



Dapagliflozin restores autophagy and attenuates apoptosis via the AMPK/mTOR pathway in diabetic nephropathy rats and high glucose-induced HK-2 cells

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Abstract

Purpose Diabetic nephropathy (DN) is a serious microvascular complication of diabetes mellitus. Significantly reduced levels of autophagy in diabetic kidneys play an important role in the development of DN. The present study investigated the effects of dapagliflozin (DAP) on renal autophagy and AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway *in vivo* and *in vitro*.

Methods We explored the effect of DAP in streptozotocin (STZ)-induced DN rats. The anti-DN effect of DAP was assessed by body weight, kidney weight/body weight ratio, blood and urine biochemical parameters, and pathological changes of kidney tissue. Number of autophagosomes in the kidney was investigated through Transmission electron microscopy. Besides, cell viability and apoptosis of DAP alone or combined with Compound C (CC, a selective AMPK inhibitor)-treated high glucose (HG)-induced HK-2 cells were detected by Cell Counting Kit-8 (CCK-8) and flow cytometry assays. Immunohistochemistry, Western blot, Enzyme-linked immunosorbent assay (ELISA), and immunofluorescence were employed to detect the expression levels of extracellular matrix (ECM) deposition, autophagy, apoptosis, and AMPK/mTOR pathway-associated targets *in vivo* and *in vitro*.

Results The results showed that DAP ameliorated the body weight and decreased kidney weight, fasting blood glucose, and serum/urine biochemical parameters of renal damage, as well as renal pathological changes. Moreover, DAP significantly ameliorated HG-induced cell apoptosis and ECM deposition in HK-2 cells. However, these favorable effects of DAP could be abolished by co-treatment with CC in HG-induced HK-2 cells. Mechanistically, DAP can enhance autophagy in DN including increased LC3-II/I ratio, Beclin-1, p-AMPK protein levels, and decreased p62 and p-mTOR protein expressions, as well as inhibited renal fibrosis and apoptosis.

Conclusion In summary, DAP alleviated fibrosis, apoptosis, and autophagy in DN rats and HG-induced HK-2 cells by regulating the AMPK/mTOR pathway.

Keywords Diabetic nephropathy · Dapagliflozin · Autophagy · AMPK

Introduction

Diabetic kidney disease (DKD) is one of the most common chronic complications of diabetes mellitus and one of the leading causes of end-stage renal disease. The prevalence of diabetes mellitus is increasing year by year and has become the leading cause of chronic kidney disease (CKD) worldwide [1]. As of 2017, the number of people with diabetes worldwide is about 451 million, and by 2045, the number of people with diabetes worldwide will reach 693 million [2]. About 40% of these diabetic patients will develop diabetic nephropathy (DN), and end-stage renal disease (ESRD) is the final outcome of DN progression, which requires renal

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replacement therapy, such as renal transplantation and dialysis treatment [3]. It has been shown that diabetic patients with DN have a 3- to 12-fold higher mortality rate than diabetic patients alone [4]. Finding ways to delay or treat DN has been a hot topic in the field of DN research. Current treatments for DN are mainly through controlling risk factors such as blood glucose, blood pressure, and lipids, as well as the use of renin-angiotensin system (RAS) blockers to reduce urinary protein and delay the deterioration of renal function, but more than 40% of treated patients still face a high risk of progressing to ESRD [5].

In recent years, sodium-dependent glucose transporters 2 (SGLT-2) inhibitors have been shown to be renoprotective and have been used in clinical practice, leading to a new dawn in the treatment of DN [6]. SGLT-2 inhibitors are a new type of non-insulin-dependent hypoglycemic agents, which mainly exert hypoglycemic effects by blocking glucose reabsorption in proximal tubules and promoting urinary sugar excretion [7, 8]. In addition to lowering blood glucose, SGLT-2 inhibitors have been shown to reduce cardiovascular events in previous clinical trials [9–11]. Meanwhile, secondary outcomes and exploratory analyses of these clinical trials showed that SGLT-2 inhibitors could improve renal regression [9–11], but there are some uncertainties about the renoprotective effects of SGLT-2 inhibitors, and further in-depth studies on their mechanisms of action are needed.

A large number of studies have shown that the pathogenesis of DN is closely related to the inhibition of autophagy and the deposition of extracellular matrix (ECM) in renal cells [12, 13]. Studies have shown that cellular autophagy is a self-protective mechanism to maintain the self-stabilization of the intracellular environment, and its main role is to remove and degrade damaged organelles and excess biomolecules [14, 15]. In diabetes mellitus, the autophagy function of renal cells is inhibited due to the high glucose environment, which leads to damaged organelles and cytotoxic substances in renal cells that can not be removed in a timely and effective manner, which affects the normal function of the cells, and then causes renal injury [16, 17]. Therefore, autophagy has become a new target for the treatment of diabetic nephropathy [18, 19].

AMP-activated protein kinase (AMPK) is a nutrient-sensitive kinase activated under conditions of energy depletion, and the mammalian target of rapamycin (mTOR) is a key regulatory molecule in the process of autophagy, and the activation of mTOR blocks autophagy under physiological conditions [20, 21]. The activation of AMPK has an inhibitory effect on mTOR, which is one of the classical pathways activated by autophagy signaling, and the AMPK/mTOR signaling pathway is believed to play an important role in the regulation of cellular autophagy and senescence [12, 22]. Among SGLT-2 inhibitors, Dapagliflozin (DAP) has a variety of pharmacological effects. Recent hotspot studies have

found that DAP has a good renoprotective effect [23, 24], but whether it exerts its DN prevention through regulating the autophagy signaling pathway mediated by the AMPK-mTOR axis has not been investigated. In the present study, to address these issues, an animal model of DN established by a high-fat diet (HFD) combined with streptozotocin (STZ) injection and also the high glucose-induced human renal tubular epithelial cell line (HK-2) were used to explore whether the AMPK-mTOR pathway is involved in the protective effect of DAP in vitro and in vivo.

