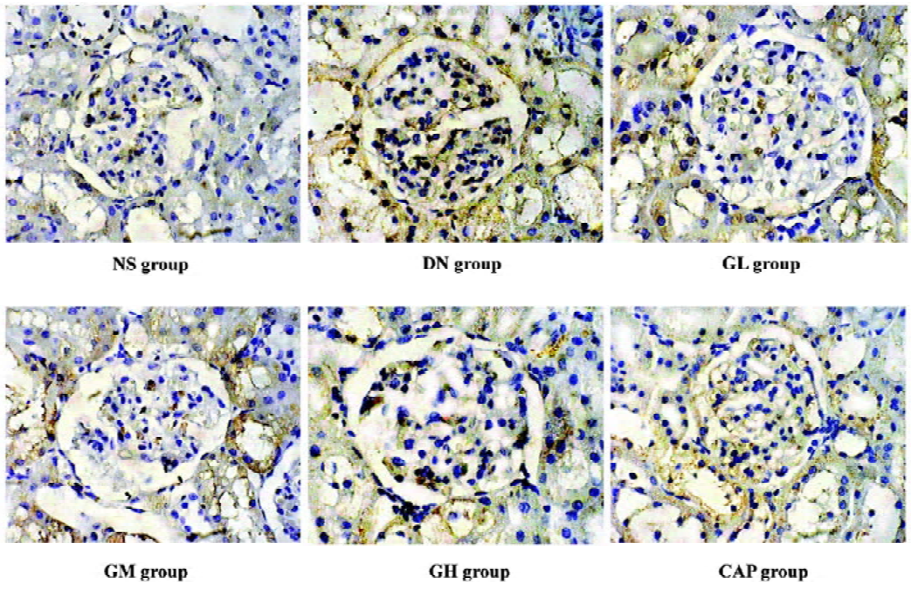

Figure 4. Immunocytochemical micrographs of TIMP-2 in kidney cortex. TIMP-2 was stained brown. GL, GM, and GH: *Gb*E 50, 100, and 200 mg/kg. CAP: captopril 10 mg/kg ×400.

lation, and eventually chronic fibrosis. Several decades of extensive research has elucidated various pathways implicated in the development of diabetic kidney disease.

Oxidative stress has been known to play an important role in the development and progression of DN, and the formation of ROS is a direct consequence of hyperglycemia. It has been shown that ROS activate the protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and JAK-STAT pathways[13,14], which lead to the activation of redox-sensitive transcription factors including NF-κB, AP-1 (Fos and Jun proteins), STAT, and Egr-1[15]. All of these enhance the transactivation of genes coding for cytokines such as TGF-β1 and CTGF, which upregulate ECM protein expression[16]. Therefore, antioxidant treatment is a potential antifibrotic

