

Therapeutic effect of sodium-glucose cotransporter 2 inhibitor and benazepril on diabetic nephropathic rats

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Abstract. The present study aimed to compare the therapeutic effect of sodium/glucose cotransporter 2 (SGLT2) inhibitor and benazepril on diabetic nephropathy (DN) rats and provide a potential novel agent for the clinical treatment of DN. The DN model was established on rats. Animals were dosed orally with SGLT2 and benazepril daily for 4 weeks. The pathological state of renal tissues were evaluated using hematoxylin and eosin, Masson and periodic acid-Schiff staining. The change in the morphology of renal tissues was observed through transmission electron microscopy. Western blotting was utilized to determine the expression level of TGF- β , N-terminal fragment of the B-type natriuretic peptide precursor (NT-proBNP) and matrix metalloproteinase-9 (MMP-9). The expression level of endothelin 1 (ET-1), von Willebrand factor (vWF), collagen (col)-I and α smooth muscle actin (α -SMA) in renal tissues was visualized using immunohistochemical assay. Significant pathological changes in the glomerular basement membrane, mesangial membrane, renal tubules, lumen, renal interstitial region and renal tubular epithelial cells were observed in DN rats, accompanied by increased collagen fibers. SGLT2 inhibitor treatment demonstrated more alleviatory effects on the pathological changes of renal tissues compared with benazepril. Compared with control, TGF- β and NT-proBNP were upregulated in DN rats, accompanied by the downregulation of MMP-9, ET-1, vWF, col-I and α -SMA, which were markedly reversed by treatment with SGLT2 inhibitor and benazepril. Compared with benazepril, the effects of SGLT2 inhibitor on the expression level of TGF- β , NT-proBNP, MMP-9, ET-1, vWF, col-I and α -SMA were more significant. Overall, SGLT2 inhibitor demonstrated an increased therapeutic effect against DN rats compared with benazepril by regulating cytokines, renal fibrosis and extracellular matrix degradation.

Introduction

Diabetic nephropathy (DN) is a serious microvascular complication of diabetes, which will contribute to severe renal failure in the advanced stage, posing a great threat to the life and health of patients. Some studies have found that China has the largest diabetes population, with a prevalence rate of ~10.9% among adults, among which the prevalence rate of DN is as high as 33.6% (1,2). The pathogenesis of DN is complex and is closely related to genetic metabolic factors, inflammatory response, oxidative stress, glucose and lipid metabolism, and blood flow (3). At present, symptomatic treatments are available for DN, but they cannot fundamentally reverse the progression of the disease.

Sodium/glucose cotransporter 2 (SGLT2) inhibitors are a new class of oral hypoglycemic agents, which can reduce blood glucose levels, prevent renal injuries, and repress glucose reabsorption in proximal convoluted tubules. In patients with poor blood glucose control, significant hypoglycemic effects have been observed by administering SGLT-2 inhibitors because of the increasing glucose excretion in urine and improving renal hyperfiltration (4). Studies have revealed that (5) renal dysfunction can be alleviated by the SGLT-2 inhibitor by inhibiting the expression level of renal tubular damage markers and neutrophil gelatinase-associated lipocalin, which further represses renal tubular interstitial fibrosis. Furthermore, a reduction of high-glucose-induced o-junction n-acetyl glucosamine glycosylation will be triggered by SGLT-2 inhibitors through the HIF pathway, which further modulates renal tubular response to hypoxia. In recent years, SGLT-2 inhibitors have shown renal protection by improving the high filtration condition, reducing proteinuria, ameliorating renal hypoxia, decreasing weight, suppressing blood pressure, reducing uric acid levels, and alleviating inflammation and oxidative stress, which have been used in the clinical practice. At present, the SGLT2 inhibitors approved in China primarily include empagliflozin, canagliflozin, and dapagliflozin. A large number of clinical tracking data, represented by CANVAS, EMPA-Reg, and DAPA-HF trial, have proven that blood glucose levels, body weights, and blood pressure levels of patients can be significantly repressed by the administration of empagliflozin, canagliflozin, and dapagliflozin (6,7). Furthermore, benazepril is widely reported for the treatment of diabetes. Xue *et al* (8) found that benazepril hydrochloride could alleviate DN by

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reducing the expression of ANGPTL-4, which is considered as an effective drug to treat DN and reduce proteinuria. Benazepril can also improve renal function and reduce renal injury in DN rats (9).

In the present study, DN was established in rats by high-fat diet and injecting streptozotocin (STZ) through the tail vein, followed by observation on the pathological changes in renal tissues. The difference in the expression level of fibrosis and inflammation-related proteins was also determined to compare the therapeutic effect of SGLT2 inhibitor and benazepril on DN rats.

Materials and methods

Establishment of DN model in rats. Thirty-eight-week-old rats were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China), and divided into the control and DN model groups. Animals in the control group were fed with normal diets. Rats in the DN model group were fed with high-fat diets for 8 weeks, followed by intraperitoneal injection with 1% STZ solution. Blood glucose was detected using a glucometer (Roche,) 3, 7 and 14 days after injection. If the blood glucose was all higher than 16.7 mmol/l, then the DN model was successfully established in rats. For *in vivo* experiments, rats were divided into four groups. Animals in the control and DN groups were intraperitoneally injected with normal saline. Rats in the SGLT2 inhibitor and benazepril groups were dosed orally with 1 mg/kg/day of SGLT2 inhibitor (10) and 62.5 mg/kg/day of benazepril for 4 weeks, respectively. Four weeks later, rats were sacrificed by intraperitoneal injection of 1% pentobarbital sodium (45 mg/kg) for follow-up detection. No breathing, no heartbeat, and pupils dilated were used to confirm death following euthanasia with sodium barbitol. Clinical symptoms, such as unmitigable severe pain and incapable of maintaining normal activities or eating on their own, were humane endpoints used in order to determine when the animals should be sacrificed to minimize suffering.

Hematoxylin and eosin (HE) staining. After collecting renal tissues from each animal, they were washed and dehydrated with 70, 80 and 90% ethanol solution, followed by incubation with equal quality of ethanol and xylene for 15 min. Subsequently, the samples were incubated with equal quality of xylene for another 15 min, followed by repeat incubation until the tissues looked transparent. Then, the tissues were embedded in paraffin, sectioned, and stained with HE staining, followed by randomly selecting the images from five fields at 100x magnification, and all phases of follicles and corpora lutea were counted by using an inverted microscope (Olympus).