Materials and methods

Animal experiments

A total of 70 male SD rats (Aged: 5–6 weeks, weighing 180–200 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China) and housed in the SPF-level standard environment with a 12-h light/dark cycle. All rats were allowed free access to food and water ad libitum. After a week of adaptive feeding, the rats were divided into two groups ($n = 10$ in each group): the normal group rats received a normal diet and the HFD group ($n = 60$) received a high-sugar-fat diet for 4 weeks. After that, the rats in the HFD group were intraperitoneally injected with 100 mg/kg STZ (Sigma-Aldrich, St. Louis, MO, USA). After 72 h, the fasting blood glucose (FBG) levels of rats in the HFD group more than 16.7 mmol/L were used. After STZ injection for 4 weeks, the DN rat was established, as accompanied by the uromicroprotein exceeding 30 mg/day. Subsequently, the DN rats ($n = 50$) were randomly divided into five groups ($n = 10$ in each group): model group, gliquidone (positive group, GLI, Beijing Wanhui Shuanghe Pharmaceutical Co., LTD, China), DAP low-dose (DAP-L) group, DAP medium-dose (DAP-M) group, DAP high-dose (DAP-H) group. Rats in the control and model groups received an equal amount of distilled water. GLI group rats received gliquidone at a dose of 10 mg/kg body weight per day by gavage. Meanwhile, the rats in the DAP groups were orally treated with DAP (AstraZeneca Pharmaceuticals Co., LTD, London, England) dissolved in distilled water at a dose of 1, 5, or 10 mg/kg every day, respectively for 12 weeks. The body weights were recorded weekly for each group.

Sample collection

After 12 weeks of GLI and DAP administration, the rats were maintained in metabolic cages for 24 h to collect urine samples. Next, all rats were anesthetized and sacrificed through cardiac puncture. Then, the kidneys were collected and weighted to calculate the ratio of kidney weight/body weight (also known as the renal index). The blood samples

from rat heart were collected and centrifuged for further examination. The level of FBG, urea nitrogen (BUN), and creatinine (Scr) in serum samples, as well as the level of urine protein, urinary albumin, and urinary creatinine were measured by automatic biochemical analyzer (Hitachi High-Tech Group, Japan).

Histopathological examination

Kidney tissues were fixed in 4% paraformaldehyde for 48 h at room temperature. After being embedded in paraffin, the kidney tissues were sectioned into 5 μm thick sections. Then, the sections were used for hematoxylin and eosin (H&E) staining to observe kidney structure, and periodic Acid-Schiff (PAS) to examine ECM accumulation in glomerular, Masson's trichrome to detect fibrosis. The photographs were pictured under light microscopy (Olympus, Tokyo, Japan).

Immunohistochemistry assay

The expression levels of the ECM protein, Type I collagen (COL I) and fibronectin (FN) in the renal tissues were detected by the immunohistochemistry assay. Briefly, the paraffin-embedded sections were dewaxed and underwent antigen retrieval and blocked with 5% BSA. The slices were then incubated with an anti-COL I antibody (1:500, ab270993, Abcam) or an anti-FN antibody (1:500, ab268020, Abcam) overnight at 4 °C. The sections were then incubated with the secondary goat anti-rabbit antibody (1:5000, ab97080, Abcam) for 30 min at room temperature, followed by incubating with DAB for 2 min and counterstaining with hematoxylin. At last, the sections were imaged with a light microscope (DS-U3, Nikon Corporation, Tokyo, Japan).

TUNEL assay

Paraffin sections were subjected to dewaxing, hydration, and proteinase K treatment without DNase for a duration of 30 min at 37 °C. Subsequently, the tissues were coated with a 0.5% Triton X-100 solution for a period of 15 min. The extent of apoptosis was assessed using the One-Step TUNEL Cell Apoptosis Detection Kit (C1089, Beyotime, China) according to the manufacturer's instructions. The nuclei were stained with DAPI (Beyotime) and visualized using a fluorescence microscope (Olympus, Japan).

Transmission electron microscopy

Renal tissues were washed using physiological saline and fixed in 2.5% glutaraldehyde at 4 °C overnight. Then, renal tissues were washed with PBS and post-fixed with 1% osmium tetroxide. Next, the samples were embedded in

epoxy resin, and the ultrathin sections (70 nm) were collected using an ultramicrotome (Leica, Germany). After counterstaining with uranyl acetate and lead citrate, the sample sections were examined by using a JEM-1400 Plus transmission electron microscope (Japan).

Cell treatment and Cell counting kit-8 (CCK-8) assay

Human kidney proximal tubular cells (HK-2) were purchased from the Cellverse Bioscience Technology Co., Ltd. (Shanghai, China) and cultured in DMEM/F12 containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution at 37°C in a humidified 5% CO₂ atmosphere. HK-2 cells were exposed to normal glucose (5.5 mM D-glucose) or high glucose (HG, 30 mM D-glucose) for 48 h. Also, cells cultured in 5.5 mM glucose-containing medium supplemented with 24.5 mM mannitol were used as HG controls. After that, the HG-induced HK-2 cells were treated with 25, 50, 100 $\mu\text{mol/L}$ DAP for 24 h, respectively. Besides, the HG-induced HK-2 cells were pre-treated with 10 $\mu\text{mol/L}$ Compound C (CC), an AMPK inhibitor for 3 h, followed by DAP treatment at a dose of 100 $\mu\text{mol/L}$ for 24 h. After that, 10 μl CCK-8 (Sangon, Shanghai, China) was added to each well for another 4 h. Finally, the absorbance was measured at 450 nm using a microplate reader (Molecular Devices).

