Ginkgo biloba leaf extract prevents diabetic nephropathy through the suppression of tissue transglutaminase

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Abstract. The present study aimed to investigate the preventive effects of *Ginkgo biloba* leaf extract (GBE) against extracellular matrix (ECM) accumulation in a streptozotocin (STZ)-induced rat model of diabetic nephropathy (DN), and to determine its underlying molecular mechanism. *In vivo*, a rat model of DN was established by intraperitoneal injection of STZ, and the rats were subsequently administered GBE. The results demonstrated that GBE significantly decreased blood glucose, the urine protein excretion rate and ECM accumulation in DN rats. In addition, the development of DN significantly induced tissue transglutaminase (tTG) protein expression, which was detected by immunohistochemistry, western blotting and PCR analyses, while GBE administration decreased tTG expression in the diabetic kidney. *In vitro*, rat glomerular mesangial cells (HBZY-1 cells) cultured with high glucose were also treated with GBE. The concentrations of tTG, fibronectin, type IV collagen, transforming growth factor (TGF)-β and connective tissue growth factor (CTGF) were detected via ELISA. The results demonstrated that GBE notably decreased the concentration of these proteins, and tTG expression was positively associated with TGF-β. GBE also suppressed tTG expression of high glucose-treated HBZY-1 cells in a concentration-dependent manner. Furthermore, tTG protein expression was detected in high glucose-treated HBZY-1 cells transfected with small interfering RNA (siRNA) oligonucleotides against TGF-β and CTGF to investigate a possible mechanism of GBE-mediated inhibition of tTG. The results demonstrated that the tTG levels remained unchanged in CTGF siRNA-transfected cells, but were decreased in the GBE + CTGF siRNA group compared with the control siRNA group, suggesting that tTG may not be regulated by CTGF, and the inhibitory effect of GBE on tTG may not be associated with the direct inhibition of CTGF. However, tTG expression was decreased following the transfection with TGF-β siRNA, in which levels of tTG were similar compared with both the GBE group and GBE + TGF-β siRNA group, indicating that tTG may be regulated by TGF-β, and that the GBE-induced repression of tTG expression may be associated with the downregulation of TGF-β. Taken together, the results of the present study suggest that GBE prevented ECM accumulation by suppressing tTG expression in DN, which was predominantly mediated by TGF-β.

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Introduction

Diabetes is a chronic progressive disease associated with endocrine and metabolic disorders, and common clinical characteristics include proteinuria, progressive renal damage, hypertension and oedema (1). Diabetes is considered a serious threat to human health, and is the most prevalent disease, apart from cardiovascular disease and cancer (2). There are a number of complications associated with diabetes, such as an increased risk of a cerebrovascular accident, coronary heart disease and retinopathy (3). Diabetic nephropathy (DN) with extracellular matrix (ECM) accumulation is a common characteristic of diabetes (1). Physiologically, ECM synthesis and degradation is balanced; however, under several pathophysiological conditions, the balance is disrupted, which results in ECM accumulation, glomerular structure damage and glomerular sclerosis, ultimately causing DN (4).

Tissue transglutaminase (tTG) is a member of the Ca^{2+}-dependent TG family, which catalyzes the formation of the γ-glutamyl-ε-lysine isopeptide bond and introduces a covalent crosslink between the protein and peptide, thus inducing resistance to enzymatic degradation (5). tTG is predominantly located in the cytoplasm, while small amounts exist in the nucleus and nuclear membrane under normal physiological conditions (6). Under high levels of glucose, tTG expression increases, the protein translocates to the outside of the cell

and crosslinks with type IV collagen (Col IV) and fibronectin (FN), which are major constituents of the ECM (7). The crosslinked product is highly stable and difficult to degrade (8). In a previous study, the administration of specific inhibitors of tTG to streptozotocin (STZ)-induced DN rats was found to significantly decrease the abundance of the extracellular crosslinked product (9). Therefore, tTG is considered a regulator of the ECM and is involved in the development of DN.

Transforming growth factor (TGF)-β is a peptide that reacts with different types of cells and is involved in numerous different biological functions, such as promoting cell hypertrophy, accelerating apoptosis and improving the content of the ECM (10). TGF-β mediates the synthesis of matrix proteins, such as Col IV and FN, under high glucose conditions and promotes the adhesion between cells and the ECM by increasing the expression of the ECM receptor (11). A previous study have demonstrated that TGF-β expression was notably increased in STZ-induced diabetic kidney tissues of rats, which was positively associated with the degree of renal fibrosis (12). Connective tissue growth factor (CTGF) is a downstream target of TGF-β, and the biological effect of TGF-β is partially mediated by CTGF (13). Another previous study demonstrated that CTGF may be associated with the pathogenesis of DN, as patients with DN had increased concentrations of CTCF in peripheral blood and urine. CTCF was also found to be associated with the albuminuria excretion index (14). Furthermore, CTGF is expressed at significantly high levels in the mesangial area of patients and animals with DN compared with normal tissues, and in high glucose-cultured mesangial cells (15). It has also been reported that the concentrations of FN and collagen are significantly increased in CTGF-treated mesangial cells (16). Taken together, these results suggest that TGF-β and CTGF serve important roles in the process of ECM accumulation. However, to the best of our knowledge, whether there is an association between tTG and TGF-β or CTGF in DN remains unclear.

Ginkgo biloba leaf extract (GBE) has been widely used to prevent and treat cardiovascular diseases due to its reported ability to induce vasodilation, inhibit the development of atherosclerosis and inflammation, and repress free radicals (17). Analogous effects have also been observed in mesangial cells cultured in high glucose medium, where GBE was demonstrated to decrease the expression levels of TGF-β and CTGF, as well as the expression levels of Col IV (18). Our previous study indicated that GBE can be used to prevent and treat renal fibrosis in rats, which may inhibit the Angiotensin (Ang) II-induced upregulation of the mRNA expression levels of TGF-β and CTGF (19). However, to the best of our knowledge, the effects of GBE on tTG have not yet been elucidated. Thus, the present study aimed to investigate the effects of GBE on tTG expression in the diabetic kidneys of rats and high glucose-treated mesangial cells to determine whether GBE exerts protective mechanisms.