Masson staining. Sections were stained with Weigert hematoxylin iron for 10 min, followed by differentiation for 5-15 sec using acid ethanol. Slides were stained with Masson blue solution for 5 min, followed by washing and staining with Ponceau dye for 8 min. Sections were washed using a weak acid working solution and then directly stained in aniline blue for 2 min. After quick dehydration using 95% ethanol and 100% ethanol successively, the images were collected using an inverted microscope (Olympus).

Periodic acid-Schiff (PAS) staining. Sections were mixed with oxidants for 15-20 min, washed three times, and added with Schiff reagents, followed exhaust dyeing in the dark for 10-20 min. After two washes using the sodium sulfite solution, sections were stained with Mayer-hematoxylin dyes for 1-2 min. Finally, images were taken using an inverted microscope (Olympus).

Transmission electron microscopy (TEM). Renal tissues were collected and washed using PBS buffer and then fixed in 4.0% glutaraldehyde in PBS overnight. Subsequently, samples were embedded in epoxy resin, and ultrathin sections (50-70 nm) were collected on copper grids. After counterstaining with aqueous uranyl acetate for 1 h, phosphotungstic acid for 1 h, and Reynolds' lead citrate for 20 min successively, the samples were examined using a transmission electron microscope (JEOL).

The sections prepared from tissues were fixed in 2.5% glutaraldehyde PBS (v/v, pH 7.2), post-fixed in 1% osmium tetroxide (v/v), and stained with 4.8% uranyl acetate after dehydration. Subsequently, the sections were washed in propylene oxide and impregnated with epoxy resins and then contrasted with uranyl acetate and lead citrate. Finally, the sections were observed under a transmission electron microscope (JEOL).

Immunohistochemical analysis. Renal tissues isolated from animals were fixed in 2% formaldehyde solution and embedded in paraffin and then cut into 4- μ m-thick sections. The sections were deparaffinated and rehydrated, which were further incubated with 3% H₂O₂ for 15 min. Subsequently, the sections were blocked with 5% BSA in TBST for 30 min, followed by incubating with primary antibody against ET-1 (1:100, Affinity), VWF (1:200, Abcam), col-I (1:200, Proteintech), or α -AMA (1:200, Abcam). After washing, the sections were incubated with HRP-conjugated secondary antibody (CST), and then images were taken using a light microscope (Olympus). Based on the images taken under a microscope, Image J was used to read the IOD (gray value) and area (Area), and finally the IOD/area ratio was used to make histogram display (11).

Western blot assay. After isolating the total proteins from renal tissues, proteins were quantified using a BCA kit (ZIKER), and ~40 μ g of proteins was loaded onto the SDS PAGE, followed by separation for 2 h. Subsequently, the proteins were transferred to the PVDF membrane (Invitrogen; Thermo Fisher Scientific, Inc.), and the membrane was further incubated with 5% BSA. Then, the membrane was incubated with the primary antibody against NT-proBNP (1:100, Affinity), TGF- β (1:1,000, Affinity), MMP-9 (1:1,000, Proteintech), and GAPDH (1:1,000, Affinity), followed by incubation using a secondary antibody (1:2,000, Affinity). Finally, the membrane was exposed to ECL solution, and the bands were visualized and quantified using Image J.

Statistical analysis. The data in the present study were presented by mean \pm SD and analyzed using GraphPad (GraphPad Software, Inc.). The difference among groups was analyzed using one-way ANOVA and Tukey's test. $P < 0.05$ was considered as a significant difference.

All animal experiments involved in this manuscript were approved by the ethical committee of The Third Affiliated Hospital of Nanchang University and carried out in accordance with the guidelines for care and use of laboratory animals and the principles of laboratory animal care and protection.

Results

Establishment of the DN model in rats. After modeling, blood glucose was detected. As shown in Table I, the level of blood glucose in DN rats was significantly elevated compared with control ($^*P<0.05$ vs. control).

Impacts of SGLT2 inhibitor on 24-h urine protein in DN rats. As shown in Table II and Fig. 1, the 24-h urine protein in DN rats was significantly elevated compared with control, which was dramatically suppressed by the administration of SGLT2 inhibitor or benazepril ($^*P<0.05$ vs. control, $^{\#}P<0.05$ vs. model). In addition, compared with the SGLT2 inhibitor group, significantly higher 24-h urine protein was observed in the benazepril group ($^{\Delta}P<0.05$ vs. SGLT2 inhibitor).

SGLT2 inhibitor significantly ameliorated the pathological state of renal tissues in DN rats. Based on the images taken in the HE, Masson, and PAS staining assay, compared with control, the glomerular basement membrane and mesangial membrane thickened; a protein tube was formed, and renal tubular hypertrophy, lumen dilation, diffuse monocytes, and infiltrated cells were observed in DN rats, accompanied with infiltration of focal inflammatory cells in the renal interstitial area, vacuolar degeneration or exfoliation of renal tubular epithelial cells, significant increase of collagen fibers, and positive glycogen reaction (Fig. 2). After the treatment of SGLT2 inhibitor, the lumen and renal tubules became smaller, and the number of protein tubules decreased remarkably. However, in the benazepril group, weak alleviatory effects on the renal injury were observed, accompanied with small amounts of glomerular basement membranes and infiltration of inflammatory factors.

In addition, pathological analysis was performed, and the results are shown in Fig. 2. Scoring was determined in accordance with the following rules: score 0, no specific variation under a light microscope and normal basement membrane under an electron microscope; score 1, slight non-specific changes under a light microscope and thickening of basement membrane under an electron microscope; score 2, higher than 25% of mesangial lengthening and less mesangial growth area compared with capillary area; score 3, at least one nodular sclerosis; score 4, 50% of glomerular progressive diabetic glomerular sclerosis. As shown in Fig. 3A, the HE score in the DN model group increased significantly compared with the control group, which was greatly repressed by the addition of SGLT2 inhibitor. As shown in Fig. 3B, the proportion of collagen fiber area in the DN model group increased significantly compared with the control group, which was greatly reversed by the SGLT2 inhibitor and benazepril. As shown in Fig. 3C, the proportion of PAS-positive area and glomerular area in the glomerular matrix of the DN model group increased significantly compared with the control group, which were dramatically

Table I. Level of blood glucose in rats.