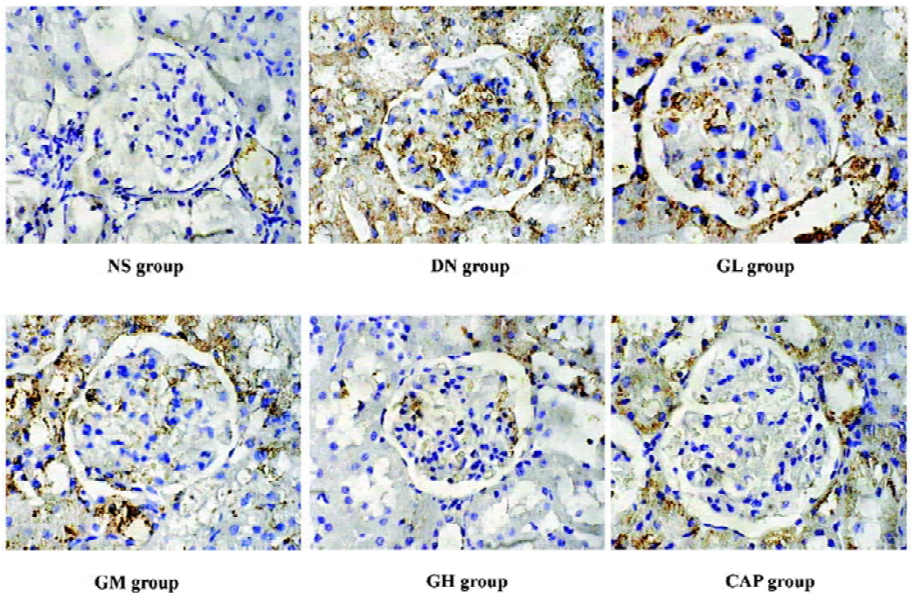

Figure 5. Immunocytochemical micrographs of CTGF in kidney cortex. CTGF was stained brown. GL, GM, and GH: *Gb*E 50, 100, and 200 mg/kg. CAP: captopril 10 mg/kg. ×400.

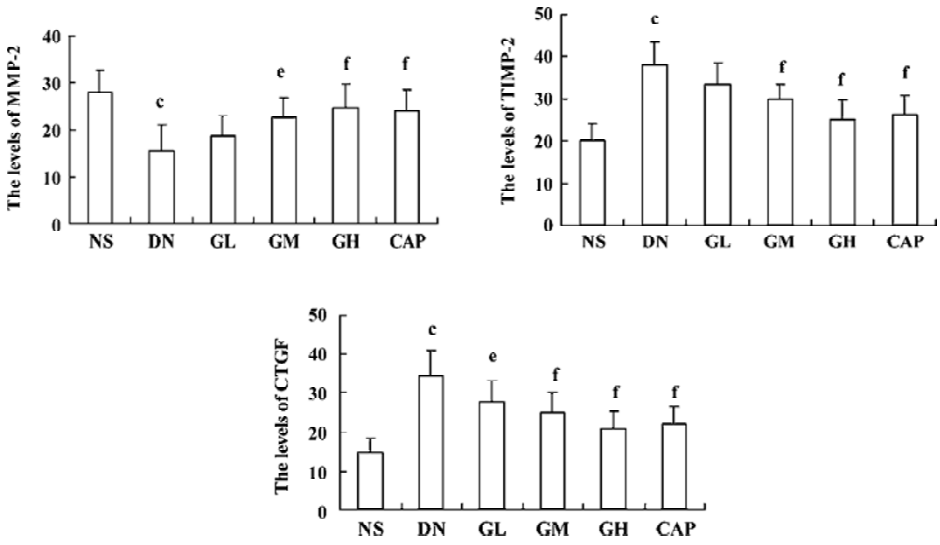

Figure 6. Effects of *Gb*E on the relative quantity of MMP-2, TIMP-2, and CTGF in kidney cortex by gray scale analysis. NS group received 6 mL/kg citrate buffer (n=13). DN group were treated with 1% CMC solution (n=13). GL group: DN rats treated with GbE 50 mg/kg (n=12); GM group: with GbE 100 mg/kg (n=14); GL group: with GbE 200 mg/kg (n=14); CAP group: the DN rats were treated with 10 mg/kg of captopril (n=11). Mean±SD. ^{c}P<0.01 *vs* NS. ^{e}P<0.05, ^{f}P<0.01 *vs* DN.

therapy for DN. Moreover, the intensity and durability of oxidative stress facilitate the formation of AGE. AGE are the biochemical end products of non-enzymatic glycosylation that are formed irreversibly. AGE are elevated in serum and in many tissues in patients with diabetes[17,18]. They can covalently crosslink and biochemically modify protein structure and affect protein functions, particularly collagen. Additionally, in recent years, cell surface receptors for AGE (RAGE) have been identified[19], and post-receptor signaling pathways are being defined[20]. Through an AGE receptor-dependent mechanism, AGE induction of cytokines and growth factors has been implicated in contributing to end-organ changes that occur in tissues of patients with diabetes[21]. At the same time, the interactions between AGE and

