Jujuboside A ameliorates tubulointerstitial fibrosis in diabetic mice through down-regulating the YY1/TGF-β1 signaling pathway

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[ABSTRACT] Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus, which is characterized in renal tubulointerstitial fibrosis (TIF). The current study was designed to investigate the protective effect of Jujuboside A (Ju A) on TIF in type 2 diabetes (T2DM) mice, and explore its underlying anti-fibrosis mechanism. A mouse T2DM model was established using high fat diet (HFD) feeding combined with intraperitoneal injection of streptozotocin (STZ). Then, diabetic mice were treated with Ju A (10, 20 and 40 mg·kg−1·d−1, i.g.) for 12 weeks. Results showed that administration of Ju A not only down-regulated fasting blood glucose (FBG) levels, but also improved hyperlipidemia and renal function in diabetic mice. Moreover, the reduced ECM accumulation was observed in the renal cortex of Ju A treated diabetic mice, while the TIF progression was also attenuated by Ju A through blocking the epithelial-to-mesenchymal transition (EMT) of renal tubular epithelial cells (RTECs). Further mechanism studies showed that Ju A treatment effectively down-regulated the protein expression and subsequent nuclear translocation of Yin Yang 1 (YY1) in the renal cortex of diabetic mice, and reduced the levels of transforming growth factor-β1 (TGF-β1) in the serum and renal cortex of Ju A treated mice. According to in vitro studies, the up-regulated YY1/TGF-β1 signaling pathway was restored by Ju A in high glucose (HG) cultured HK-2 cells. Taken together, these findings demonstrated that Ju A can ameliorate the TIF of DN through down-regulating the YY1/TGF-β1 signaling pathway.

[KEY WORDS] Diabetic nephropathy; Jujuboside A; Tubulointerstitial fibrosis; Extracellular matrix; YY1/TGF-β1 signaling pathway


Introduction

Diabetic nephropathy (DN) is one of the most common complications of diabetes. About 30%~40% of type 1 and 20% of type 2 diabetes (T2DM) patients will eventually develop into DN [1]. The main pathological features of DN are glomerular hypertrophy, thickening of the glomerular basement membrane (GBM), extracellular matrix (ECM) accumulation, and renal fibrosis [2]. In particular, renal fibrosis is an irreversible characteristic of DN, which has been significantly observed in the glomerulus and tubulointerstitium of advanced DN [3, 4]. Currently, most of the clinical treatment strategies for DN focus on hypoglycemia, with few or almost no drugs to alleviate renal fibrosis. Therefore, it is of great importance to explore the underlying mechanism of renal fibrosis, and develop new drugs to inhibit renal fibrosis and delay the progression to DN.

Recent studies have indicated the important role of the glomerulus, particularly the mesangium, in the pathological process of DN [5]. Furthermore, increasing attention has been
drawn on the pathological mechanism of glomerular fibrosis in the process of DN \[^6\]. It was reported that glomerular fibrosis appeared early in the progressive stage of nephropathy \[^7\]. As one of the important underlying pathologies of renal fibrosis, tubulointerstitial fibrosis (TIF) is an end-stage pathological process of DN, with a closely relationship with impairment of renal function \[^8\]. Currently, a large number of studies have demonstrated that YY1 is a key factor in the pathogenesis of DN \[^9\]. Thus, while developing hypoglycemic agents, attenuating TIF is an adjuvant strategy for DN treatment.

Yin Yang 1 (YY1), a nuclear transcription factor with dual transcriptional activity, is widely distributed in eukaryotic cells, and plays an important role in regulating cell proliferation, differentiation, apoptosis and mammalian embryo development \[^10\]. YY1 was found to be involved in the regulation of cellular metabolisms, including glycol-metabolism, lipid metabolism, and bile acid metabolism, which are closely associated with metabolic-related diseases \[^11\]. Recent studies have suggested that YY1 is a novel profibrotic mediator in multiple diseases (such as interstitial pulmonary fibrosis, cancer, liver fibrosis, and cardiac fibrosis) via various signaling pathways \[^12-19\]. Furthermore, YY1 participated in chronic renal failure through regulating renal tubular epithelial cells (RTECs), mesangial cells and podocytes \[^16-18\]. It is well-known that the epithelial-mesenchymal transition (EMT) of RTECs is one of the potential mechanisms of TIF, with the characteristics of loss of epithelial proteins (such as E-cadherin and ZO-1) and acquisition of mesenchymal proteins (such as vimentin and α-SMA) \[^19\]. According to recent studies, YY1 acted as a novel therapeutic target for DN-related renal fibrosis through dysregulating EMT-associated proteins \[^16, 20\]. Hyperglycemia can lead to over-secretion of transforming growth factor-β1 (TGF-β1), which is considered as an important promoter of DN-related fibrosis \[^21, 22\]. It was reported that YY1 over-expression was correlated with TGF-β1 mRNA levels in human brain gliomas, and YY1 was closely related to EMT and pro-fibrogenesis induced by TGF-β1 in alveolar epithelial cells \[^23, 24\]. Moreover, another study showed that YY1 was a potent transcriptional regulator of TGF-β1 in human renal mesangial cells \[^25\]. However, the role of the YY1/TGF-β1 signaling pathway in TIF induced by T2DM remained largely unknown.