Groups	N	Time point	Blood glucose (mmol/l)
Control	4	3 weeks after STZ injection	6.28±0.38
		Before sacrifice	8.27±2.35
Model	12	3 weeks after STZ injection	24.23±2.71 ^a
		Before sacrifice	23.45±3.71 ^a

^a $P<0.05$ vs. control at the same timepoint. STZ, streptozotocin.

Table II. Level of 24-h urine protein in rats.

Groups	N	Urine protein (mg/24 h)
Control	4	5.68±1.58
Model	4	38.30±7.49 ^a
SGLT2 Inhibitor	4	12.04±2.38 ^b
Benazepril	4	19.49±2.74 ^{b,c}

^a $P<0.05$ vs. Control; ^b $P<0.05$ vs. Model; ^c $P<0.05$ vs. SGLT2 inhibitor. SGLT2, sodium/glucose cotransporter 2.

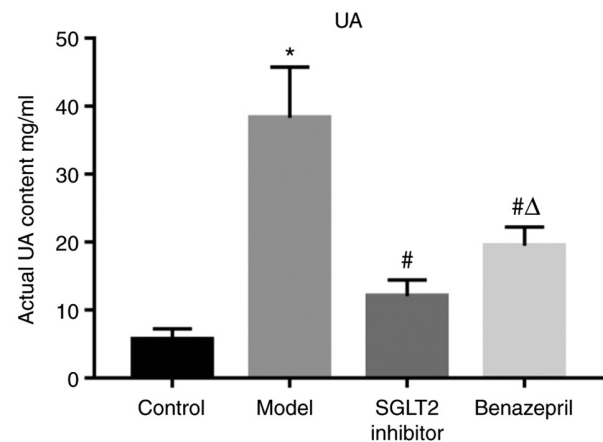


Figure 1. Level of urine protein in rats at 24 h after administration. $^*P<0.05$ vs. control; $^{\#}P<0.05$ vs. model; $^{\Delta}P<0.05$ vs. SGLT2 inhibitor. UA, urea; SGLT2, sodium/glucose cotransporter 2.

reversed by the SGLT2 inhibitor and benazepril ($^*P<0.05$ vs. control; $^{\#}P<0.05$ vs. model).

Impact of SGLT2 inhibitor on the morphology of renal tissues in DN rats. The ultrastructure of renal tissues is shown in Fig. 4. Compared with control, the epithelial cells of renal tubules in DN rats were damaged; the deposition of lumps of electron density in endothelial cells and basal membrane was observed, and the capillary lumen in the nephron was blocked. In addition, the severe injury on the mitochondria was induced, which was characterized by swelling, deformation, vacuolation, disorder, and blurred or disappeared mitochondrial crest. The increased endoplasmic reticulum phagocytes, enlarged filtration

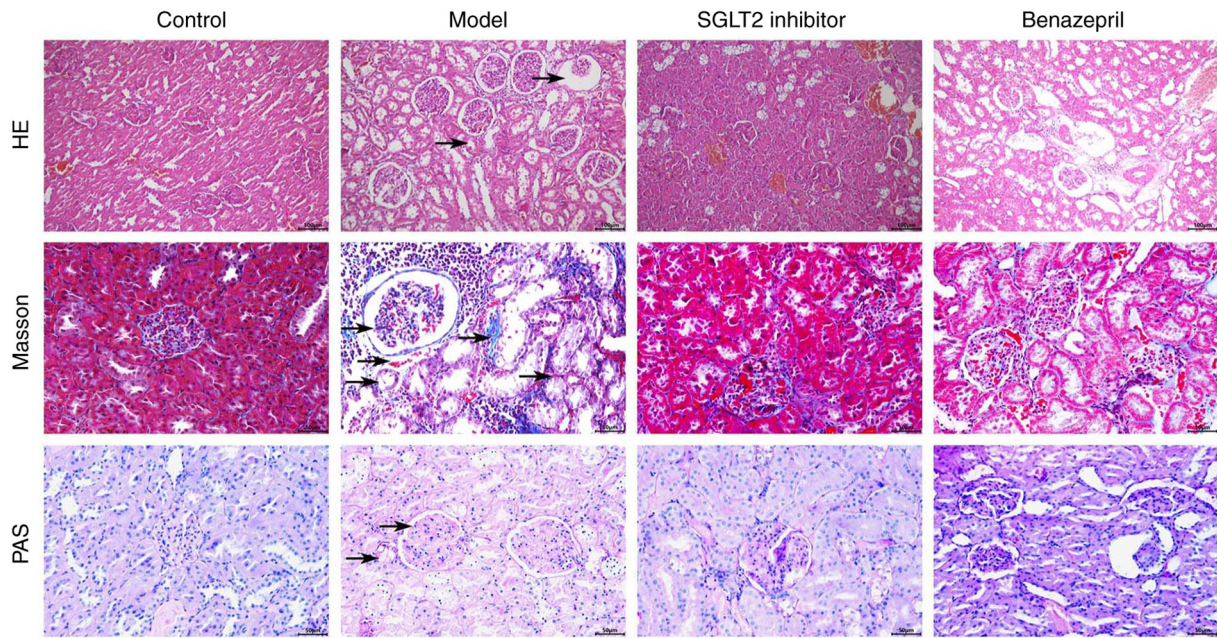


Figure 2. Images of HE (magnification, x200), Masson (magnification, x400) and PAS (magnification, x400) assays were captured to evaluate the pathological changes in renal tissues. HE, hematoxylin and eosin; SGLT2, sodium/glucose cotransporter 2; PAS, periodic acid-Schiff.