Materials and methods

GBE extract. GBE was purchased from JiangSu Xuzhou Huakang Biological Products Co., Ltd. The extract was obtained through ethanol extraction method and the contents of total *Ginkgo* flavonol glycosides and terpene lactones in GBE were detected with high-performance liquid chromatography by the aforementioned company. According to the Chinese Pharmacopoeia (2015) (20), the total *Ginkgo* flavonol glycosides content in GBE is 25.3% (recommended, >24.0%) and the terpene lactones content in GBE is 6.37% (recommended, >6.0%), its moisture content is 4.6% (recommended, ≤5.0%).

Chemical reagents. Streptozotocin (STZ) was purchased from MilliporeSigma. Ginaton (GBE injection) was from Dr Willmar Schwabe GmbH & Co. KG. Rabbit anti-tTG polyclonal antibody (cat. no. 121495) was from Abcam. Rabbit anti-CTGF polyclonal antibody (cat. no. 323092), anti-TGF-β polyclonal antibody (cat. no. 324045), HRP-anti-rabbit IgG and 3,3'-diaminobenzidine (DAB) were from OriGene Technologies, Inc. TRIzol® reagent, molecular weight protein marker, enhanced chemiluminescence (ECL), Lipofectamine® 2000 and SuperScriptII were all from Thermo Fisher Scientific, Inc. Primers were purchased from RuiJie Biological. PVDF membranes were from EMD Millipore. Rabbit anti-FN (cat. no. 0666R) and anti-Col IV (cat. no. 0553R) polyclonal antibodies were from BIOSS. Mouse anti-GAPDH polyclonal antibody (cat. no. 365062) and peroxidase-conjugate secondary antibody (cat. no. sc2004) were from Santa Cruz Biotechnology, Inc. Rat mesangial cells (HBZY-1) were purchased from Wuxi BioHermes Biological Co., Ltd. DMEM was supplied by Gibco; Thermo Fisher Scientific, Inc. TGF-β ELISA kit (cat. no. BMS623-3) was from Thermo Fisher Scientific, Inc., FN ELISA kit (cat. no. EK0350) was from Wuhan Boster Biological Technology, Ltd., and Col IV ELISA kit (cat. no. H145) and BCA kit were from Nanjing Jiancheng Bioengineering Institute. CTGF and tTG ELISA kits (cat. nos E90010Ra and E90053Ra) were from YouerSheng Technology Co., Ltd. Mayer's hematoxylin solution and periodic acid-schiff (PAS) staining were from Beijing Dingguo Changsheng Biotechnology Co., Ltd.

DN animal model protocol. Healthy male Wistar rats (weight, 180-220 g; age, 6 weeks) were provided by the Laboratory Animal Center of Jilin University. All the rats were housed in a pathogen-free facility with free access to a standard dried chow diet and water throughout the period of study. During the present study, the animals were housed in a temperature of 22-26°C and a humidity of 50-65% in a controlled environment with a 12-h light/dark cycle. The padding was changed twice a week and the health status was observed with no mortalities. All the animal experiments were conducted following internationally recognized guidelines proposed by the World Association for the Protection of Animals on animal welfare and the regulations on the administration of laboratory animals in China. All the *in vivo* experiments were approved by the Animal Experimental Ethical Inspection Committee of Jilin University, School of Pharmaceutical Sciences (ethical permission code 20190014; Changchun, China).

A single intraperitoneal injection of 50 mg/kg STZ was used to induce diabetes in the rats. The blood and urine glucose levels of each rat were tested 3 days later. The DN animal model standard was as follows: Blood glucose level ≥16.7 mmol/l and 24-h urinary albumin excretion >20-200 μg/min (21). The DN rats were randomly divided into two groups, ten for DN group

and ten for GBE group. Ten healthy rats for the control group. The GBE group rats were intragastrically administered with 100 mg/kg GBE once a day (dissolved in 0.5% carboxymethylcellulose sodium; Beijing Dingguo Changsheng Biotechnology Co., Ltd.), an equal volume of vehicle was given in the control and DN groups for 12 weeks. The rats were subsequently euthanized using pentobarbital sodium (150 mg/kg body weight; Beijing Dingguo Changsheng Biotechnology Co., Ltd.) through the intraperitoneal route, followed by cervical dislocation.

Measurement of blood glucose and renal function. Blood and urine of all the rats were collected before the animals were sacrificed at the end of study, and the 24-h urinary albumin excretion and blood glucose were examined using biuret (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) and 7150 Automatic Biochemical Analyzer (Hitachi, Ltd.), respectively.

PAS staining for mesangial matrix expansion. Rats were sacrificed following GBE administration as aforedescribed. Then the whole kidneys were removed completely and fixed in 10% buffered formalin at room temperature for 24 h, kidney tissues were cut longitudinally in the same position and embedded in paraffin for a light microscopic study (Olympus Corporation). The 5 μm kidney tissue sections were all stained with PAS reagent. The paraffin sections were incubated in periodate alcohol solution (0.088 mol/l periodic acid, 0.05 mol/l sodium acetate and 4.30 mol/l ethyl alcohol) at 17-20˚C for 10 min. Afterwards, they were washed with 70% ethanol and transferred into reductant (0.125 mol/l sodium thiosulfate, 10.187 mol/l ethyl alcohol, 0.02 mol/l HCl and 0.12 mol/l KI) at 17-20˚C for a 10-min incubation. Subsequently, the sections were washed with 70% alcohol again and soaked in the PAS solution for 1-1.5 h at room temperature. The sections were rinsed with running water for 10 min. The nuclei of cells were stained with Mayer's hematoxylin for 3-5 min at room temperature followed by 1% hydrochloric acid alcohol (1% concentrated hydrochloric acid and 99% ethyl alcohol) wash. Finally, the sections were washed with running water for 3 min, and then dehydrated to reinforce the diaphaneity and sealing. Glycogen and mucin in the glomerulus were stained purple by PAS, whereas the nuclei were stained blue by Mayer's hematoxylin. PAS-positive staining areas were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.) analysis software, and total glomerular tuft areas were analyzed using ImageJ software (version 1.51d; National Institutes of Health). The glomerulosclerosis area (%) was calculated using the following formula: (PAS-positive staining area/total glomerular tuft area) x100%.