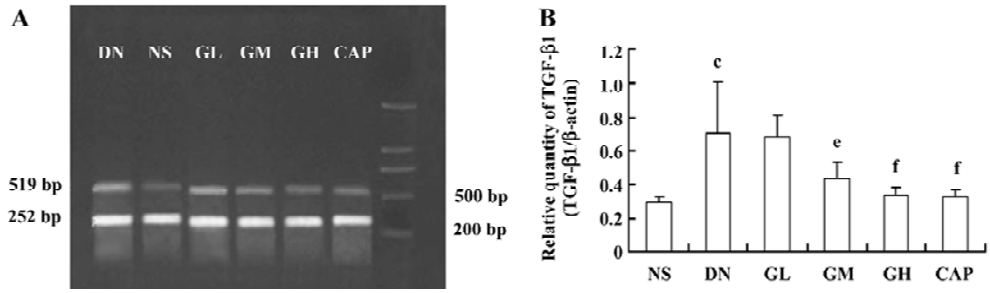

Figure 7. Effects of *Gb*E on the TGF-β mRNA expression. (A) Agarose electrophoresis of RT-PCR products amplified from the total RNA extracts of kidney cortex, beta-actin was used as the internal standard in each sample. (B) RT-PCR data for relative quantity of TGF-β mRNA performed by densitometric analysis. n=6. Mean±SD. ^{c}P<0.01 *vs* NS group. ^{e}P<0.05, ^{f}P<0.01 *vs* DN group. GL, GM, and GH represented *Gb*E 50, 100, and 200 mg/kg. CAP: captopril 10 mg/kg.

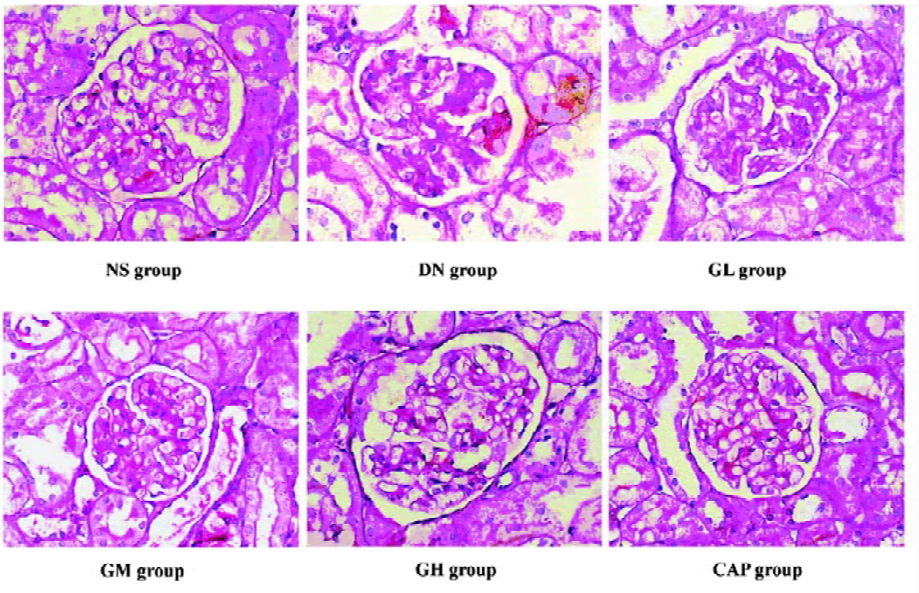

Figure 8. Effects of *Gb*E on glomerular mesangial hyperplasia in kidneys by light microscopy. NS group: the capillary loops are opened and portion of the glomerular is relatively small; DN group: the capillary loops are opened but narrowed and portion of the glomerular is relatively large; GL group (*Gb*E 50 mg/kg): the mesangial hyperplasia is moderate; GM, GH groups (*Gb*E 100 and 200 mg/kg) and CAP (captopril 10 mg/kg) group: the mesangial hyperplasia is at low-grade. PAS stain, ×40.