Flow cytometry assay

The apoptosis of DAP alone and CC in combination-treated HK-2 cells was measured with an Annexin V-FITC/PI apoptosis kit (#556,547, BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Briefly, the treated HK-2 cells were collected and washed with PBS. And then, the cells were incubated with 5 μl Annexin V-fluorescein isothiocyanate (FITC) and 10 μl propidium iodide (PI) in the dark for 15 min. Finally, the cell apoptosis of treated HK-2 cells was estimated using a flow cytometer (BD Biosciences) and FlowJo software.

Enzyme-linked immunosorbent assay (ELISA)

After the treatment of DAP alone and CC in combination in HG-induced HK-2 cells, the cell culture medium were collected and centrifuged at 3000 rpm for 10 min 4 °C. Then, the supernatant was collected. The concentrations of collagen I and fibronectin secreted from treated HK-2 cells in the supernatant were detected using commercial ELISA kits (ml025998 and ml023933, mlbio, Shanghai, China) following the manufacturer's instructions.

Immunofluorescence assay

The paraffin sections of kidney tissues were de-paraffinised, retrieved, washed with PBS, and then blocked with 5% goat serum for 1 h at room temperature. Subsequently, the sections were incubated with anti-LC3 (1:100, AF5402, Affinity) antibody, anti-p-AMPK (1:100, AF6423, Affinity), and anti-p-mTOR (1:100, AF3308, Affinity) in a wet box overnight at 4 °C. After washing with PBS for three times, the sections were incubated with rabbit 488 fluorescent (green) secondary antibody (1:500, ab150077, Abcam) for 1 h in the dark at room temperature. Next, the cell nuclei were stained using DAPI for 15 min. Then, the sections were covered with an anti-fluorescence quenching mounting plate and observed by a fluorescence microscope (Nikon, Japan). To determine the expression of LC3 and p-AMPK in treated HK-2 cells, the immunofluorescence staining was also performed. Briefly, the treated HK-2 cells were fixed with 4% paraformaldehyde and blocked with 1% BSA containing 0.3% Triton X-100 for 30 min at room temperature. Next, the treated HK-2 cells were incubated with primary anti-LC3 (1:200, ab192890, Abcam) and anti-p-AMPK (1:200, ab92701, Abcam) at 4 °C overnight, followed by being stained with Alexa Fluor 594 (1:500, ab150080, Abcam) for 1 h in the dark. The nuclei were then stained with DAPI. Finally, images were taken on a Ts2-FC fluorescence microscope (Nikon Corporation).

Western blot analysis

Total protein samples from the kidney tissues and the treated HK-2 cells were prepared by using cooled RIPA buffer (Beyotime, China) and the total protein concentration was detected by BCA Protein Assay Kit (pc0020, Beyotime). An equal amounts of protein (30 µg) samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (GE Healthcare Life). The membranes were then blocked with 5% nonfat milk in Tris

buffered saline (TBST) for 1 h. After washed with TBST, the membranes were incubated with primary antibody of TGF-β1 (1:1000, BF8012, Affinity), α-SMA (1:1000, AF1032, Affinity), p62 (1:1000, AF5384, Affinity), LC3 (1:1000, 4108 s, Cell Signaling Technology, Danvers, MA, USA), Bax (1:1000, AF0120, Affinity), Bcl-2 (1:1000, AF6139, Affinity), p-AMPK (1:1000, AF3423, Affinity), AMPK (1:1000, AF6423, Affinity), p-mTOR (1:1000, AF3308, Affinity), mTOR (1:1000, AF6308, Affinity), E-cadherin (1:1000, 3195 T, Cell Signaling Technology), Vimentin (1:1000, R22775, Zenbio, Suzhou, China), Beclin-1 (1:1000, AF5128, Affinity), and GAPDH (1:5000, 10494-1-AP, Proteintech) overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies (1:5000, 7074, Cell Signaling Technology). Then, the membranes were exposed to BeyoECL Plus chemiluminescence reagent (P0018S, Beyotime) and visualized using a ChemiScope6100 instrument (Clinx Science instrument Co. Ltd., Shanghai, China). Finally, the semi-quantitative analysis of protein bands was performed using ImageJ software (National Institutes of Health, USA).

Statistical analysis

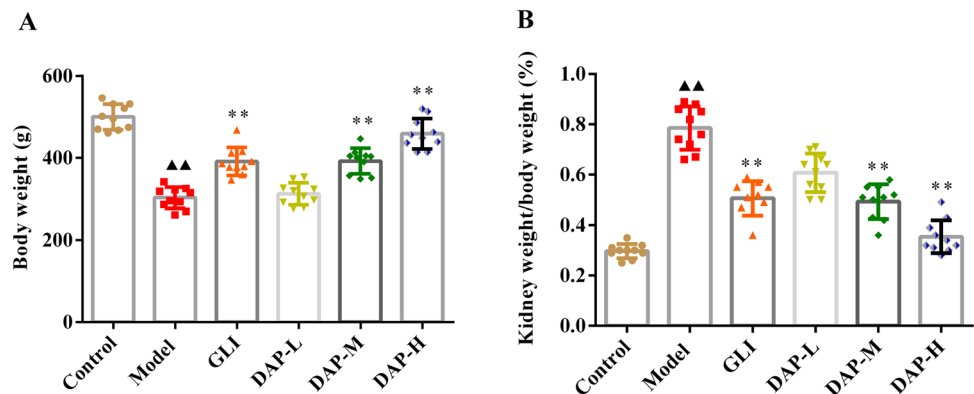
All data are shown as the mean ± standard deviation (SD). Statistical significance was estimated using Student's *t* test between two groups. Additionally, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests was used among multiple groups. *P* < 0.05 was considered significant.