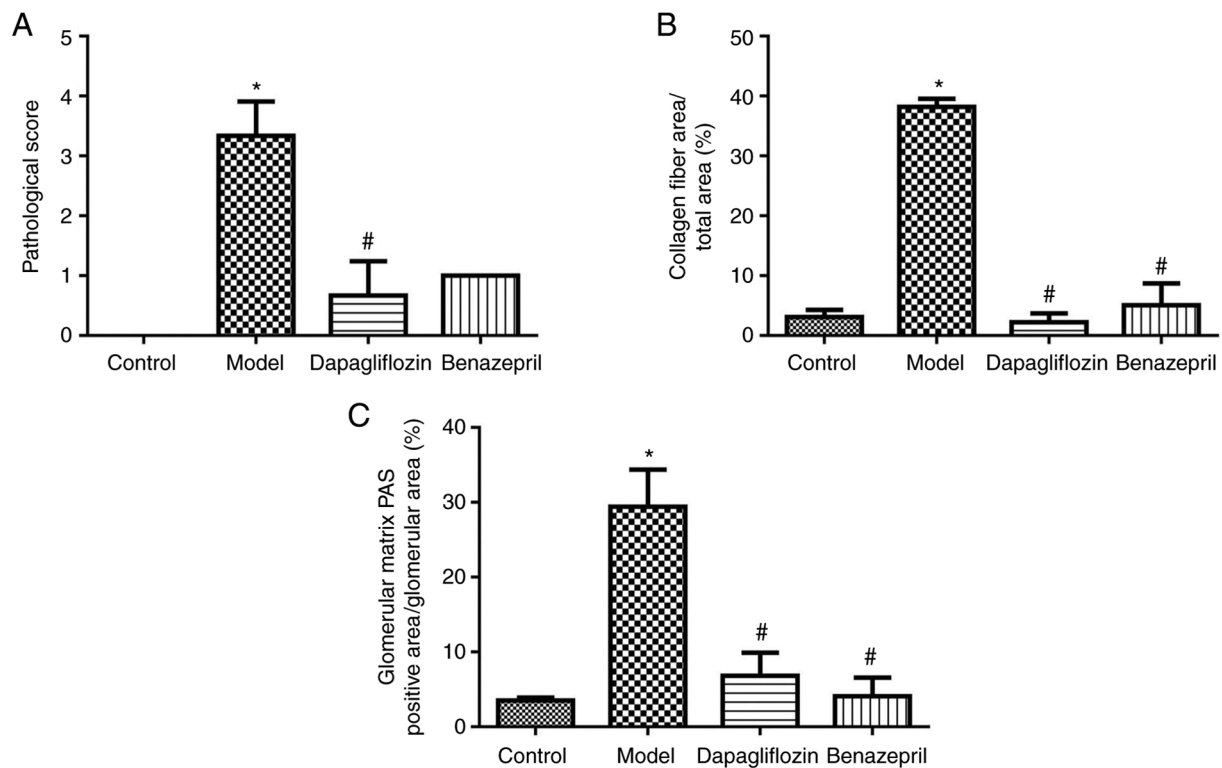


Figure 3. Pathological analysis on rat renal tissues were measured using (A) HE scoring, (B) Masson scoring and (C) PAS scoring. * $P < 0.05$ vs. control; # $P < 0.05$ vs. model. HE, hematoxylin and eosin; SGLT2, sodium/glucose cotransporter 2; PAS, periodic acid-Schiff.

membrane space, and deciduous basal membrane and endothelial cells were also observed. After the treatment of SGLT2 inhibitor, the pathological state was significantly alleviated, and the deposition in the basal membranes and endothelial cells disappeared. In addition, the deformation and vacuolation in the mitochondria were not observed. However, after the treatment of benazepril, the alleviated effect was not significant.

Impact of SGLT2 inhibitor on the expression level of TGF- β , NT-proBNP, and MMP-9 in renal tissues of DN rats. As shown in Fig. 5, the expression level of TGF- β and NT-proBNP was significantly promoted, and the expression level of MMP-9 remarkably declined in DN rats compared with control (* $P < 0.05$ vs. control). After the treatment of SGLT2 inhibitor and benazepril, TGF- β and NT-proBNP were significantly downregulated,

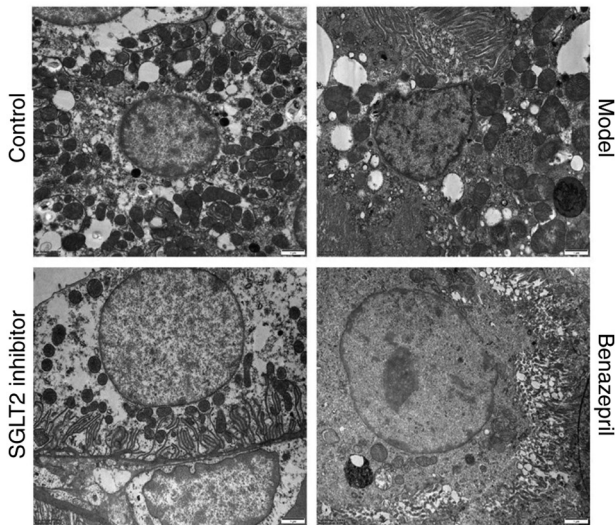


Figure 4. Change of morphology in renal tissues was observed using transmission electron microscopy (magnification, x9300). SGLT2, sodium/glucose cotransporter 2.

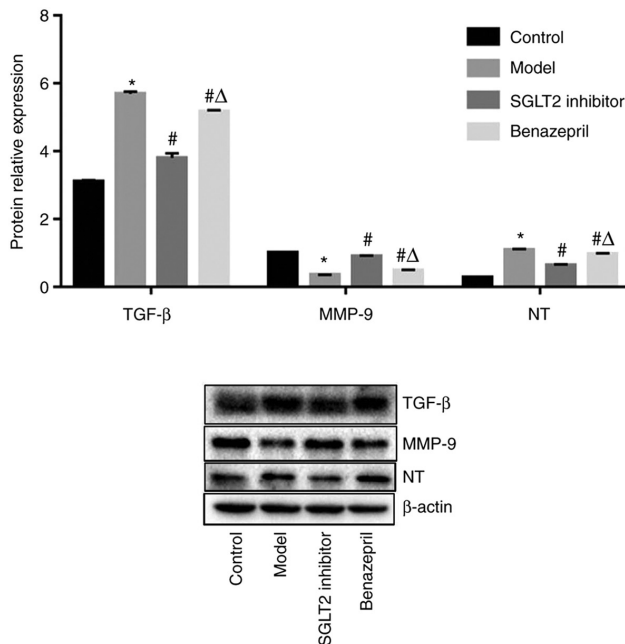


Figure 5. Expression level of TGF-β, NT-proBNP and MMP-9 in renal tissues was determined using western blotting. *P<0.05 vs. control; #P<0.05 vs. model; ΔP<0.05 vs. SGLT2 inhibitor. NT-proBNP, N-terminal fragment of the B-type natriuretic peptide precursor; MMP-9, metalloproteinase-9; NT, N-terminal; SGLT2, sodium/glucose cotransporter 2.

and MMP-9 was significantly upregulated (#P<0.05 vs. model). However, compared with SGLT2 inhibitor, the impact of benazepril on the expression level of TGF-β, NT-proBNP, and MMP-9 was less significant (ΔP<0.05 vs. SGLT2 inhibitor).