Given the findings mentioned above, we hypothesized that chronic persistent hyperglycemia exposure may lead to up-regulated YY1 expression, which then promotes EMT process through increasing TGF-β1 levels, leading to TIF in DN. Compounds that down-regulate the levels of blood glucose may inactivate the YY1/TGF-β1 signaling pathway, and the EMT process of RTECs, resulting in the delayed progression of DN-related TIF.

Jujuboside A (Ju A) is a triterpene saponin isolated from Semen Ziziphi Spinosae, and exerts a wide range of pharmacological activity, including anti-oxidant, anti-inflammatory, anti-apoptotic, anti-proliferative and neuro-protective effect, which are closely related with the pathogenesis of DN \[^25-28\]. Reports showed that Ju A ameliorated DN through suppressing oxidative stress and apoptosis, and enhancing autophagy \[^29\]. In the present study, Ju A exerted significant anti-hyperglycemic effect on diabetic mice, while remarkably improved dyslipidemia. Ju A also effectively restored renal function, attenuated morphological changes and reduced collagen accumulation in the renal cortex of diabetic mice. Further mechanism studies indicated that Ju A ameliorated the TIF of DN partly through down-regulating the YY1/TGF-β1 signaling pathway, so as to block the EMT of RTECs during DN.

**Methods and Materials**

**Materials and reagents**

Jujuboside A standard (YM-Y0334) was purchased from Shanghai YuanMu Biological Technology Co., Ltd. (Shanghai, China). Streptozocin (T1507) was obtained from Shanghai TopScience Biological Technology Co., Ltd. (Shanghai, China).

Primary antibodies against E-cadherin (3195, 3195S), vimentin (5741), and snail (3879) were from Cell Signaling Technology (MA, USA). Antibodies against YY1 (ab109228, 22156-1-AP), twist (ab175430), α-SMA (ab32575, AF1032), lamin B (ab133741), laminin (ab11575) and collagen IV (ab6586, AF0510) were from Abcam (CA, USA) or/and Affinity Biosciences (OH, USA). Antibody against TGF-β1 (AF1027) was from Affinity Biosciences (OH, USA). Antibody against β-actin (AP0060) was from Bioworld (St. Louis, USA). Donkey anti-rabbit antibody (DyLight® 594) was purchased from EarthOx Life Sciences (CA, USA). RIPA lysis buffer was purchased from Beyotime (Nanjing, China). IRDye® 40 lysis buffer was purchased from Beyotime (Nanjing, China). IRDye® 800CW goat anti-mouse IgG (926-32210) and anti-rabbit IgG (926-32211) were purchased from LI-COR Biosciences (NE, USA).

**Animals**

C57BL/6J mice, aged eight weeks, half male and half female, were purchased from Jinan Pengyue Laboratory Animal Breeding Co., Ltd. (Jinan, China). The animals were housed in the accredited barrier facility of Jiangsu Key Laboratory of New Drug Research and Clinical Pharmacy, with free access to standard chow and water before and during the experiments. The animal procedures were approved by the Animal Ethics Committee of Xuzhou Medical University (No. L20210226359) and in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China).

**Experimental design**

A mouse T2DM model was established according to previous reports \[^30\]. Briefly, after acclimation for two weeks, mice were divided into a control group and diabetic groups. Mice in the control group were fed with normal diet. Meanwhile, the remaining mice were fed with high fat diet (HFD)
for 12 weeks before intraperitoneal injection of STZ (100 mg kg\(^{-1}\) in 0.1 mol·L\(^{-1}\) sodium citrate buffer, pH 4.5). HFD (45% kcal fat; D12451) was purchased from Jiangsu Xietong Pharmacological Bio-Engineering Co., Ltd. (Nanjing, China). The fasting blood glucose (FBG) levels were measured three days later, and the model was successfully established when FBG levels reached 13.9 mmol·L\(^{-1}\) as described \(^{[31]}\). Diabetic mice were randomly divided into four groups (\(n \geq 6\)): a diabetic mice group (DM), a diabetic mice with low-dose Ju A group (Ju A, 5 mg kg\(^{-1}\)), a diabetic mice with medium-dose Ju A group (Ju A, 10 mg kg\(^{-1}\)) and a diabetic mice with high-dose Ju A group (Ju A, 20 mg kg\(^{-1}\)). All mice were intragastrically administrated for 12 weeks. The control group was treated with normal diet, while HFD was given to the DM and Ju A treatment groups throughout the experiments. Then, the mice were sacrificed, and renal tissues and blood samples were collected for further studies.