Results

DAP treatment ameliorated renal function of DN rats

To clarify the roles of DAP on DN, a rat model of DN was established through STZ injection. As shown in Fig. 1A, B,

Fig. 1 Effect of DAP treatment on body weight and kidney index in HFD/STZ-induced DN rats. **A** Body weight of six groups of rats at 12 weeks after DAP and GLI treatment (*n* = 10). **B** The kidney weight/body weight (kidney index) of six groups of rats at 12 weeks after DAP treatment (*n* = 10). ▲▲ *P* < 0.01 vs. control group. ***P* < 0.01 vs. model group



we observed that the body weight in DAP and GLI groups was significantly increased than those of the model group, while the ratio of kidney weight/body weight was greatly reduced in DAP and GLI groups when compared with the model group. Compared with the model group, DAP and GLI treatment significantly suppressed the increase in FBG (Fig. 2A), serum BUN (Fig. 2B), Scr (Fig. 2C), 24-h urinary protein (Fig. 2D), urinary albumin (Fig. 2E), and urinary creatinine (Fig. 2F). These results suggested that DAP effectively ameliorated renal function in STZ-induced DN rats.

DAP treatment improved histopathological changes in DN rats

To further investigate the histopathological changes in the kidneys from DN rats with DAP treatment, the H&E, PAS, and Masson staining were performed. H&E staining showed that renal tubular epithelial vacuolar degeneration, irregular glomerular morphology, and renal tubular edema with partial necrosis in the model group (Fig. 3A). As illustrated in Fig. 3B, PAS staining showed that the mesangial and glomerular basement membranes were slightly thickened in DN rats. Additionally, the Masson staining revealed a significant increase of collagen fibers in the kidneys of DN

rats (Fig. 3C). However, the abnormal pathological changes were restored by GLI and DAP treatments (Fig. 3). Moreover, Immunohistochemical staining and Western blotting showed that COL-I, FN, TGF- β 1, and α -SMA were significantly upregulated in the kidneys of DN rats, which were observably downregulated by the treatment of GLI or DAP (Fig. 4A, B). These results collectively suggested that DAP indeed has anti-renal fibrosis effects against DN.

DAP treatment attenuated apoptosis and promoted autophagy in DN rats

The effect of DAP or GLI on cell apoptosis in the kidneys of DN rats was further investigated by TUNEL staining. The results showed that, compared with model group, DAP treatment attenuated cell apoptosis levels in the kidneys of DN rats in a dose-dependent manner (Fig. 5A). To investigate the effect of DAP on cell autophagy, the number of autophagosomes in the kidneys of DN rats was detected by transmission electron microscopy. The results showed that DAP treatment remarkably increased the number of autophagosomes in DAP-treated groups in a dose-dependent manner compared with model group (Fig. 5B). To further confirm the occurrence of cell apoptosis and

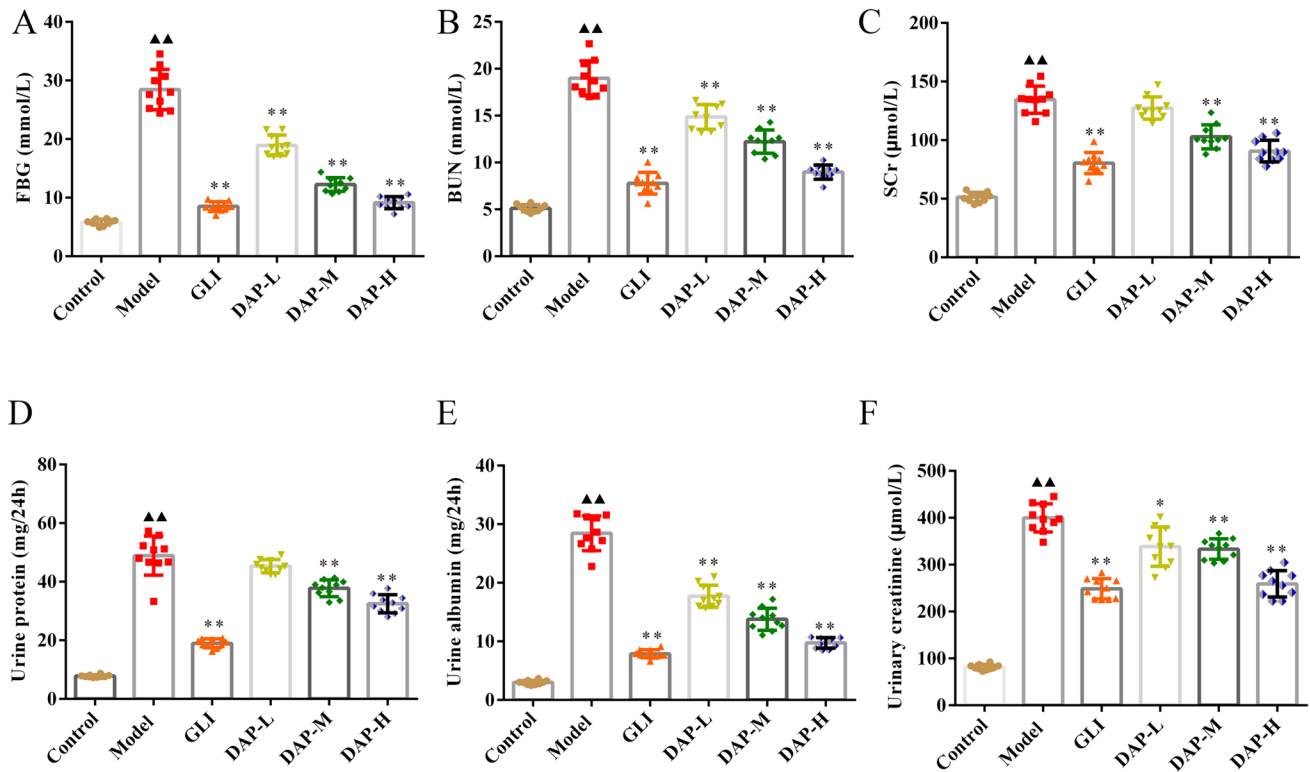


Fig. 2 Effect of DAP treatment on biochemical index in HFD/STZ-induced DN rats. A–C Serum FBG, BUN, and Cr levels of six groups of rats at 12 weeks after DAP and GLI treatment ($n=10$). (D–F) Urine protein, urine albumin, and urinary creatinine levels of

six groups of rats at 12 weeks after DAP and GLI treatment ($n=10$). $\blacktriangle\blacktriangle P<0.01$ vs. control group. $*P<0.05$, and $**P<0.01$ vs. model group