SGLT2 inhibitor significantly downregulated the expression level of ET-1, vWF, col-I, and α-SMA. As shown in Fig. 6, ET-1 was distributed in the glomeruli and tubules. However, α-SMA and col-I were primarily expressed in the basal membrane of tubular cells and partly distributed in the mesangial area of the glomerulus. In addition, vWF was highly expressed

in renal tubular epithelial cells and in the renal interstitium and partly distributed in endothelial cells and mesangial cells of the glomerulus. Compared with control, ET-1, vWF, col-I, and α-SMA were significantly upregulated in DN rats, which were dramatically downregulated by the treatment of SGLT2 inhibitor or benazepril (*P<0.05 vs. control, #P<0.05 vs. model). However, compared with the SGLT2 inhibitor group, significantly high expression levels of ET-1, vWF, col-I, and α-SMA were observed in the benazepril group (ΔP<0.05 vs. SGLT2 inhibitor).

Discussion

Similar to other diabetic microvascular complications, the pathogenesis and process of DN have not been fully elucidated, whereas hyperglycemia is the key factor leading to the onset of DN. At present, DN animal models are established on the basis of a diabetes model. With disease progression, the established diabetes model will gradually develop into an appropriate DN model, and the establishment of an appropriate DN model is the basis for studying the pathogenesis of DN. At present, the commonly used method to establish a DM model is the injection of STZ. The standards for DN modeling using STZ are different. In the present study, the methods for modeling and identification are referred to the description reported previously (12). Animals were fed with high-fat diets for 8 weeks, followed by intraperitoneal injection with 1% STZ solution to establish the DN model. The results indicated that the average blood glucose level within 3 weeks after the injection of STZ was higher than 16.7 mmol/l, and the 24 h urine protein level was higher than 30 mg, which indicated that the DN model was successfully established.

DN is a common complication of diabetes. In the early stage of DN, the development of proteinuria will be induced by renal enlargement and damage of glomerular filtration function (13). Some studies have shown that SGLT2 inhibitors can improve the glomerular filtration rate of patients with type 2 diabetes, thereby reducing the occurrence of glomerular toxicity (14). In the present study, 24 h urinary protein levels decreased significantly after treatment with SGLT2 inhibitors and benazepril.

DN is a common complication of diabetes, which is primarily characterized by microcirculation disturbance, micro-hemangioma formation, and microvascular basement membrane thickening. At the early stage, the primary renal pathological changes of DN include renal hypertrophy, mesangial area dilation, and glomerular basement membrane thickening. Wang reported that (15) 1 week after STZ injection, increased tubular lumen and mild necrosis of tubular epithelial cells were observed in the DN group using the HE staining assay because of significant renal toxicity. Extensive tubular vacuolation was observed at the 12th week. In the present study, we found that the glomerular volume in DN rats was significantly larger than that in normal rats, accompanied by the thickened basal membrane of glomerular capillaries, widened mesangial area, increased collagen fibers, and positive PAS staining on the mesangial matrix. These observations indicated that a certain renal damage was induced in DN rats. In addition, based on the TEM results, in renal tissues of DN rats, epithelial cells in renal tubules were