Immunohistochemical analysis of tTG expression. The kidney paraffin sections, prepared as described in the previous section, were dewaxed at 60˚C for 30 min, and washed with boiled 0.01 mol/l PBS (pH 7.4, 0.0203 mol/l Na_2HPO_4, 0.00167 mol/l NaH_2PO_4, 0.1367 mol/l NaCl) three times (3 min each), blocked in 5% bovine serum albumin (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) at room temperature for 1 h. Afterwards, the samples were incubated with rabbit anti-tTG antibody for 24 h at 4˚C (1:300 dilution), the bound antibodies were subsequently detected with HRP-anti-rabbit IgG for 20 min at room temperature (1:1,000 dilution) and DAB, followed by counterstaining with Mayer's hematoxylin for 2 min at room temperature, and negative controls were incubated with PBS. Finally, images were captured under a light microscope (Nikon TE-2000U; Nikon Corporation), and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.) analysis software.

Culture of HBZY-1 cells. HBZY-1 cells were cultured in low glucose (5.5 mM) DMEM containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 10% newborn calf serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C with 5% CO_2 and 95% air. Confluent cells were used for experiments between passages 3 and 7. HBZY-1 cells at 85-90% confluence were seeded into six-well plates ($1x10^6$ cells/well) and incubated with high glucose (30 mM) DMEM without serum and different concentrations (0, 3.125, 6.25, 12.5, 25 and 50 μg/ml) of Ginaton at 37˚C for 24 h. Control group cells were cultured with low glucose (5.5 mM) DMEM without serum at 37˚C for 24 h.

Semi-quantitative reverse transcription-PCR (RT-PCR). Total mRNA was extracted with TRIzol following the manufacturer's protocol. RT-PCR reactions were prepared according to the manufacturer's instructions of SuperScriptII reverse transcriptase (10,000 units total, at 200 U/μl; 5 X first-strand buffer, 250 mol/l Tris-HCl, 375 mol/l KCl, 15 mol/l $MgCl_2$); 100 mol/l DTT), using 1 μg of total mRNA as the template. The upstream and downstream primers were designed for rat tTG mRNA, and GAPDH mRNA was used for sample normalization. The sequences of the primers used were as follows: tTG forward, 5'-GGCAATGACTTTGACGTGTTTG-3' and reverse, 5'-ATACAGGGAATCAGAAAGTGGGTTC-3'; and GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. The molecular sizes of the amplification products were 396 and 452 bp, respectively. The following thermocycling conditions were used for semi-quantitative PCR with DNA polymerase (Thermo Fisher Scientific, Inc.): Initial denaturation at 94˚C for 5 min; 30 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec; and a final extension at 72˚C for 10 min. PCR products (5 μl) were used for gel electrophoresis (1% agarose gel) and target bands visualized by ethidium bromide, the results were utilized to quantify the intensity of nucleic acid bands with ImageJ 1.25 software and calculate the ratio of tTG/GAPDH.

Western blotting. Fresh frozen renal cortical tissues (100 μg) were homogenized in 100 μl lysis buffer [0.01 mol/l Tris-HCl (pH 7.5), 0.1 mol/l NaCl, 0.001 mol/l EDTA, 100 μg/ml PMSF, 1 μg/ml Aprotinin] for 30 min and thereafter centrifuged at 12,000 x g for 15 min at 4˚C. The BCA method was used to quantify the level of protein in each sample to ensure equal protein loading. Proteins were heated with 2X SDS-PAGE sample buffer [100 mol/l Tris-HCl (pH 6.8), 200 mol/l DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerin] for 5 min and separated by SDS-PAGE according to their molecular weight. Briefly, 50 μl proteins, along with a molecular

weight protein marker, were subjected to SDS-PAGE (12% acrylamide gel) and electroblotted onto PVDF membranes. The membranes were blocked with 5% non-fat milk in TBS containing 0.1% Tween-20 for 1 h at room temperature and then probed at 4˚C with anti-tTG (1:300 dilution) or anti-GAPDH antibody (1:500 dilution) overnight. After incubation with peroxidase-conjugated secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature, membranes were developed using ECL. In addition, rat mesangial cells (HBZY-1) were cultured *in vitro* for further study, confluent HBZY-1 cells were harvested from 6-well plates, lysed on ice with 100 μl lysis buffer (Beijing Dingguo Changsheng Biotechnology Co., Ltd.) per well and centrifuged at 12,000 x g for 15 min at 4˚C. The supernatant was collected and western blotting was performed as described above. All the band intensities were analyzed using ImageJ 1.25 software.

MTT assay. HBZY-1 cells in the logarithmic growth phase were dissociated by trypsinization at 37˚C for 2 min and seeded into 96-well plates at a density of $2x10^4$ cells/ml and 200 μl cell suspension/well overnight. Cells were treated with different concentrations of GBE injection, as described previously, under high glucose (30 mM) DMEM at 37˚C for 72 h in order to estimate whether this agent induced cell injury. Subsequently, 20 μl MTT (5 mg/ml in PBS) solution was added into each well and the samples were incubated at 37˚C for 4 h. A total of 150 μl dimethylsulfoxide was added to each well and the plates were placed on a shaker at room temperature for 10 min. The absorbance at 570 nm was measured with a microplate reader (SpectraMax Plus384; Molecular Devices, LLC). The percentage of surviving cells was calculated as a fraction of the negative control group cells which were treated with an equal volume of low glucose (5.5 mM) DMEM.

ELISA for testing the contents of FN, Col IV, tTG, TGF-β and CTGF. The cells were treated as described in previous sections, then culture supernatant of each sample was collected and diluted with a coating buffer (pH 9.6, 0.06 mol/l carbonate) at a dilution of 1:9. A total of 100 μl diluent was added to each well in the ELISA plate at 4˚C overnight and the coating buffer without supernatant fluid was used as the control. The coating buffer was removed and samples washed three times with PBS with 5% Tween-20 (3 min each). The wells were blocked with PBS containing 2% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) for 1.5 h at 37˚C. The plate was subsequently washed again and incubated with antibodies against tTG, TGF-β, CTGF, FN and Col IV (1:1,000 dilution) for 1.5 h at 37˚C. After incubation with a HRP-conjugated secondary antibody (1:2,000 dilution) for 1 h at 37˚C the supernatant fluid was disposed of and O-phenylenediamine was added and incubated in the dark at room temperature for 5 min. H_2SO_4 was added to stop the reaction and the absorbance value was detected at a wavelength of 490 nm with a DG5033A Automatic Microplate ELISA Analyzer (Nanjing Huadong Electronics Group Medical Equipment Co., Ltd.).