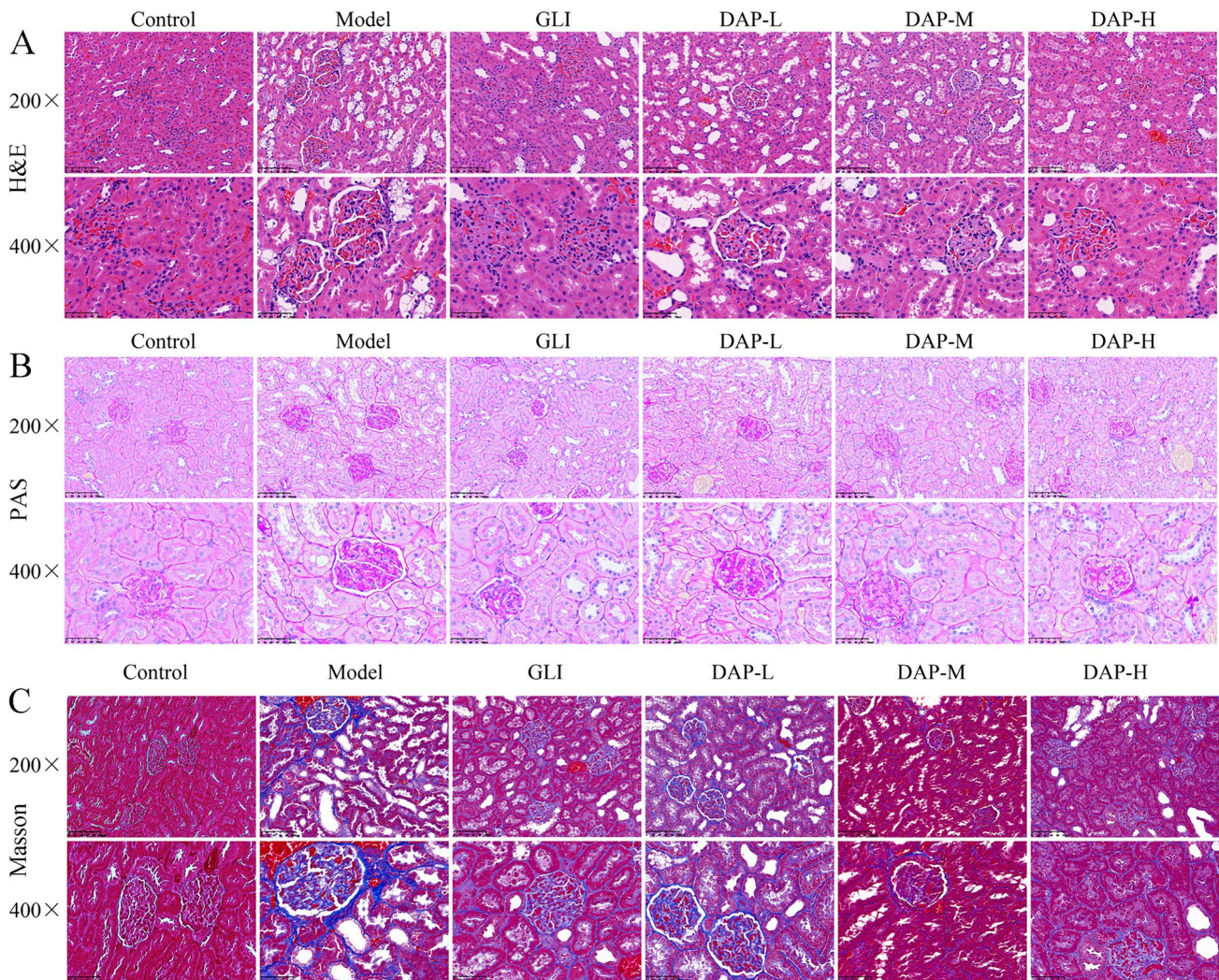


Fig. 3 Effect of DAP treatment on renal pathological changes in the kidney of HFD/STZ-induced DN rats. **A** H&E, **B** PAS, and **C** Masson's trichrome staining of rat kidney tissues. Scale bar, 100 μm , 50 μm

autophagy, we then detected apoptosis- and autophagy-related proteins. The results of immunofluorescence staining showed that, following DAP treatment, the protein expression of LC3 was increased (Fig. 5C). Similarly, the protein expression levels of P62 and Bax were markedly down-regulated in the DAP-treated DN rats, while the levels of LC3 and Bcl-2 were significantly up-regulated after DAP administration (Fig. 5C). Research has shown that AMPK/mTOR signaling pathway is associated with cell autophagy in the development of DN. To test this, the AMPK and mTOR levels in the kidneys of DN rats were measured after DAP treatment using immunofluorescence staining and Western blotting. The results showed that the phosphorylation levels of AMPK were higher in DAP-treated DN rats than in model rats; however, the phosphorylation levels of mTOR were repressed by DAP treatment (Fig. 6A-6C). These results suggested that DAP

inhibited cell apoptosis and promoted cell autophagy in the kidneys of DN rats.