RAGE induce the activation of oxidative stress and stimulate the production and release of cytokines, which amplify tissue damage. Thus, oxidative stress and AGE interact mutually and upregulate each other, which can lead to ECM accumulation and mesangial cell hypertrophy. In our study, after the DN rats were treated with *Gb*E, the activities of T-AOC, T-SOD, CAT, and GSH-Px (common indicators of changes in the anti-oxidation system) all increased significantly, strongly suggesting that *Gb*E has a potent antioxidative capability *in vivo*. Furthermore, we also found that *Gb*E, even in low doses, significantly decreased AGE levels both in the kidney cortex and in the serum. These results are consistent with a former report in which *Gb*E inhibited oxidized low density lipoprotein (LDL)-stimulated fibronectin production through an antioxidant action in rat mesangial cells[22]. These data strongly suggest that *Gb*E has the characteristics of antioxidant and anti-AGE, and this could be of benefit for the prevention of DN.

Of the complicated mechanisms, the pathway of hyperglycemia-oxidative stress TGF-β1-ECM is assuredly very important. ROS are considered the activators of overall signaling pathways. TGF-β1 is the key cytokine mediating the

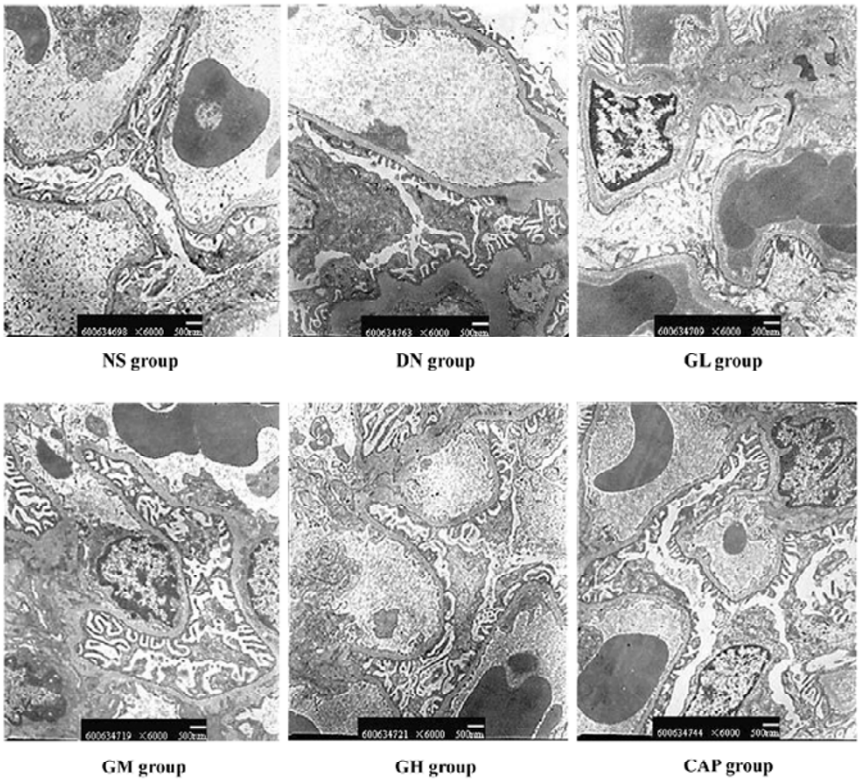

Figure 9. Effects of *Gb*E on the ultrastructural changes in kidneys by the transmission electron microscopy. NS group: the thickness of glomerulus basement membrane was normal. DN group: the glomerular basement membrane was wrinkled, and thickened partly, with effacement of some visceral epithelial cell foot processes and microvillous transformation. GL group (*Gb*E 50 mg/kg): a few of visceral epithelial cell foot showed microvillous transformation. The glomerular basement membrane, pedicelsa and glomerular capillary loops were almost normal. GM, GH groups (*Gb*E 100 and 200 mg/kg) and CAP (captopril 10 mg/kg) group: the glomerular capillary loops, glomerular basement membrane, pedicelsa and mesangial matrix were normal. ×6 000. Bar=500 nm