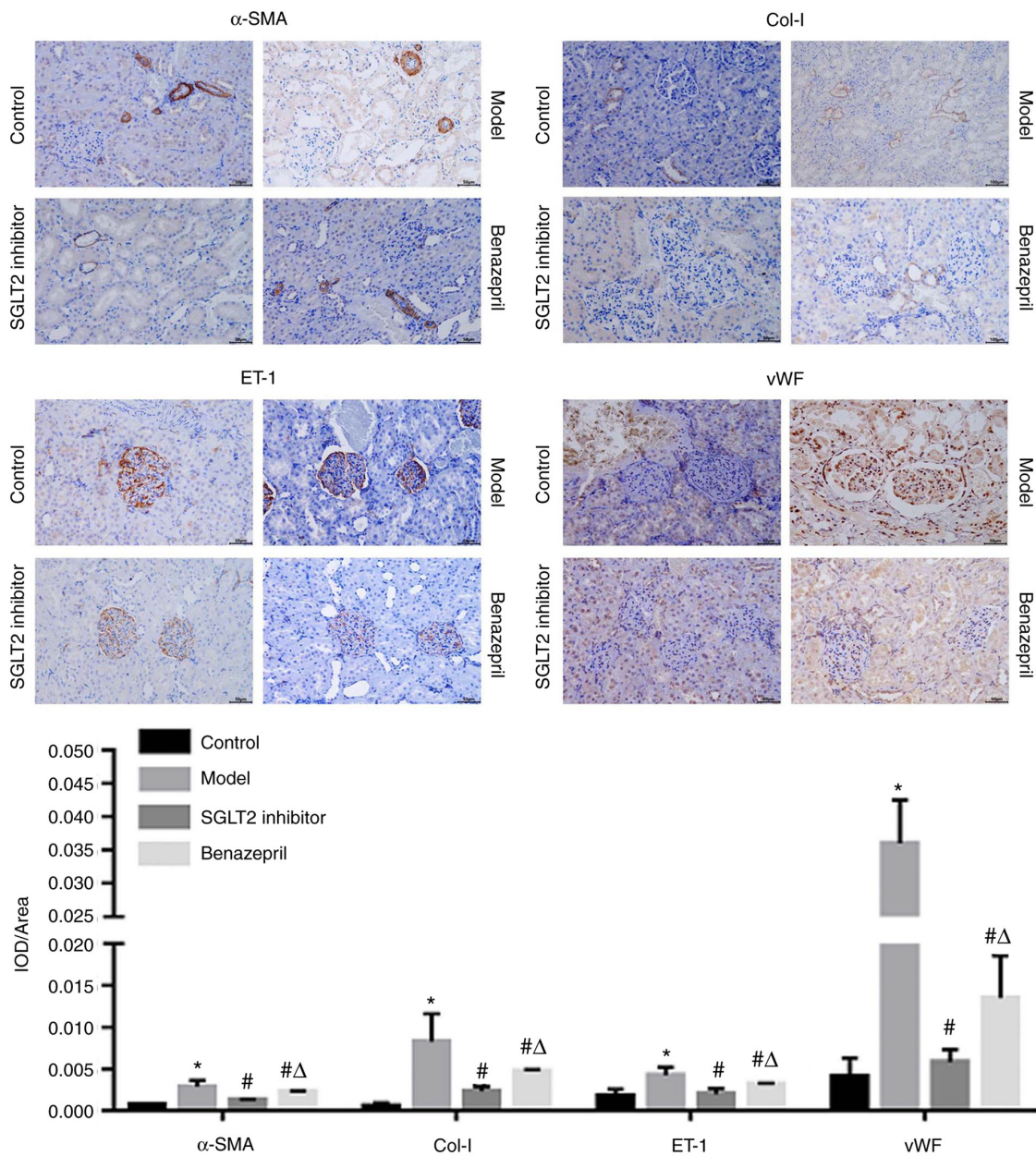


Figure 6. Expression levels of ET-1, vWF, col-I and α-SMA in renal tissues were determined using immunohistochemical assays. * $P < 0.05$ vs. control; # $P < 0.05$ vs. model; Δ $P < 0.05$ vs. SGLT2 inhibitor. ET-1, endothelin 1; vWF, von Willebrand factor; col-I; α-SMA, α smooth muscle actin; Col, collagen; SGLT2, sodium/glucose cotransporter 2; IOD, integrated optical density.

damaged; endothelial cells and basal membrane were deposited; the capillary lumen in renal unit was blocked, and the basal membrane and endothelial cells were shed. After the treatment of SGLT2 inhibitor and benazepril, the pathological state in DN rats was significantly alleviated. Moreover, the therapeutic effect of SGLT2 inhibitor was superior to that of benazepril, which provided a reliable pathological basis for replacing benazepril with SGLT2 inhibitor for the clinical treatment of DN.

In the mechanism study of DN, significantly induced macrophage accumulation and activation could be induced by high glucose levels, which further trigger the glomerular immune complex deposition and increase the production of chemokines and inflammatory factors. Furthermore, excessive release of ROS will be induced by renal hemodynamics,

disordered renal metabolic pathway, and oxidative stress, which further stimulates the opening of related signaling pathways, resulting in declined degradation of the extracellular matrix (ECM) and a large accumulation of ECM in the kidney. Consequently, renal fibrosis and renal dysfunction will be induced (16).

TGF-β1 is involved in glomerular and tubulointerstitial damage during the development of DN by inducing the synthesis of ECM in epithelial cells, mesangial cells, proximal convoluted tubular epithelial cells, and fibroblasts. In the early stage of DN, the expression level of TGF-β1 is significantly elevated. The blood glucose and expression level of TGF-β1 is positively correlated (17). In the present study, the upregulation of TGF-β1 was accompanied by the elevation of blood glucose levels. In addition, the development of renal

fibrosis and DN is mediated by TGF- β 1 (18). Compared with DN rats, TGF- β 1 was significantly downregulated by the treatment of SGLT inhibitor, indicating that the SGLT inhibitor might alleviate renal fibrosis by regulating TGF- β 1, which finally contributed to the alleviation of DN symptoms.

Proliferation will be facilitated, and the trans differentiation of mesangial cells, renal tubular epithelial cells, fibroblasts, and pericellular cells into myofibroblasts (MFB) will be stimulated. In addition, the expression of α -SMA will be induced by TGF- β 1, the expression level of which is used to indirectly reflect the degree of renal fibrosis. Pathological secretion of collagens (primarily Col I and Col III) can be observed in MFB, which further contributes to the excessive deposition of ECM. Studies have found that the renal cell phenotypic transformation, secretion of ECM, and inflammatory response induced by high glucose levels can be repressed by salvianolic acid B, which further alleviates renal blood flow (18). In the present study, based on the results of the immunohistochemical assay, α -SMA and Col I were highly expressed in the mesangial area of the glomerulus in DN rats, particularly in vessels. After treatment of SGLT2 inhibitor and benazepril, the expression of α -SMA and Col I was significantly decreased. Matrix metalloproteinase (MMP) is a zinc- and calcium-dependent proteolytic enzyme that degrades ECM. The expression level of the tissue inhibitor of metalloproteinase and plasminogen activator inhibitor will be facilitated by TGF- β 1 by inhibiting the degradation of ECM against MMPs. In the MMP family, MMP-9 is an important proteolytic enzyme (19). In the present study, deposition was found in endothelial cells and basal membranes, and collagen fibers were significantly increased in DN rats. The expression level of MMP-9 was significantly declined. After the treatment of SGLT2 inhibitor and benazepril, MMP-9 was significantly upregulated, and the number of collagen fibers was decreased, indicating that the therapeutic effect of SGLT2 inhibitor and benazepril on DN might be related to the degradation of ECM mediated by MMP-9.

NT-proBNP is a neuroendocrine hormone secreted by ventricular muscles primarily metabolized by the kidney. In addition, NT-proBNP is closely associated with the development of microangiopathy. DN is an important complication of microangiopathy during the development of diabetes (20,21). Studies have shown that NT-proBNP is involved in kidney diseases. NT-proBNP expands vascular diuretic sodium discharge and inhibits the sympathetic nerve and renin-angiotensin-aldosterone system, which is a potential indicator of the glomerular overpass rate by alleviating the declined glomerular overpass rate to delay the progression of chronic kidney disease (22). In the present study, we found that NT-proBNP was significantly upregulated in DN rats, which was consistent with previous reports (23).