DAP improved the cell viability and inhibited cell apoptosis in HG-induced HK-2 cells

The cell viability and cell apoptosis of HG-induced HK-2 cells were detected by CCK-8 and flow cytometry, respectively, following the administration of DAP at a concentration of 25, 50, and 100 $\mu\text{mol/L}$ alone or in combination with 10 $\mu\text{mol/L}$ of CC for 24 h. The results showed that the cell viability of HG-induced HK-2 cells was increased at both concentrations (Fig. 7A). As demonstrated in Fig. 7B, the cell apoptosis rate of HG-induced HK-2 cells was decreased in the DAP-treated HK-2 cells (Fig. 7B). Meanwhile, the ELISA revealed that DAP treatment led to decreased collagen I and Fibronectin content in the culture supernatant

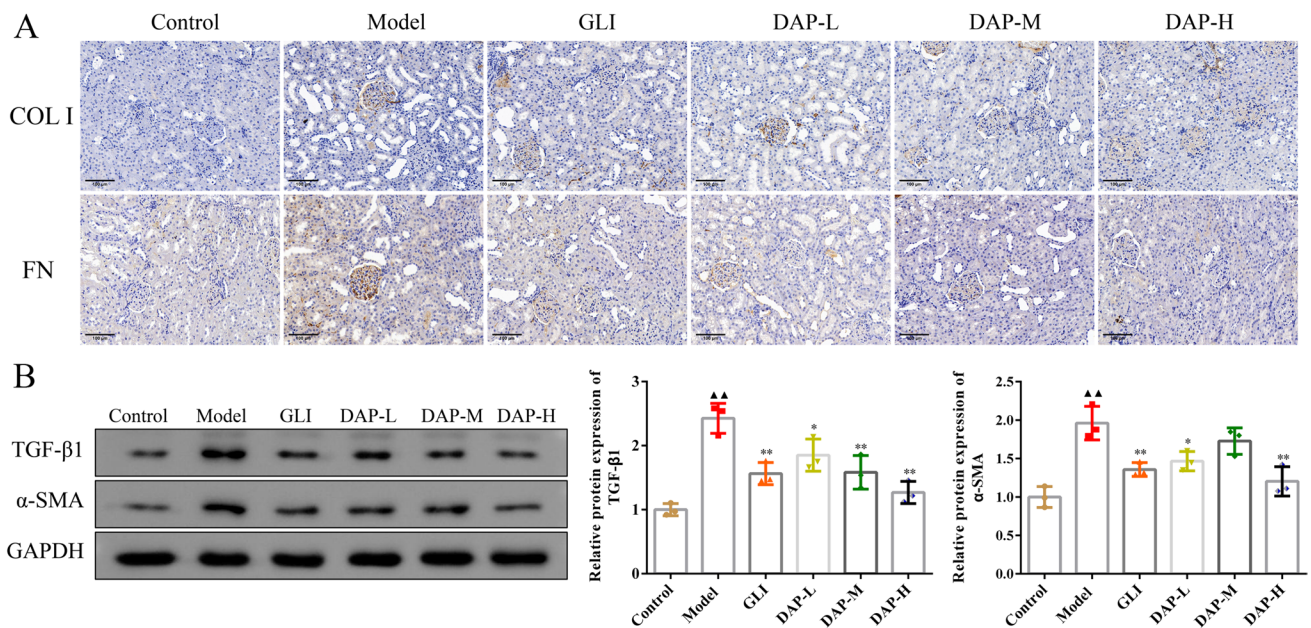


Fig. 4 Effect of DAP treatment on ECM accumulation in the kidney of HFD/STZ-induced DN rats. **A** Immunohistochemical staining of Type I collagen (COL I) and FN (magnification 200 \times). **B** Protein

expression of TGF- β 1 and α -SMA in the kidney tissues was detected by Western blot assay. $\blacktriangle\blacktriangle P < 0.01$ vs. control group. $*P < 0.05$, and $**P < 0.01$ vs. model group

of HG-induced HK-2 cells (Fig. 7C). Consistent with the in vivo results, the protein expression levels of Bcl-2 and E-cadherin were higher in DAP-treated HK-2 cells than in HG-induced HK-2 cells. Conversely, DAP-treated HG-induced HK-2 cells had weakened protein expression levels of Bax, vimentin, and α -SMA (Fig. 7D). More importantly, the results of immunofluorescence staining and Western blotting demonstrated that treatment of DAP could up-regulate the decreased levels of LC3, beclin-1, p-AMPK, while down-regulate the increased levels of p-mTOR in HG-induced HK-2 cells (Fig. 8A, C). However, these effects were partly abolished by CC treatment (Figs. 7, 8). Collectively, the results suggested that the anti-DN effect of DAP involved the activation of autophagy in vitro and in vivo through the AMPK/mTOR pathway.

Discussion

DN is a serious microvascular complication of diabetes mellitus, which ultimately leads to the decline of renal function, with a high rate of disability and lethality [25, 26]. Studies have confirmed that the AMPK/mTOR signaling pathway has an important role in regulating renal tissue autophagy during DN and that AMPK/mTOR signaling pathway-mediated renal tissue autophagy can significantly alleviate renal tissue injury in the DN environment [27, 28]. Therefore, in the present study, we established a DN rat model and used the SGLT-2 inhibitor (DAP) to observe the effects of DAP

on body weight, renal index, FBG, BUN, Scr, urine protein, urine albumin, urinary creatinine, and renal histopathological changes, as well as on the fibrosis-associated proteins, COL I, FN, TGF- β 1, and α -SMA, the autophagy-associated proteins, LC3 II, Beclin 1, and AMPK/mTOR signaling pathway-related protein expressions, to discuss its therapeutic effect, as well as to compare it with GLI, a commonly used drug in the clinical treatment of DN, in order to provide a reference for the degree of efficacy. The results showed that, compared with the model group, the body weight of rats in the DAP groups was increased; renal index, FBG, BUN, Scr, urine protein, urine albumin, urinary creatinine content were decreased; renal histopathological damage was reduced; LC3II, Beclin 1, Bcl-2 and AMPK-related protein expressions were up-regulated, and the expression of COL I, FN, TGF- β 1, α -SMA, Bax, mTOR-related proteins were down-regulated, indicating that DAP has an anti-diabetic nephropathy effect by promoting autophagy.