production of ECM proteins. Hyperglycemic conditions generate ROS. ROS and AGE interact with and upregulate each other and activate the TGF-β1/Smad signaling pathway[23]. Furthermore, ROS generated intracellularly from the glucose metabolism, and the AGE-RAGE interaction also activate PKC (together with DAG) and MAPK pathways. These, together with activated Smads, coordinate the transcription of a wave of genes, including angiotensinogen, thrombospondin-1 (TSP-1), and CTGF. Angiotensin II (Ang (II) stimulates further generation of ROS and the expression of TGF-β1. More recently, Ang II blockade is rapidly becoming a standard antifibrotic therapy in renal diseases because ACE inhibitors block TGF-β1 induced by Ang II[24]. In our study, we also found that an ACE inhibitor (captopril) decreased the relative quantity of TGF-β1 mRNA and the level of CTGF. CTGF is another prosclerotic cytokine and has also been shown to be involved in both the early and later stages of DN[25]. Secreted CTGF works in concert with TGF-β1 activated by TSP-1 and ROS to transactivate subsequent waves of genes, including those encoding structural proteins whose accumulation leads to glomerulosclerosis in DN. It is becoming clear that the coordinated expression of TGF-β1 and CTGF is crucial for the induction of ECM proteins and thus, for the development of DN. Numerous studies indicate that hyperglycemia induces an increase in TGF-β1 expression at both the mRNA and protein levels in experimental and human diabetes, as well as in cultured mesangial cells, and that increased signaling by TGF-β1 is also markedly influenced by CTGF. However, the expression of some ECM proteins, such as fibronectin, is CTGF-dependent, and its promoter region does not contain any Smad-binding elements. Thus, CTGF may mediate the induction of the ECM protein expression both directly and indirectly by potentiating the TGF-β1/Smad signaling pathway. In other words, CTGF is a crucial mediator for the TGF-β1-stimulated matrix protein expression. In our experiment, after the DN rats were treated with *Gb*E, the relative quantity TGF-β1 mRNA in the kidney cortex of the DN rats decreased significantly, strongly sug-

gesting that *Gb*E has an inhibitive effect on TGF-β1 mRNA in the kidney cortex. This is consistent with a previous report about the effect of *Gb*E on TGF-β1[26]. We also found that the expression of CTGF on *Gb*E-treated DN rats markedly decreased. Therefore, in this aspect, *Gb*E can be a prime candidate for the prevention and treatment of DN.

The ECM is a complex structure that influences the behavior of its resident cells, and its accumulation correlates closely with renal impairment in diabetes and ultimately leads to glomerular scarring. Therefore, a balance between ECM synthesis and degradation is a prerequisite for maintaining the structural and functional integrity of the glomerulus. MMP are a group of zinc dependent endopeptidases with similar biochemical natures that are capable of degrading all components of ECM and basement membranes. Thus, changes in the MMP expression or activity will directly translate into altered ECM turnover. MMP-2 is the main MMP responsible for the degradation of collagen IV. It is secreted in an inactive form that becomes activated on the cell surface by a membrane type 1 MMP. The activity of MMP-2 is also regulated by specific tissue inhibitors of MMP (TIMP-1 and TIMP-2), that were modulated directly and indirectly by TGF-β1. So the balance between MMP and TIMP is important in the fibrotic process. MMP is one of the main proteinases that participates in the degradation of ECM. In our study, the levels of MMP-2 and TIMP-2 in the DN group conspicuously changed. The expression of MMP-2 greatly decreased and TIMP-2 increased in the DN group. After treatment with *Gb*E, the level of MMP-2 increased and TIMP-2 decreased. This result strongly suggests that *Gb*E has a potent inhibitory effect on the accumulation of ECM.

For the complexity of the mechanisms of DN, it is necessary to develop new drugs to deal with more than 1 pharmacological target. In our STZ–induced, early DN rat model, we found that *Gb*E reduced the blood glucose level, decreased the level of AGE, the intensity of oxidative stress, the level of TGF-β1 mRNA, TIMP-2, CTGF, increased MMP-2, further lowered the levels of collagen IV and laminin in the kidney cortex, and deceased the thickness of GBM, and therefore ameliorated the morbidity in physical behavior and morphology. Therefore, *Gb*E has protective effects on several pharmacological targets in the complicated pathology mechanism of DN. This means that *Gb*E may exert a protective effect on the early development of DN in STZ-induced diabetic rats.

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