Transfection with small interfering RNA (siRNA) against TGF-β and CTGF. HBZY-1 were cultured in 6-well plates to 85-90% confluence, at which point they were transfected with four separate gene phosphorylated double-stranded siRNA oligonucleotides targeting TGF-β (5'-GCAACAAUUCCUGGCGUUA-3', 5'-GCAACAACGCAAUCUAUGA-3', 5'-GGACUACGCCAAAGAA-3' and 5'-GAACCAAGGAGACGGAAUA-3') or CTGF (5'-GAAGACGCGUUUGGCCCUG-3', 5'-GACAAUACCUUCUGCAGGC-3', 5'-GUGAAGACCUACCGGGCUA-3' and 5'-CCAAAGCAGUUGCAAAUAC-3') as well as non-specific pooled duplex negative control siRNA (5'-AUGAACGUGAAUUGCUCAAUU-3' and 5'-UUGAGCAAUUCACGUUCAUUU-3') using Lipofectamine® 2000, according to the manufacturer's protocol. HBZY-1 at density of $1x10^6$ cells/well were plated for at least 24 h before transfection, and transfected with 5 μl siRNA at 37˚C for 6 h. A total of 6 h after transfection, the HBZY-1 were exposed to 12.5 μg/ml of GBE for 72 h, and cultured with high glucose (30 mM) DMEM, the control siRNA group were transfected with 5 at 37˚C for 6 h and exposed only to high glucose DMEM. Finally, tTG protein expression levels were detected by western blotting, as described above.

Statistical analysis. Statistical analysis was performed using the SPSS 22.0 statistical package (IBM Corp.). The results are presented as the mean ± SD, and three repeats were performed for each experiment. Differences among all groups were evaluated using one-way analysis of variance with Tukey's post hoc test, correlation analysis were evaluated using Pearson's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GBE decreases blood glucose levels and relieves renal injury in a rat model of DN. In the present study, the rat models of DN had blood glucose levels of ≥13.8 mmol/l and a 24-h urinary albumin excretion of >20-200 μg/min (28.8-288 mg/24 h), which were significantly higher compared with those in the control group. In addition, the kidney weights of rats with DN were significantly elevated compared with those in the control group (all $P<0.01$; Fig. 1A-C), and exhibited an increase in the glomerulosclerosis area ($P<0.01$; Fig. 1D). These results indicated that the STZ-induced rat model of DN had been successfully established. Decreased 24-h urinary albumin excretion levels and lower kidney weights were observed in the GBE group compared with those in the DN group ($P<0.05$), and the glomerulosclerosis area was significantly decreased ($P<0.01$), suggesting that GBE may not significantly reduce glucose levels, while significantly decrease the renal injury of DN rats.

GBE inhibits tTG expression in rat models of DN. In the present study, histological examination was performed to detect tTG expression levels in kidney tissues, and western blot and PCR analyses were further performed to detect tTG protein and mRNA expression levels, respectively. Immunohistochemistry analysis demonstrated that tTG expression was significantly upregulated in the kidney glomeruli and tubules of rat models of DN compared with that in the control group ($P<0.01$; Fig. 2A). Furthermore, tTG protein ($P<0.01$; Fig. 2B) and mRNA levels ($P<0.01$; Fig. 2C) were significantly higher in DN rats compared with those in the

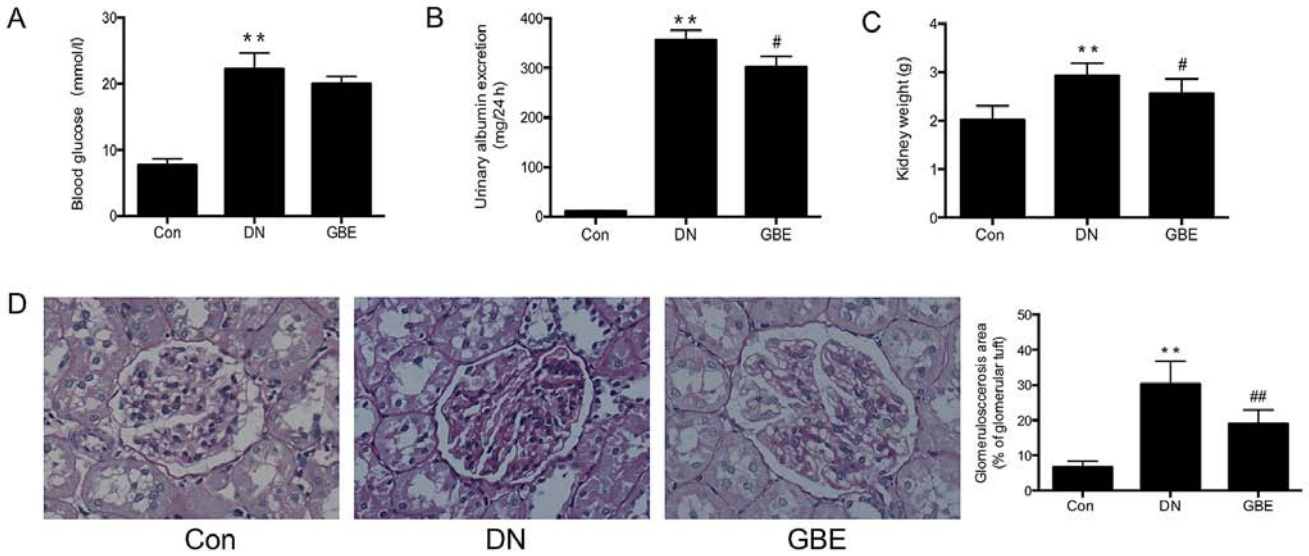

Figure 1. Effect of GBE on blood glucose and renal dysfunctions of rats with DN. (A) Blood glucose levels. (B) Urinary albumin excretion. (C) Kidney weights of rats. (D) Glomerulosclerosis area (magnification, x400; scale bar, 100 μm). Data are presented as the mean ± SD (n=10 rats/group). **P<0.01 vs. Con group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. DN group. Con, control; DN, diabetic nephropathy; GBE, *Ginkgo biloba* leaf extract.