Autophagy is a highly conserved lysosomal degradation pathway, which is an important regulatory mechanism for maintaining intracellular homeostasis [29, 30]. Previous studies have found that persistent high glucose in DN inhibits the expression of autophagy-related protein (LC3, Beclin-1) expression in DN, reducing autophagy levels and impeding the clearance of damaged proteins and cytotoxic products produced by accumulation in the organelles, thus leading to renal injury and renal parenchymal cell dysfunction in DN [31, 32]. Activation of cellular autophagy can significantly inhibit high glucose-induced renal tubular

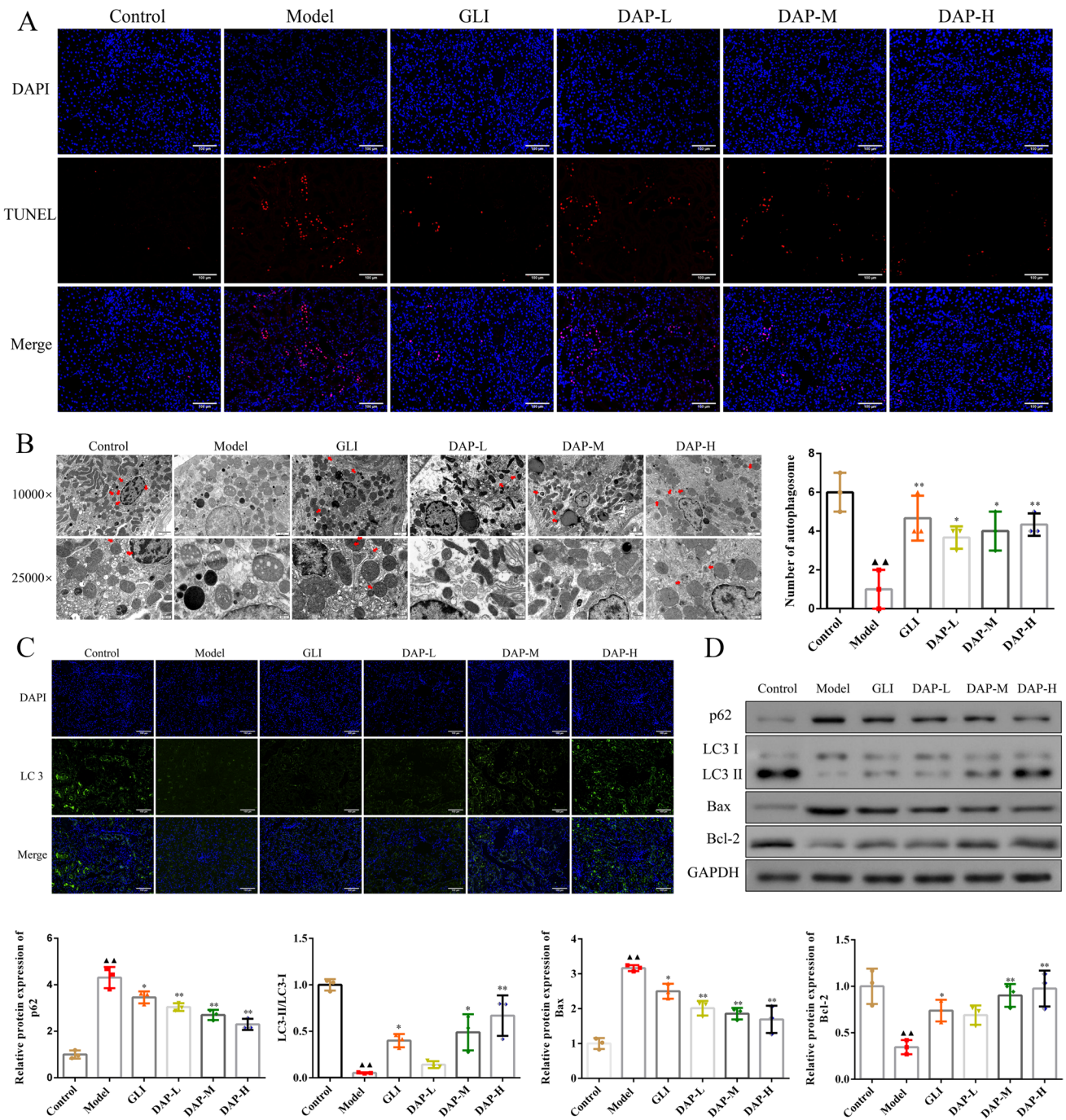


Fig. 5 Effect of DAP treatment on cell apoptosis and autophagy in the kidney of HFD/STZ induced DN rats. **A** Apoptotic cells in kidney tissue sections were measured with TUNEL staining, Scale bar, 100 μ m. **B** Representative TEM images of autophagosomes (red) in the kidney tissue sections of DAP-treated DN rats are presented (magnification 10,000 \times , 25,000 \times). **C** Immunofluorescence stain-

ing was used to detect LC3 expression in the kidney tissue sections of DAP-treated DN rats (Scale bar, 100 μ m). **D** Western blot was employed to assess the protein level of apoptosis and autophagy-related genes (Bax, Bcl-2; p62, LC3) in DAP-treated DN rats. $\blacktriangle\blacktriangle P < 0.01$ vs. control group. $*P < 0.05$, and $**P < 0.01$ vs. model group

epithelial cell injury and improve cellular function [33]. These studies suggest that the insufficient level of autophagy may be an important mechanism leading to the progression of DN.