Cell culture

Being purchased from American Type Culture Collection (Manassas, VA, USA), human proximal tubular epithelial cell line HK-2 cells were kind gift from prof. KONG Ling-Dong (State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University), and cultured in DMEM containing 5.56 mmol·L\(^{-1}\) D-glucose (normal glucose, NG), supplemented with 100 U·mL\(^{-1}\) penicillin, 100 μg·mL\(^{-1}\) streptomycin and 10% FBS. The confluent cells were grown in serum-free DMEM containing 5.56 mmol·L\(^{-1}\) D-glucose for 24 h prior to the experiments. The cells were divided into the following groups: a normal group, a high glucose group (HG, 60 mmol·L\(^{-1}\)), a high glucose + low-dose Ju A group (Ju A, 1.25 μmol·L\(^{-1}\)), a high glucose + medium-dose Ju A group (Ju A, 2.5 μmol·L\(^{-1}\)) and a high glucose + high-dose Ju A group (Ju A, 5 μmol·L\(^{-1}\)). HK-2 cells were pre-treated with different concentrations of Ju A for 30 min, followed by treatment of HG for another 24 h according to the literature \(^{[32]}\).

CCK-8 assay

HK-2 cells were cultured in 96 well microplates at a density of 8000 cells/well. Then, 24 h after cultivation, the cells were serum starved and treated with HG (60 mmol·L\(^{-1}\)) and different concentrations of Ju A. After exposed to Ju A for 24 h, a cytotoxicity assay was performed using a CCK-8 assay kit (DoJinDo, ShangHai, China). The absorbance was measured at 450 nm using a 96-well plate reader (BioTek, USA), and the OD\(_{450}\) absorbance was proportional to the number of viable cells.

Measurement of renal function and biochemical parameters

The levels of FBG were measured by test strips from LifeScan (CA, USA). The levels of blood urea nitrogen (BUN) and blood creatinine (Cr) which can assess renal function, were examined by commercial kits from Jiancheng (Nanjing, China). The total triglyceride (TG), total cholesterol (T-CHO), high-density lipoprotein (HDL-C) and low-density lipoprotein (LDL-C) were measured using an automated analyzer in Xuzhou Oriental People’s Hospital (Xuzhou, China). The kidney index (mg·g\(^{-1}\)) was calculated as the ratio of the weight of the two kidneys to the body weight of a mouse.

ELISA analysis

The concentration of TGF-β1 was detected by mouse immunoassay ELISA kits (LanpaiBio, ShangHai, China) according to the manufacturer’s instructions. Then, the absorbance was measured by a microplate reader (BioTek, USA).

Renal morphology assessment

Renal tissue was first fixed in 4% formaldehyde and embedded in paraffin. The paraffin-embedded tissue blocks were cut into 4 μm-thick sections perpendicularly to the longitudinal axis of the kidney. Then, the sections were baked in a 60 °C incubator for 2 h, before stained with hematoxylin and eosin (H&E) to assess renal morphology. Periodic acid-Schiff (PAS) staining was used to assess glycogen accumulation. Periodic acid-silver metheramine (PASM) staining was conducted to assess the thickening of the glomerular basement membrane. Sirius red staining and Masson’s trichrome staining were used to demonstrate collagen deposition in the renal tubules. The sections were observed under an Olympus BX43F microscope (OLYMPUS, Tokyo, Japan). Quantitative image analysis was performed using Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD).

Immunohistochemical analysis

Renal tissue was first cut into 4 μm-thick sections, before deparaffinized in xylene and hydrated in graded alcohol and water. Then, the sections were placed in 3% H\(_2\)O\(_2\) to eliminate endogenous peroxidase activity. Next, the sections were blocked with normal goat serum, followed by incubation with anti-laminin (1∶100), anti-collagen IV (1∶100), anti-YY1(1∶100) and anti-TGF-β1 (1∶100) antibodies at 4 °C overnight. The sections were stained using a polymer HRP detection system (ZSGBBIO, Beijing, China) and counterstained with hematoxylin. The sections were observed under an Olympus BX43F microscope (OLYMPUS, Tokyo, Japan). The three most central sections of each defect were analyzed. Linear measurements were obtained using an image analysis system (Image-Pro Plus 6.0, Media Cybernetics, Silver Spring, MD).

Immunofluorescence analysis

Renal tissue and HK-2 cells were analyzed by immunofluorescence staining, as previously described \(^{[33]}\). Then, immunofluorescence images were captured using an Olympus BX43F fluorescence microscope (OLYMPUS, Tokyo, Japan).