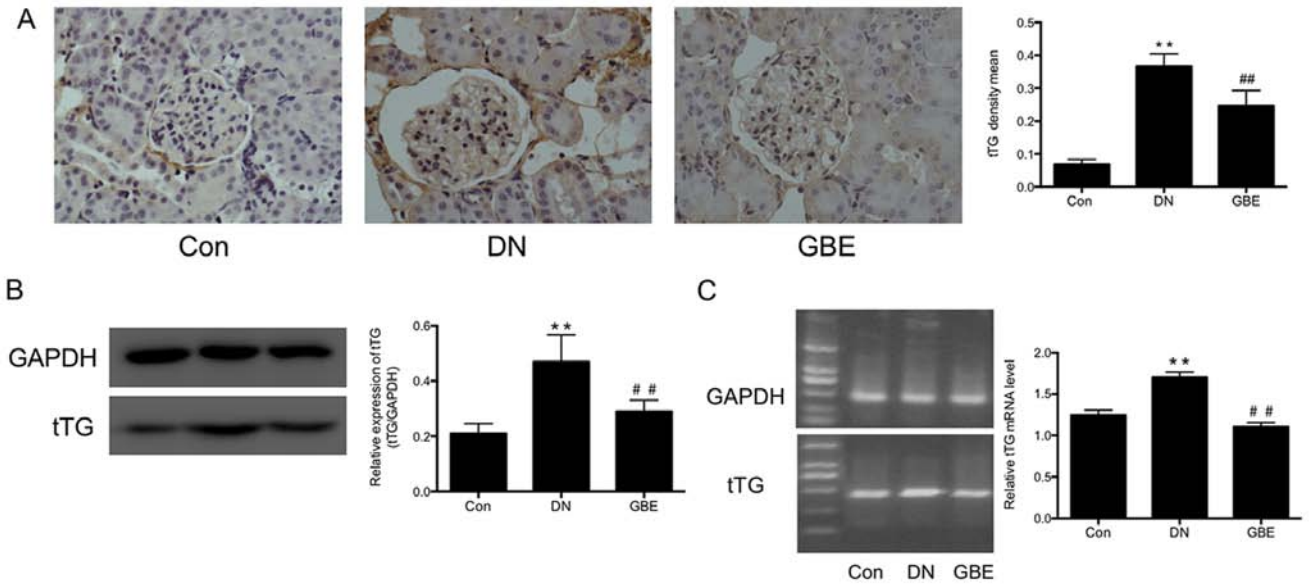

Figure 2. GBE suppresses the expression of tTG in diabetic kidney. (A) Representative immunohistochemistry staining of tTG in kidney (magnification, x400; scale bar, 100 μm). (B) Expression of tTG detected by western blotting in kidney tissues, the protein level was semi-quantified by densitometric analysis. (C) mRNA level of tTG in kidney samples. GAPDH was used as the internal standard in each sample, all values are presented as the mean ± SD. **P<0.01 vs. Con group; $^{\#\#}$P<0.05 vs. DN group. Con, control; DN, diabetic nephropathy; GBE, *Ginkgo biloba* leaf extract; tTG, tissue transglutaminase.

control rats. Notably, following treatment with GBE, tTG protein (P<0.01) and mRNA (P<0.01) levels were significantly decreased compared with those in the DN group.

GBE decreases the expression levels of FN, Col IV, tTG, TGF-β and CTGF in high glucose-induced HBZY-1 cells. To determine whether GBE exerted protective effects on HBZY-1 cells, cell viability was assessed using an MTT assay. HBZY-1 cells were treated with different concentrations of GBE for 72 h. The results demonstrated that GBE failed to significantly alter the viability of HBZY-1 cells compared with the control group (Fig. 3A).

The results of the present study demonstrated that Col IV and FN protein levels were increased in HBZY-1 cells treated with high glucose (P<0.05; Fig. 3B). Following treatment with different concentrations of GBE (3.125, 6.25, 12.5, 25 and 50 μg/ml) the levels of Col IV and FN were decreased (P<0.01).

The protein expression levels of tTG, TGF-β and CTGF were increased in high glucose-treated HBZY-1 cells compared with those in low glucose-treated HBZY-1 cells (P<0.01; Fig. 3C). Furthermore, GBE significantly downregulated the expression levels of tTG, TGF-β and CTGF (P<0.01). Correlation between tTG and TGF-β or CTGF was assessed, and the results demonstrated that tTG expression was closely