Endothelin-1 (ET-1), encoded by the EDN1 gene, is primarily expressed in glomerular endothelial cells (GENs), and it plays an important role in DN (24). ET-1 acts by binding to endothelin receptor subtypes, endothelin A receptor (ETAR), and endothelin B receptor (ETBR). In ETBR^{-/-} GENs exposed to high glucose levels, suppression of ET-1 binding to ETBR activated the NF- κ B pathway to secrete large amount of ET-1. Given the communication between GENs and mesangial cells in diabetes, ET-1 binding

to ETAR in mesangial cells promoted the RhoA/ROCK pathway, thereby accelerating mesangial cell proliferation and ECM accumulation (25). Studies have shown that vascular endothelial injury plays an important role in the development of DN (26). Von Willebrand factor (vWF) is a glycoprotein that plays an important role in platelet formation. The lack of vWF will lead to von Willebrand disease and result in bleeding, whereas the increased expression level of vWF will create an environment that promotes thrombosis. Increased serum vWF level indicates the vascular endothelial cell injury and blood hypercoagulability (27). Some studies have found that increased vWF in patients is related to DN-related arteriosclerosis and blood hypercoagulability, and reducing the vWF level significantly improves patients' renal function (28). In the present study, in DN rats, significant injuries in endothelial cells and renal tubular epithelial cells were observed. Compared with control, the expression level of vWF in DN rats was significantly promoted. After the treatment of SGLT2 inhibitor and benazepril, the expression level of vWF was significantly suppressed, indicating that the therapeutic effect of SGLT2 inhibitor and benazepril on DN might be related to the downregulation of vWF.

To confirm whether side effects could be induced in normal mice by the administration of SGLT2 inhibitor. UA content in 24 h and HE staining were performed between the control rats and SGLT2 inhibitor treated normal rats. As shown in Fig. S1A, no significant difference on the actual UA content was observed between the control and the SGLT2 inhibitor treated normal rats. Furthermore, in SGLT2 inhibitor treated normal rats, the glomerular basement membrane was normal and the structure of the renal tubule was regular, with rare infiltration of inflammatory cells (Fig. S1B), indicating that SGLT2 inhibitor did not induce any pathological changes on renal tissues of normal rats. In addition, it is widely reported (29-31) that the risk of hypoglycemia cannot be induced by the treatment of SGLT2 inhibitor.