Nuclear and cytoplasmic extraction

The cytoplasmic and nuclear fractions were separated using an NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Thermo-Scientific, Rockford, IL, USA) according to the manufacturer’s instructions. The detailed method of nuclear and cytoplasmic extraction was described in our previous study \(^{[34]}\).

Western blot analysis

Total proteins in the renal cortex and HK-2 cells were
harvested in lysis buffer and the protein concentrations were determined by the BCA Protein Assay Kit (Thermo-Scientific, Rockford, IL, USA) according to the manufacturer’s instructions. Then, 40–80 μg of proteins were loaded into 8% or/and 10% SDS-PAGE before transferred onto NC membrane (Millipore, USA), as previously described [30]. After the membrane was incubated with secondary antibodies at room temperature for 1 h, the blots were scanned using a LI-COR Odyssey infrared scanner (LI-COR, USA), and target bands were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

**Transmission electron microscopy**

For transmission electron microscopy (TEM), renal samples were pre-treated as described in our previous study [35]. Images were captured using Tecnai G2 TEM (FEI, Hillsboro, OR, USA).

**Molecular docking**

YY1 amino acid sequence (NP_003394.1) was extracted from the NCBI database. Based on this sequence, YY1 homology modeling was imitated by SWISS-MODEL, and this modeling was saved as PDB format file. The molecular structure of Ju A was drawn by ChemDraw 14.0. Sybyl-x2.0 was used to conduct molecular docking between Ju A and YY1. Energy minimization of Ju A ligand was carried out by the Powell method using the MINIMIZE module in the Sybyl-x2.0 program. Subsequently, YY1 homology models were pre-treated by the Surflex-Dock module, and the obtained YY1 was saved as mol2 format file. Molecular docking was performed using the pre-treated YY1 and the energy-optimized Ju A. At last, the docking results were visualized by PyMOL.

**Statistical analysis**

All animal experiments were repeated at least six times, and the experimental data are expressed as the mean ± SD (standard deviation). Statistical analysis was performed by Graphpad prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). The least significant difference test was performed to compare the differences between multiple groups by independent sample t-test or variance analysis; P values ≤ 0.05 were considered statistically significant.

**Results**

**Ju A reversed the alterations in blood biochemical indexes in diabetic mice**

After administration with Ju A for 12 weeks, we examined the FBG, body weight and kidney index of the experimental mice. Compared with normal mice, diabetic mice in the DM group showed significantly increased FBG levels, which then decreased after treatment with Ju A in a dose-dependent manner (Fig. 1A). Furthermore, the DM group showed declined body weight, which then significantly increased after Ju A treatment, as shown in Fig. 1B. In contrast, the significantly increased kidney index of diabetic mice was reduced by high-dose Ju A (Fig. 1C).

The renal function of mice was examined by detecting the levels of BUN and C₇, according to our previous study [30]. Compared with normal mice, diabetic mice in the DM group showed obviously elevated levels of BUN and C₇, which were then significantly reduced by treatment with Ju A in a dose-dependent manner (Fig. 1D). Meanwhile, the up-regulated levels of T-CHO and TG in diabetic mice were down-regulated by Ju A treatment, especially at the middle and high doses (Fig. 1E). Furthermore, compared with normal mice, the levels of LDL-C increased, but HDL-C decreased in diabetic mice of the DM group, which were then reversely changed after treatment with Ju A (Fig. 1F). These data suggested that Ju A improved renal function in diabetic mice.

**Ju A attenuated morphological changes and reduced collagen accumulation in the kidneys of diabetic mice**

H&E staining was used to observe the pathological changes. Results showed that compared with the control group, the DM group presented aggravated glomerular hypertrophy and renal tubular distension (Fig. 2A). However, these pathological changes were alleviated in the Ju A treatment groups. Compared with normal mice, diabetic mice in the DM group presented thickened GBM after PASM staining, which was relieved in Ju A treated mice (Fig. 2B). Sirius red staining and Masson’s trichrome staining were used to indicate collagen deposition. As shown in Figs. 2C and 2D, collagen accumulation increased in both the glomerular and renal interstitium of the DM group. However, administration of Ju A dose-dependently reduced collagen deposition to some extent. Glycogen is an important component of ECM. According to PAS staining, the increased glycogen deposition in the renal cortex of diabetic mice was reduced by treatment with different doses of Ju A (Fig. 2E). These results demonstrated that Ju A prominently improved the morphological lesion and reversed collagen deposition in the renal cortex of diabetic mice.