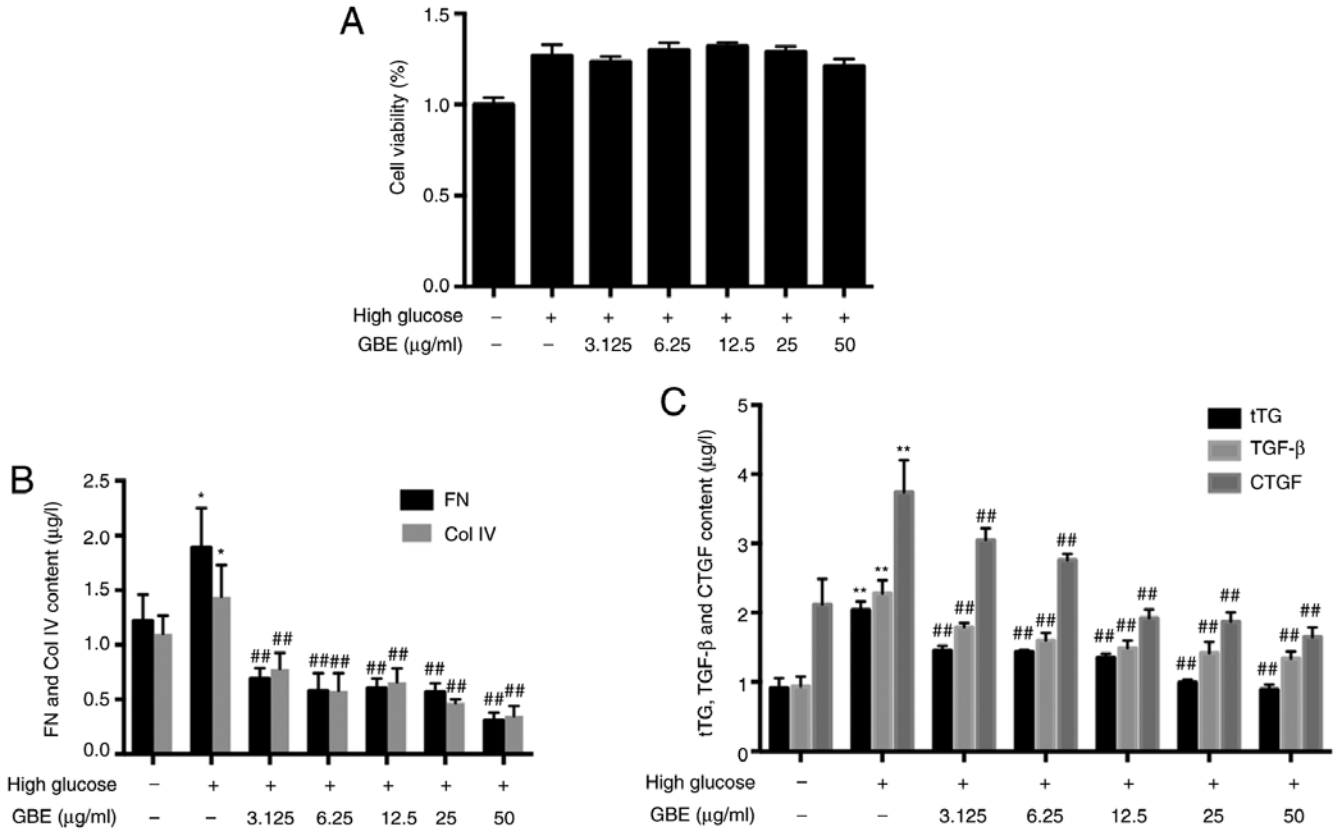

Figure 3. Effects of GBE on secretion of FN, Col IV, tTG, TGF-β and CTGF. (A) Effect of GBE on cell viability. (B) Protein levels of FN and Col IV. (C) Protein levels of tTG, TGF-β and CTGF. Data are presented as the mean ± SD. *P<0.05 and **P<0.01 vs. low glucose control group; $^{\#\#}$P<0.01 vs. high glucose group. tTG, tissue transglutaminase; FN, fibronectin; Col IV, type IV collagen; TGF-β, transforming growth factor-β; CTGF, connective tissue growth factor; GBE, *Ginkgo biloba* leaf extract.

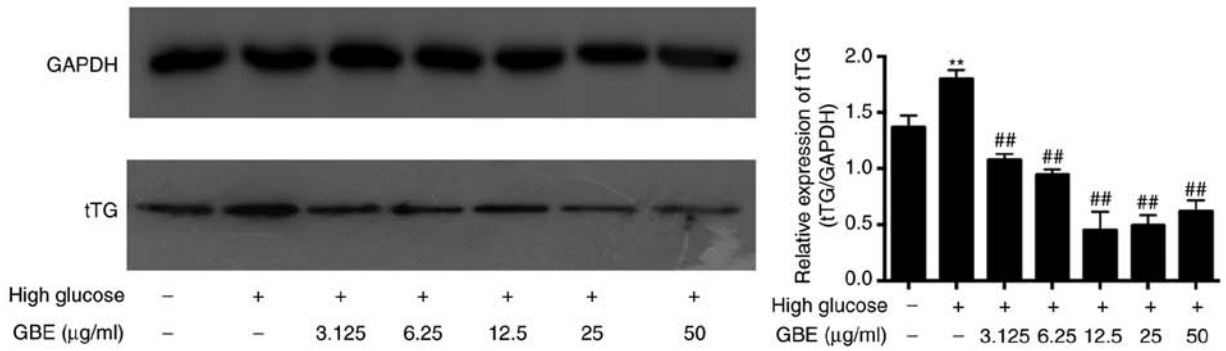

Figure 4. GBE suppresses the expression of tTG in high glucose-treated HBZY-1. GAPDH was used as the internal standard in each sample, the protein level semi-quantification was conducted by densitometric analysis. Data are presented as the mean ± SD. **P<0.01 vs. low glucose control group; $^{\#\#}$P<0.01 vs. high glucose group. tTG, tissue transglutaminase; GBE, *Ginkgo biloba* leaf extract.

positive associated with both TGF-β and CTGF expression (P<0.0001; Table I).

GBE inhibits tTG expression in high glucose-cultured HBZY-1. Western blot analysis was performed to detect tTG protein expression in high glucose-treated HBZY-1 cells and in cells following treatment with different concentrations of GBE. The results demonstrated that tTG protein expression was significantly increased in high glucose-treated HBZY-1 cells compared with that in low glucose-treated control cells (P<0.01; Fig. 4). Notably, tTG expression decreased following treatment with GBE, in a concentration-dependent manner (P<0.01). Compared with the high glucose group, tTG expression levels in the 12.5, 25 and 50 μg/ml GBE-treated groups were reduced by >50%, and no statistically significant differences were observed between the three groups. Thus, 12.5 μg/ml GBE was selected for subsequent experimentation. Collectively, these results suggest that GBE may decrease tTG expression in DN.