In the present study, DN was successfully established in rats by STZ. Western Blot and immunohistochemistry experiments showed that SGLT2 inhibitor and benazepril reduced the expression of α -SMA and Col I by inhibiting TGF- β 1. In addition, the expression of MMP-9 was upregulated, which inhibited the accumulation of ECM and improved renal fibrosis and interstitial fibrosis. Furthermore, the vascular endothelial injury was repaired, and blood flow dynamics was regulated by SGLT2 inhibitors and benazepril by reducing the protein expression levels of ET-1, vWF, and NT-proBNP, which finally achieved the effect of improving vascular lesions. As a SGLT2 inhibitor, dapagliflozin improved renal pathology and morphology of rats by reducing urinary protein content. The pathogenesis of DN is complex. Therefore, an in-depth study on the renal protective mechanism of SGLT2 inhibitors will provide a solid foundation for personalized drug usage and precision therapy. Collectively, SGLT2 inhibitors have demonstrated renal protective ability in clinical and basic studies, and their application will change the treatment of DN, whereas studying the renal protective mechanism of SGLT2 inhibitors will provide the possibility for the wide application of SGLT2 inhibitors in other nephropathy. The present study provides preliminary findings on the molecular mechanism of SGLT2 inhibitors, particularly dapagliflozin, in the treatment of DN.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LH and FL confirm the authenticity of all the raw data. LH conceived and designed the study. YaZ and FL performed the experiments. JH and HC analyzed and interpreted the data. CC, DX and YiZ performed the statistical analysis. LH drafted the manuscript. FL revised the manuscript for important intellectual content. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments involved in this manuscript were approved by the Ethical Committee of The Third Affiliated Hospital of Nanchang University (approval no. 2020031701) and carried out in accordance with the guidelines for care and use of laboratory animals and the principles of laboratory animal care and protection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Weng JP and Bi Y: Epidemiological status of chronic diabetic complications in China. *Chin Med J (Engl)* 128: 3267-3269, 2015.
- Wang L, Gao P, Zhang M, Huang Z, Zhang D, Deng Q, Li Y, Zhao Z, Qin X, Jin D, *et al*: Prevalence and ethnic pattern of diabetes and prediabetes in China in 2013. *JAMA* 317: 2515-2523, 2017.
- Cho NH, Shaw JE, Karuranga S, Huang Y, da Rocha Fernandes JD, Ohlrogge AW and Malanda B: IDF diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Res Clin Pract* 138: 271-281, 2018.
- Zaccardi F, Webb DR, Htike ZZ, Youssef D, Khunti K and Davies MJ: Efficacy and safety of sodium-glucose co-transporter-2 inhibitors in type 2 diabetes mellitus: Systematic review and network meta-analysis. *Diabetes Obes Metab* 18: 783-794, 2016.
- Hodrea J, Balogh DB, Hosszu A, Lenart L, Besztercei B, Koszegi S, Sparding N, Genovese F, Wagner LJ, Szabo AJ and Fekete A: Reduced O-GlcNAcylation and tubular hypoxia contribute to the antifibrotic effect of SGLT2 inhibitor dapagliflozin in the diabetic kidney. *Am J Physiol Renal Physiol* 318: F1017-F1029, 2020.
- Rådholm K, Figtree G, Perkovic V, Solomon SD, Mahaffey KW, de Zeeuw D, Fulcher G, Barrett TD, Shaw W, Desai M, *et al*: Canagliflozin and heart failure in type 2 diabetes mellitus: Results from the CANVAS program. *Circulation* 138: 458-468, 2018.
- Sattar N, McLaren J, Kristensen SL, Preiss D and McMurray JJ: SGLT2 inhibition and cardiovascular events: Why did EMPA-REG outcomes surprise and what were the likely mechanisms? *Diabetologia* 59: 1333-1339, 2016.
- Xue L, Feng X, Wang C, Zhang X, Sun W and Yu K: Benazepril hydrochloride improves diabetic nephropathy and decreases proteinuria by decreasing ANGPTL-4 expression. *BMC Nephrol* 18: 307, 2017.
- Li H, Wang Y, Zhou Z, Tian F, Yang H and Yan J: Combination of leflunomide and benazepril reduces renal injury of diabetic nephropathy rats and inhibits high-glucose induced cell apoptosis through regulation of NF- κ B, TGF- β and TRPC6. *Ren Fail* 41: 899-906, 2019.
- Arab HH, Al-Shorbagy MY and Saad MA: Activation of autophagy and suppression of apoptosis by dapagliflozin attenuates experimental inflammatory bowel disease in rats: Targeting AMPK/mTOR, HMGB1/RAGE and Nrf2/HO-1 pathways. *Chem Biol Interact* 335: 109368, 2021.
- Lin L, Hou G, Han D, Yin Y, Kang J and Wang Q: Ursolic acid alleviates airway-vessel remodeling and muscle consumption in cigarette smoke-induced emphysema rats. *BMC Pulm Med* 19: 103, 2019.
- Yang Y, Zhang Z, Su K, Chen Z and Huang S: Methodological study of streptozotocin-induced diabetic nephropathy model in rats. *West China Medicine* 20: 299-300, 2005.
- Chang TT and Chen JW: The role of chemokines and chemokine receptors in diabetic nephropathy. *Int J Mol Sci* 21: 3172, 2020.
- Scheen AJ: SGLT2 inhibitor empagliflozin reduces renal outcomes and dampens the progressive reduction in glomerular filtration rate in patients with type 2 diabetes and antecedents of cardiovascular disease. *Evid Based Med* 22: 69-70, 2017.
- Wang W, Li Z, Chen Y, Wu H, Zhang S and Chen X: Prediction value of serum NGAL in the diagnosis and prognosis of experimental acute and chronic kidney injuries. *Biomolecules* 10: 981, 2020.
- Sifuentes-Franco S, Padilla-Tejeda DE, Carrillo-Ibarra S and Miranda-Díaz AG: Oxidative stress, apoptosis, and mitochondrial function in diabetic nephropathy. *Int J Endocrinol* 2018: 1875870, 2018.
- Garcia FA, Rebouças JF, Balbino TQ, da Silva TG, de Carvalho-Júnior CH, Cerqueira GS, Brito GA and Viana GS: Pentoxifylline reduces the inflammatory process in diabetic rats: Relationship with decreases of pro-inflammatory cytokines and inducible nitric oxide synthase. *J Inflamm (Lond)* 12: 33, 2015.
- Navarro-González JF, Mora-Fernández C, Muros de Fuentes M, Chahin J, Méndez ML, Gallego E, Macía M, del Castillo N, Rivero A, Getino MA, *et al*: Effect of pentoxifylline on renal function and urinary albumin excretion in patients with diabetic kidney disease: The PREDIAN trial. *J Am Soc Nephrol* 26: 220-229, 2015.
- Dimas GG, Didangelos TP and Grekas DM: Matrix gelatinases in atherosclerosis and diabetic nephropathy: Progress and challenges. *Curr Vasc Pharmacol* 15: 557-565, 2017.
- Lu R and Liang G: Changes and significance of serum homocysteine, uric acid and blood lipid levels in patients with chronic heart failure. *Hebei Med J* 39: 2113-2116, 2017.
- Zhao Huihui XD: The value of NT-proBNP in the evaluation of curative effect and prognosis of patients with heart failure. *Modern Instruments Med* 21: 101-102, 2015.
- Chang LH, Hwu CM, Chu CH, Lin YC, Huang CC, You JY, Chen HS and Lin LY: The combination of soluble tumor necrosis factor receptor type 1 and fibroblast growth factor 21 exhibits better prediction of renal outcomes in patients with type 2 diabetes mellitus. *J Endocrinol Invest* 44: 2609-2619, 2021.
- Svensson M, Gorst-Rasmussen A, Schmidt EB, Jorgensen KA and Christensen JH: NT-pro-BNP is an independent predictor of mortality in patients with end-stage renal disease. *Clin Nephrol* 71: 380-386, 2009.
- Gagliardini E, Zoja C and Benigni A: Et and diabetic nephropathy: Preclinical and clinical studies. *Semin Nephrol* 35: 188-196, 2015.
- Zou HH, Wang L, Zheng XX, Xu GS and Shen Y: Endothelial cells secreted endothelin-1 augments diabetic nephropathy via inducing extracellular matrix accumulation of mesangial cells in ETBR(-/-) mice. *Aging (Albany NY)* 11: 1804-1820, 2019.
- Karasek D, Spurna J, Kubickova V, Krystynik O, Cibickova L, Schovanek J and Goldmannova D: Association of pigment epithelium derived factor with von Willebrand factor and plasminogen activator inhibitor 1 in patients with type 2 diabetes. *Physiol Res* 68: 409-418, 2019.

27. Favalaro EJ, Henry BM and Lippi G: Increased VWF and decreased ADAMTS-13 in COVID-19: Creating a milieu for (micro)thrombosis. *Semin Thromb Hemost* 47: 400-418, 2021.
28. Tang S, Wang X, Deng T, Ge H and Xiao X: Identification of C3 as a therapeutic target for diabetic nephropathy by bioinformatics analysis. *Sci Rep* 10: 13468, 2020.
29. Ferrannini E, Seman L, Seewaldt-Becker E, Hantel S, Pinnetti S and Woerle HJ: A phase IIb, randomized, placebo-controlled study of the SGLT2 inhibitor empagliflozin in patients with type 2 diabetes. *Diabetes Obes Metab* 15: 721-728, 2013.
30. Stenlöf K, Cefalu WT, Kim KA, Alba M, Usiskin K, Tong C, Canovatchel W and Meininger G: Efficacy and safety of canagliflozin monotherapy in subjects with type 2 diabetes mellitus inadequately controlled with diet and exercise. *Diabetes Obes Metab* 15: 372-382, 2013.
31. Ferrannini E, Ramos SJ, Salsali A, Tang W and List JF: Dapagliflozin monotherapy in type 2 diabetic patients with inadequate glycemic control by diet and exercise: A randomized, double-blind, placebo-controlled, phase 3 trial. *Diabetes Care* 33: 2217-2224, 2010.