**Ju A ameliorated TIF in diabetic mice**

The protective effect of Ju A on the ultra-structure of the renal tubules in diabetic mice was examined by TEM. Compared with normal mice, diabetic mice in the DM group presented abnormal subcellular structure in RTECs, including atypical mitochondria, swelling mitochondria, cristolysis, partial resolution of the cytoplasm and vacuolar changes in cytoplasm (Fig. 3). However, compared with the DM group, the vacuolar changes and resolution of the cytoplasm of RTECs were significantly improved in the Ju A treatment groups. Meanwhile, Ju A treatment (especially at the high dose) greatly protected the ultra-structure of mitochondria in RTECs, including swelling and cristolysis, indicating that Ju A exhibited protective effect against the ultrastructural changes of RTECs in diabetic mice.

Next, to investigate the progression of TIF in diabetic mice, the expression of type IV collagen and laminin, two major components of ECM, were examined in vivo. As shown in Figs. 4A and 4B, the remarkably up-regulated expression of both type IV collagen and laminin were blocked by middel- and high-dose Ju A in the renal tubular of diabet-
ic mice. To further verify the protective effect of Ju A on TIF in diabetic mice, the EMT of RTECs was examined through detecting EMT protein markers. As shown in Fig. 4C, the up-regulated expression of α-SMA and the decreased expression of E-cadherin in the renal cortex of diabetic mice were blocked by Ju A to various degrees, especially in the high-dose Ju A group. Furthermore, the immunofluorescence results of α-SMA and E-cadherin confirmed that the improved expression of the two proteins induced by Ju A treatment mainly occurred in RTECs (Fig. 4D). The up-regulated expression of EMT-associated proteins, vimentin and twist, induced by T2DM were also down-regulated by Ju A treatment to various degrees (Fig. 4E). Therefore, our results demonstrated that Ju A ameliorated TIF in diabetic mice through blocking the EMT of RTECs.

Ju A improved TIF through down-regulating the YY1/TGF-β1 signaling pathway in diabetic mice

According to our previous study, YY1 was a key regulator in the renal fibrosis of DN through dysregulation of EMT-associated proteins. Another study about alveolar epithelial cells also indicated that EMT and pro-fibrogenesis induced by TGF-β1 were mediated by YY1. As shown in Fig. 5A, immunohistochemical staining illustrated increased YY1 that was mainly distributed in RTECs compared with that in the control group, which was then down-regulated by Ju A in diabetic mice in a dose-dependent manner. The above...
The findings were confirmed by immunoblot analysis of the renal cortex. As shown in Fig. 5B, the expression of total YY1 protein significantly increased in the renal cortex of diabetic mice, which was then relieved in Ju A treated diabetic mice. Furthermore, the redistribution of YY1 from the cytoplasm to the nucleus in the renal cortex of diabetic mice were reversed by Ju A treatment in a dose dependent manner (Figs. 5C and 5D).

The levels of TGF-β1 remarkably increased in the serum of diabetic mice, which then decreased by Ju A treatment (Fig. 5E). Similarly, the expression of TGF-β1 increased in the renal tubular of diabetic mice and was ameliorated by middle- and high-dose Ju A, as demonstrated by immunohistochemical staining (Fig. 5F). Immunoblot analysis of the renal cortex was used to further confirm these changes. As shown in Fig. 5G, the levels of TGF-β1 protein increased in the renal cortex of diabetic mice, which then significantly decreased after treatment with Ju A in a dose-dependent manner. Taken together, these results indicated that the YY1/TGF-β1 signaling pathway was up-regulated in the renal tubular of diabetic mice, where Ju A may improve TIF, at least in part through down-regulating the YY1/TGF-β1 signaling pathway.

Ju A down-regulated the YY1/TGF-β1 signaling pathway in HG cultured HK-2 cells

To further evaluate whether Ju A can down-regulate the YY1/TGF-β1 signaling pathway, CCK-8 assay was used to examine cell viability after treatment with Ju A in HG-cultured HK-2 cells. Results showed that the suppressed cell vi-
ability induced by HG was enhanced after treatment with Ju A, ranging from 1.25 to 5 \( \mu \text{mol} \cdot \text{L}^{-1} \) (Fig. 6A). Thus, 1.25, 2.5 and 5 \( \mu \text{mol} \cdot \text{L}^{-1} \) Ju A were used to examine its effect on the YY1/TGF-\( \beta \)1 signaling pathway in vitro. As shown in Fig. 6B, the expression of total YY1 was significantly up-regulated in HG-cultured HK-2 cells, which was then remarkable down-regulated by Ju A treatment. Meanwhile, the redistribution of YY1 from the cytoplasm to the nucleus induced by HG was blocked by Ju A treatment in HK-2 cells in a dose-dependent manner (Fig. 6C). The changes of HK-2 cells were further strengthened by immunofluorescence staining YY1 (Fig. 6D). Eventually, the levels of TGF-\( \beta \)1 protein were examined by Western blot. As shown in Fig. 6E, exposure to HG significantly up-regulated TGF-\( \beta \)1 levels compared with the control, and Ju A treatment dose-dependently decreased TGF-\( \beta \)1 levels in HG-cultured HK-2 cells. These results revealed that Ju A down-regulated the YY1/TGF-\( \beta \)1 signaling pathway in HG-cultured HK-2 cells.