Role of TGF-β and CTGF in the downregulation of tTG expression levels following GBE treatment. Subsequent

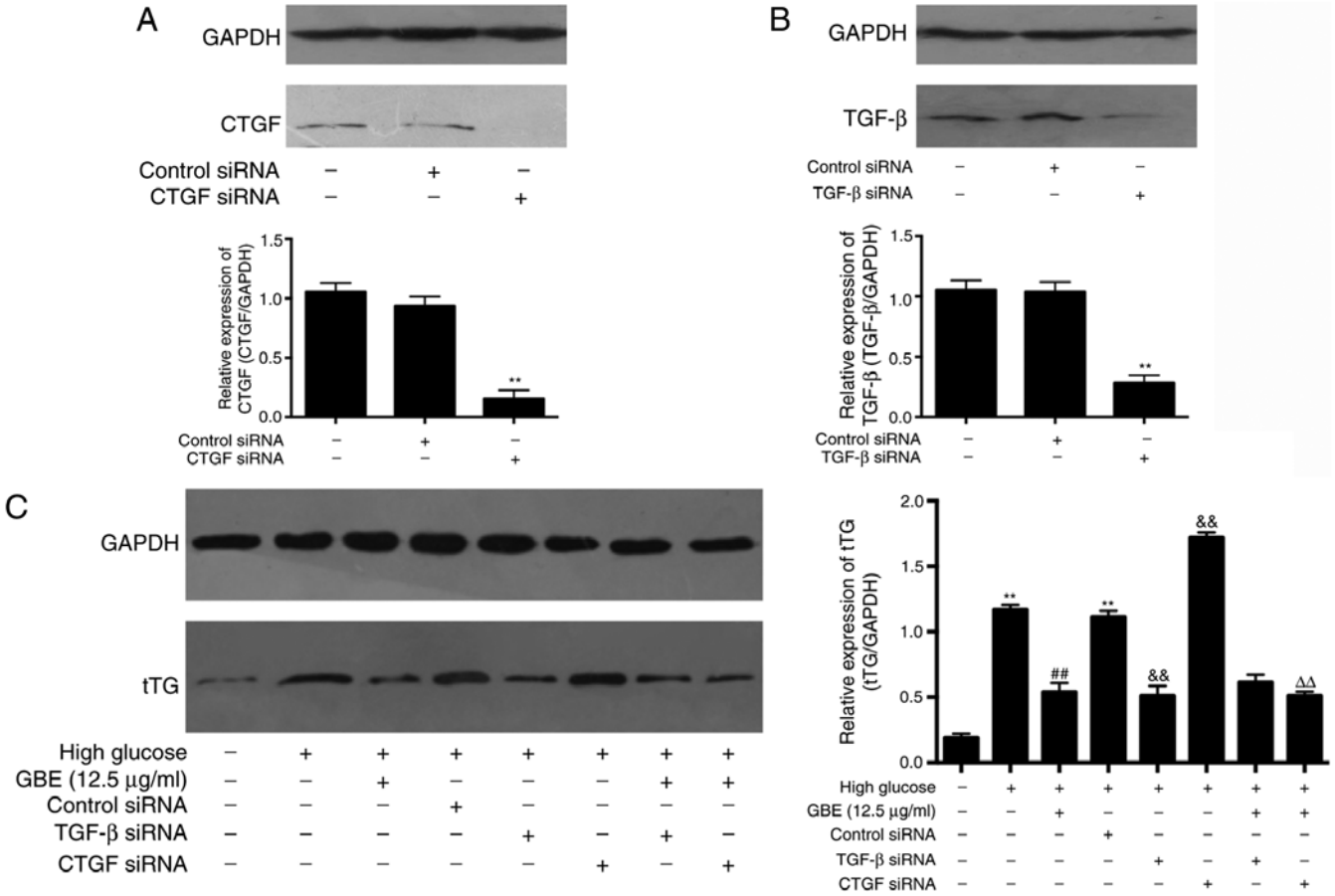

Figure 5. Role of TGF-β and CTGF in the GBE-mediated downregulation of tTG protein expression. Effect of (A) CTGF and (B) TGF-β siRNA transfection on the protein expression levels of CTGF and TGF-β, respectively. **P<0.01 vs. control siRNA. (C) tTG expression was measured by western blotting in cells transfected with CTGF, TGF-β or control siRNA and treated with high glucose and 12.5 μg/ml GBE. GAPDH was used as the internal standard in each sample, the protein level was semi-quantified by densitometric analysis. Data are presented as the mean ± SD. **P<0.01 vs. low glucose control group; ##P<0.01 vs. high glucose group; &&P<0.01 vs. control siRNA group; ΔΔP<0.01 vs. CTGF siRNA without GBE group. tTG, tissue transglutaminase; GBE, *Ginkgo biloba* leaf extract; TGF-β, transforming growth factor-β; CTGF, connective tissue growth factor; siRNA, small interfering RNA.

Table I. Correlation analysis between tTG and TGF-β and CTGF protein expression levels.

	tTG expression	
Parameter	Pearson's correlation (r values)	n
TGF-β expression	0.867[a]	48
CTGF expression	0.924[a]	48

[a]P<0.0001. tTG, tissue transglutaminase; TGF-β, transforming growth factor-β; CTGF, connective tissue growth factor.

experiments were performed to confirm the involvement of TGF-β and CTGF in the protective effects of GBE. Western blot analysis demonstrated that transfection with CTGF or TGF-β siRNA significantly inhibited the protein expression levels of CTGF and TGF-β compared with those in the respective control siRNA groups (Fig. 5A and B). In addition, transfection with CTGF siRNA failed to significantly decrease tTG protein expression in HBZY-1 cells, while GBE notably inhibited tTG expression in CTGF siRNA-transfected HBZY-1 cells compared with the CTGF siRNA-transfected only group (Fig. 5C), suggesting that increased tTG expression in HBZY-1 cells under high glucose levels may not be regulated by CTGF, and the inhibitory effect of GBE on tTG expression may be independent of CTGF. Transfection with TGF-β siRNA significantly decreased tTG protein expression compared with the control siRNA group (P<0.01), while tTG expression was similar in the GBE, GBE + TGF-β siRNA and GBE + CTGF siRNA groups, indicating that the GBE-induced inhibition of tTG expression may be associated with downregulating TGF-β expression.