**Molecular modeling studies of Ju A and YY1**

Molecular docking can simulate the interaction between small molecule ligands and macromolecular receptors. In order to further investigate the molecular interaction between YY1 and Ju A, molecular docking was performed by computer modeling. NCBI search results showed that Human YY1 had only one polypeptide chain, which contained 414 amino acids. Taking the complex (PDB ID: 1ubd.LC) as a template, the homology modeling of YY1 was performed by SWISS-MODEL. As shown in Fig. 7A, the main elements of YY1 were the alpha helix and random coil, where YY1 exhibited a large binding pocket. The sequence identity of this homology modeling was 100\%, and the sequence similarity was 0.64. Meanwhile, the value of GMQE was 0.81, and Qmean was 0.66. Furthermore, the modeling conformation was evaluated by Ramachandran Plot analysis, and results showed that most of amino acids distributed in the optimal region (Fig. 7B). All these results demonstrated that the simulated three-dimensional structure of YY1 had high stability and rationality.

The chemical structure of Ju A is shown in Fig. 7C. Results showed that Ju A was a heterocyclic compound with relatively complex structure. Then, molecular docking between YY1 and Ju A was conducted in our subsequent experiments. As shown in Fig. 7D, the binding pocket of YY1 was composed of TYR383, THR389, GLN396, THR378, THR372, ASN369, PHE368, LEU366, VAL346, ARG342, HIS343, LYS339, LYS332, and PHE334. In particular, THR389, THR378, ASN369 and PHE368 were the key residues to form hydrogen bonds, which contributed to the formation of YY1-Ju A complex. The best conformation score was 2.36, and polar value was 4.13. These results suggested that Ju A bound to YY1, and competitively occupied the binding pocket of YY1.

**Discussion**

As the major characteristic of T2DM, long-standing severe hyperglycemia is a direct factor which results in the development of DN. Thus, reduction of blood glucose levels by insulin injection or/and oral antidiabetic agents, and kidney protection are the current treatment strategies of DN \[36\]. Located in the nephron, the renal tubules account for about 80\% of the total renal volume, and are responsible for tubular reabsorption, where total glucose can be re-absorbed by RTECs into the blood \[37\]. Thus, chronic long-term high glucose in the blood may lead to constant accumulation of matrix proteins within RTECs, resulting in the development of TIF. Once developed, TIF is irreversible in advanced stages of DN, and eventually causes renal dysfunction \[38\]. Therefore, accompanying with hypoglycemic therapy, anti-TIF strategy is essential for the treatment of advanced DN. In the current study, we found that the elevated FBG levels in diabetic mice significantly decreased by Ju A treatment, while dyslipidemia in diabetic mice was restored by Ju A treatment. These improved renal function indexes showed that Ju A exhibited renal protective effect on diabetic mice. Moreover, we...
proposed for the first time that Ju A improved the TIF of RTECs through blocking the YY1/TGF-β1 signaling pathway in DN.

Blood glucose control directly affect the occurrence of diabetic complications and the severity of damage in target organs, especially the kidneys. Thus, hyperglycemia control is the priority of DN [39]. T2DM patients are usually accompanied with hyperglycemia and hyperlipidemia, and the later is characterized by hypercholesterolemia, hypertriglyceridemia, increases in LDL-C and decreases in HDL-C [40]. It was reported that dysregulated metabolism of blood lipids was obviously restored through effective hyperglycemia control in T2DM patients [41]. Furthermore, hyperlipidemia played a synergetic role in the pathogenesis of renal injury under diabetic conditions [42]. According to the current study, Ju A treatment not only ameliorated hyperglycemia and lowered body weight, but also improved hyperlipidemia in diabetic mice. Renal function evaluation showed that Ju A exerted renal protective effects, and down-regulated kidney index. These results suggested that Ju A may be a promising agent for the pre-treatment of DN.

It has been widely confirmed that renal fibrosis is closely related with the deterioration of renal function [43]. According to our previous studies, renal fibrosis in DN had a closely relationship with the Akt/mTOR signaling and PI3K/Akt signaling cascades, and mTOR, PI3K and Akt are the targets of Ju A using H9C2 cell studies [30, 44, 45]. As Ju A exhibited renal protective effects against DN, renal fibrosis was assessed in diabetic mice. Excessive deposition of ECM stimulated the development of renal fibrosis [46]. Pathological changes of the

[Fig. 4 Effects of Ju A on the TIF of diabetic mice. (A) Expression of type IV collagen in the renal interstitium of diabetic mice by immunohistochemical method and statistical analysis of type IV collagen expression. (B) Expression of laminin in the renal interstitium of diabetic mice by immunohistochemical method and statistical analysis of laminin expression. (C) Expression of α-SMA and E-cadherin in the renal interstitium of diabetic mice by Western blot and statistical analysis of α-SMA and E-cadherin expression. (D) Distribution and expression of α-SMA and E-Cadherin in the renal tubules by immunofluorescence, Bar = 50 μm. (E) Expression of vimentin and twist in the renal interstitium of diabetic mice by Western blot and statistical analysis of vimentin and twist expression, Bars = 50 μm. Control: normal mice; DM: diabetic mice; 5 mg·kg⁻¹: diabetic mice treated with low-dose Ju A (5 mg·kg⁻¹); 10 mg·kg⁻¹: diabetic mice treated with middle-dose Ju A (10 mg·kg⁻¹); and 20 mg·kg⁻¹: diabetic mice treated with high-dose Ju A (20 mg·kg⁻¹). Each bar represents the mean ± SD (n = 6). *P < 0.05, **P < 0.01 vs Control; ***P < 0.001, ****P < 0.001 vs DM]
renal cortex of diabetic mice were first examined by H&E and PASM staining, which showed that Ju A exerted morphological protective effect on the glomeruli and renal interstitium of diabetic mice. Then, collagen deposition was investigated by Masson trichrome staining and Sirius red staining. Our results indicated that the increased collagen and accumulated ECM in the renal cortex of diabetic mice were ameliorated by treatment with Ju A in a dose-dependent manner, which suggested that Ju A effectively reversed collagen deposition in the renal cortex of diabetic mice. As one of the important components of ECM, glycogen was examined by PAS staining. Our results demonstrated that the increased glycogen deposition in the tubular basement membrane and tubular interstitium induced by T2DM were obviously reduced by Ju A, which suggested that Ju A alleviated the renal fibrosis of DN.

The renal tubule composes a large portion of the nephron of the kidney. Therefore, in the current study, we found that the renal tubules were vulnerable in the destructive fibrogenic process of DN compared with the glomerulus. Our TEM results showed that Ju A exhibited remarkably protective effect against DN-induced disorder in the subcellular structure of RTECs. Based on these findings, the protective effect of Ju A on TIF was examined in diabetic mice by de-

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**Fig. 5** Effect of Ju A on the YY1/TGF-β1 signaling pathway in the renal cortex of diabetic mice. (A) Total YY1 expression in the renal interstitium of diabetic mice through immunohistochemical method and statistical analysis of Total-YY1 expression, Bars = 50 μm. (B) Relative levels of total YY1 protein in the experimental mice. (C) Relative levels of nuclear YY1 protein in experimental mice. (D) Relative protein levels of cytoplasm YY1 in the experimental mice. (E) Levels of TGF-β1 in the serum of experimental mice. (F) Expression of TGF-β1 in the renal interstitium of diabetic mice through immunohistochemical method and statistical analysis of TGF-β1 expression. (G) Relative levels of TGF-β1 protein in the renal cortex of mice by Western blot and statistical analysis of TGF-β1 expression. Control: normal mice; DM: diabetic mice; 5 mg·kg⁻¹: diabetic mice treated with low-dose Ju A (5 mg·kg⁻¹); 10 mg·kg⁻¹: diabetic mice treated with middle-dose of Ju A (10 mg·kg⁻¹); and 20 mg·kg⁻¹: diabetic mice treated with high-dose Ju A (20 mg·kg⁻¹). Each bar represents the mean ± SD (n = 6). *P < 0.05, **P < 0.01 vs Control; *P < 0.05, **P < 0.01 vs DM.
Detecting the expression of fibrosis-related marker proteins. Immunohistochemical staining results of collagen IV and laminin revealed that Ju A improved the TIF of diabetic mice. Our previous studies had fully confirmed that the EMT of tubular epithelial cells was an early process of renal fibrosis, especially TIF [16, 19, 20, 33, 35]. To further verify the protective effect of Ju A on TIF, the expression of EMT-related proteins was examined in the tubular interstitium of diabetic mice. According to Western blot analysis and immunofluorescence, α-SMA expression increased and E-cadherin expression decreased significantly in the tubular interstitium of diabetic mice, but these changes were reversed by Ju A treatment, which suggested that Ju A blocked the EMT of the tubular interstitium in diabetic mice. These findings were further evidenced by the changes of other EMT-related proteins, such as vimentin and twist.