Discussion

DN is a serious complication of diabetes and is closely associated with the early mortality of affected patients (1). Suppression of high blood glucose levels and high blood pressure is a basic treatment of DN (22). To a certain extent, these methods can effectively delay the onset of kidney disease; however, they fail to inhibit ECM accumulation, resulting in some patients eventually developing end-stage renal disease (23,24). Further studies are required to identify and develop novel treatments to cure DN. Currently, Traditional Chinese Medicine has become an important research focus, with promising new treatments, such as ginkgo (25). ECM accumulation in the glomerular

mesangium and tubulointerstitium is the main structural feature of DN, and the clinical diagnostic indicators include thickening of the glomerular basement membrane and broadening of the mesangial matrix, as well as albuminuria (26,27). Preliminary studies have demonstrated that GBE affects the protection of kidneys in STZ-induced diabetic rats. For example, it has been reported that blood glucose, 24-h urinary albumin excretion and ECM accumulation notably decreased following treatment with GBE (28). Earlier studies have demonstrated that extract of *Ginkgo biloba* 761 can inhibit the thickening of the basement membrane and ECM deposition in endotoxaemic rat kidneys, and increase antioxidant enzyme activity to prevent renal tissue damage (29). Lasaite *et al* (30) treated patients with type 2 diabetes with GBE for 18 months, and revealed that the patients' glycated hemoglobin levels notably decreased, while their quality of life significantly improved. GBE is a Traditional Chinese Medicine that has been previously reported to improve DN (31).

Under continuously high blood glucose levels, tTG expression increases in the tubules and glomeruli (6), which regulates the aggregation of a variety of ECM proteins, including FN, collagen and collagen peptide, thus resulting in the accumulation of the ECM (7). The results of the present study demonstrated that tTG expression increased in the kidneys of diabetic rats, while GBE effectively decreased tTG protein and mRNA levels. TGF-β is an important regulatory factor of tTG, which functions as an inducer of several cytokines and fibrosis (32). A number of stimulating factors, such as hyperglycemia, advanced glycation end products and oxidative stress can stimulate the production of TGF-β (33,34). In addition, growth factors, such as CTGF, are closely associated with the promotive effect on fibrosis of TGF-β, and can enhance the biological function of TGF-β by combining with the TGF-β domain (35). In the present study, the secretion of Col IV and FN was increased in HBZY-1 cells cultured with high glucose, while GBE notably decreased the levels of FN and Col IV, in a concentration-dependent manner. Furthermore, the levels of tTG, TGF-β and CTGF were all decreased following treatment with GBE in high glucose-cultured HBZY-1 cells, and tTG expression was positively associated with TGF-β and CTGF expression. The siRNA-mediated knockdown of TGF-β and CTGF was performed to determine the potential inhibitory mechanism of GBE on tTG. HBZY-1 cells transfected with TGF-β siRNA had a markedly impaired ability to upregulate the expression levels of tTG simulated by high glucose exposure, and tTG expression in these cells was similar to that in the GBE-treated HBZY-1 cells transfected with TGF-β siRNA. tTG expression was not significantly suppressed following the siRNA-mediated knockdown of CTGF, whereas tTG expression was notably downregulated following treatment with GBE. Taken together, these results suggested that increased tTG expression in HBZY-1 cells under high glucose environments may not be directly mediated by CTGF but by TGF-β, and GBE may repress tTG expression by inhibiting TGF-β, which is independent of CTGF. Thus, it may be hypothesized that GBE protects ECM accumulation in DN mainly by inhibiting tTG expression via regulation of TGF-β.

Cui *et al* (34) injected human recombinant TGF-β into isolated perfused rat kidneys and the results revealed that tTG mRNA expression was upregulated by 8-fold compared with that in normal rats, resulting in the accumulation of ECM (36). Furthermore, tTG expression is considered to be closely associated with CTGF expression (37). These findings may suggest potential targets for the treatment of DN. DNA methylation is an important regulatory mechanism of CTGF expression, whereby high glucose levels can induce the demethylation process of the CTGF gene promoter and increase CTGF expression in human glomerular mesangial cells or DN model mice, thus altering the expression of its downstream factor (38). In addition, several microRNA (miRNAs/miRs) play an important role in altering TGF-β expression levels; for example, upregulated miR-27a expression is closely associated with diabetes, and high glucose-induced upregulation of miR-27a expression activates TGF-β/Smad3 signaling, which contributes to the upregulated changes of CTGF in NRK-52E cells (39). In GBE serum-treated mesangial cells cultured with high glucose medium, Smad2/3 expression decreased, Smad7 expression increased, while Col IV, laminin and TGF-β mRNA levels were decreased (40). TGF-β receptor 1 has been identified as a target of miR-130b. A previous study demonstrated that miR-130b expression was significantly downregulated in mouse glomerular mesangial cells treated with TGF-β. Notably, TGF-β induced miR-130b suppression via nuclear transcription factor Y subunit γ, which subsequently upregulated TGF-β receptor 1 to increase the expression of TGF-β target fibrotic genes (such as PAI-1), along with upregulating profibrotic genes (Col IV α1 and CTGF) in the progression of DN (26).

The present experiments were the first to observe whether GBE treatment has an effect on DN. After obtaining the present results, screening experiments were performed to identify possible effective chemical elements, and it was initially revealed that the total flavonoids in GBE can notably inhibit ECM accumulation, and a study on its possible mechanism will be published in future. Meanwhile, the total lactone sof GBE, including ginkgolide A, B, C, did not significantly inhibit ECM accumulation, and ECM aggregation represents one of the mechanisms of DN. Whether total lactone level has other renal protective mechanisms requires further experiments to elucidate. In future studies, microarray detection will be performed to identify the potential miRNAs that are involved in the regulation of TGF-β by GBE, and to further verify the potential protective mechanism of GBE on DN, which may help to determine novel therapeutic strategies for the prevention and treatment of kidney injury-associated diseases.

In conclusion, the results of the present study demonstrated that GBE decreased tTG expression both in DN rats and rat mesangial cells *in vitro* under high glucose conditions, and GBE may protect rat mesangial cells by inhibiting tTG via modulating TGF-β expression, thus providing a novel strategy for the treatment of DN.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XY and QS conceived and designed the study. JG, HL, YL, JL and YS performed the experiments. XY and QS wrote the manuscript. YZ and XY analyzed the data. YZ reviewed and edited the manuscript. YZ and XY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All the *in vivo* experiments were approved by the Animal Experimental Ethical Inspection Committee of Jilin University School of Pharmaceutical Sciences (ethical permission code, 20190014; Changchun, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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