As we confirmed that Ju A ameliorated against TIF in diabetic mice through EMT process, its anti-fibrosis mechanism was further investigated. It was reported that Ju A protected isoproterenol-induced H9C2 cell injury via the mTOR signaling pathway, and our previous study showed that mTOR-mediated YY1 participated in HG-induced EMT process of RTECs, resulting in the TIF of DN [20, 26]. In another study, we found that the up-regulated YY1 promoted the EMT of RTECs in DN [16]. Moreover, our latest research demonstrated that HG or/and hyperglycemia induced the up-regulated expression and nuclear translocation of YY1 protein, which promoted the mitochondrial dysfunction of early DN,

Fig. 6 Effect of Ju A on the YY1/TGF-β1 signaling pathway in HG-cultured HK-2 cells. (A) Effect of Ju A on cell viability in a concentration-dependent manner. (B) Relative levels of total YY1 protein by Western blot in HK-2 cells. (C) Relative levels of nuclear and cytoplasm YY1 protein by Western blot in HK-2 cells. (D) Representative levels of YY1 protein through immunofluorescence in HK-2 cells. (E) Relative levels of TGF-β1 protein by Western blot in HK-2 cells. NG: HK-2 cells treated with glucose (5.56 mmol·L⁻¹); HG: HK-2 cells treated with 60 mmol·L⁻¹ glucose; HG + Ju A groups: after pretreated with Ju A (1.25, 2.5 and 5 μmol·L⁻¹) for 30 min, HK-2 cells were exposed to HG for 24 h. Each bar represents the mean ± SD (n = 3). *P < 0.05, **P < 0.01 vs NG; #P < 0.05, ##P < 0.01 vs HG.
In the current study, Ju A protected the mitochondrial damage in the renal tubules of diabetic mice. Then, we explored whether YY1 was involved in the protection of Ju A against TIF in diabetic mice. Immunohistochemical staining showed increased total YY1 that was mainly distributed in renal tubular cells, and Ju A treatment dose-dependently down-regulated its expression. These findings were further confirmed by immunoblot analysis of total YY1 in the renal cortex of mice. Results of nuclear and cytoplasmic extraction showed that the expression and nuclear translocation of YY1 participated in the protection of Ju A against TIF of DN.

Activation of TGF-β1 can initiate collagen accumulation and promote the development of TIF through promoting EMT, and the renal tubular cells of DN showed an increased levels of TGF-β1 [47-50]. According to previous reports, there was a close regulatory relationship between TGF-β1 and YY1 in different tissue, besides the kidneys [17, 23, 51]. After identifying the role of YY1 in the protective effect of Ju A against TIF in diabetic mice, the levels of TGF-β1 protein were detected in the serum and renal cortex by ELISA kits, Western blot and immunohistochemical staining. Being consistent with relevant reports, the up-regulated trends of TGF-β1 in the serum and renal tubules of diabetic mice were all blocked by the treatment of Ju A. Subsequently, HG cultured HK-2 cells were used to determine the effect of Ju A on the YY1/TGF-β1 signaling pathway. The results of in vitro stud-
ies were consistent with these in vivo. These findings suggested that the ameliorated TIF by Ju A was associated with the down-regulated YY1/TGF-β1 signaling pathway in DN.

Based on the results of pharmacological studies in vivo and in vitro, molecular docking was carried out by computer modeling to further explore the interaction between Ju A and YY1. Results of 3D diagrams showed that YY1 target and Ju A had good blinding effect, suggested that Ju A bound to YY1 and exerted protective effect on TIF of DN. Besides RTECs, glomerular mesangial cells, podocyte and glomerular endothelial cells are the important renal parenchymal cells. Although the protective effect and possible mechanisms of Ju A on TIF of DN were confirmed, our results also showed that Ju A improved the pathological changes of the glomerulus in diabetic mice. But the exact mechanism by which Ju A exerted the protective effect on the glomerulus of diabetic mice requires further investigation.

In the current study, our results showed that Ju A attenuated hyperglycemia and hyperlipidemia, and improved the renal function of diabetic mice. Ju A reduced ECM deposition and morphological changes in diabetic mice. The promoted EMT of renal tubular cells was blocked by Ju A treatment, leading to the amelioration of TIF in diabetic mice. Further mechanism studies demonstrated that the down-regulated YY1/TGF-β1 signaling pathway was involved in the amelioration of TIF induced by Ju A in DN.

**Conclusion**

In conclusion, the present study shows that Ju A can effectively control glycemia and lipidemia, and improve the renal function of diabetic mice. Accordingly, morphological changes and collagen accumulation in the renal cortex of diabetic mice are improved by Ju A treatment. Furthermore, Ju A ameliorates the TIF of DN, at least in part through suppressing the YY1/TGF-β1 signaling pathway (Fig. 8). In summary, our research provides a novel insight into the pathogenesis of TIF in DN, and Ju A may act as a promising adjuvant and preventive therapeutic agent for DN-associated TIF.

**References**