The AMPK/mTOR signaling pathway is thought to play an important role in the regulation of cellular autophagy and senescence [34]. AMPK and mTOR via protein phosphorylation can regulate cellular metabolism and autophagy

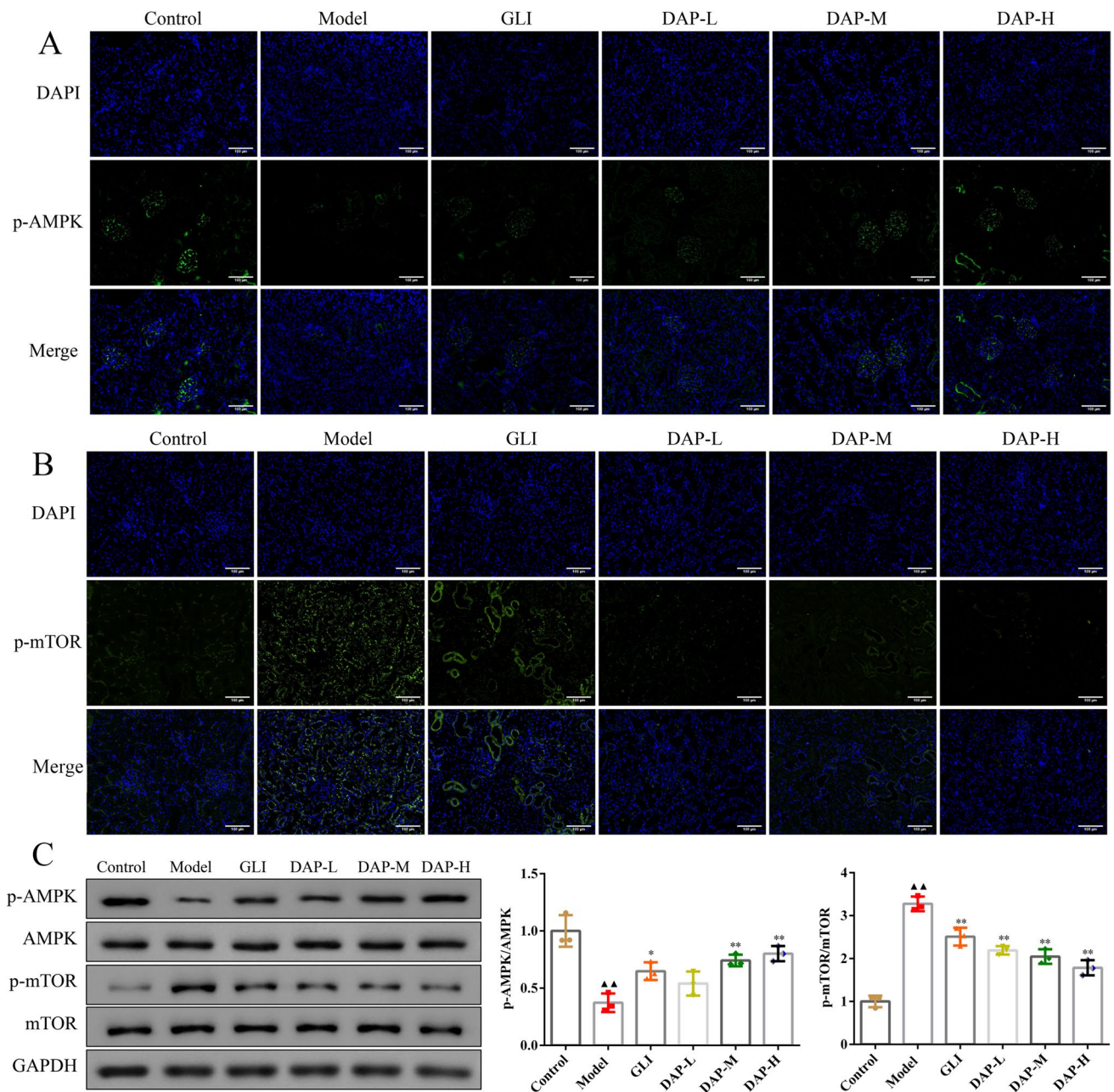


Fig. 6 Effect of DAP treatment on AMPK/mTOR pathway in the kidney of HFD/STZ induced DN rats. **A, B** Immunofluorescence staining was used to detect phosphorylation of AMPK (p-AMPK) and mTOR (p-mTOR) in the kidney tissue sections of DAP-treated

DN rats (Scale bar, 100 μ m). **C** Expressions of p-AMPK, AMPK, p-mTOR, and mTOR, as detected by Western blotting. $\blacktriangle\blacktriangle P < 0.01$ vs. control group. $*P < 0.05$, and $**P < 0.01$ vs. model group

levels, and inhibition of the mTOR kinase signaling pathway and activation of the AMPK signaling pathway can induce cellular autophagy to slow down the aging process and prolong the lifespan of the organism [35]. Ding et al. found that the level of autophagy in renal tubular epithelial cells was significantly increased in animal models of diabetes mellitus of both types 1 and 2 diabetes mellitus [36]. In STZ-induced diabetic rats, metformin increased renal

AMPK phosphorylation, reversed mTOR activation, and then inhibited renal hypertrophy. mTOR activity is regulated by growth factors, stress, energy status, and amino acids. mTOR is involved in autophagy, senses nutrient overload, and therefore inhibits autophagy. Rapamycin, a specific mTOR inhibitor, restored the autophagic activity of podocytes cultured under high glucose conditions, suggesting that mTOR may be involved in the inhibition of autophagy in

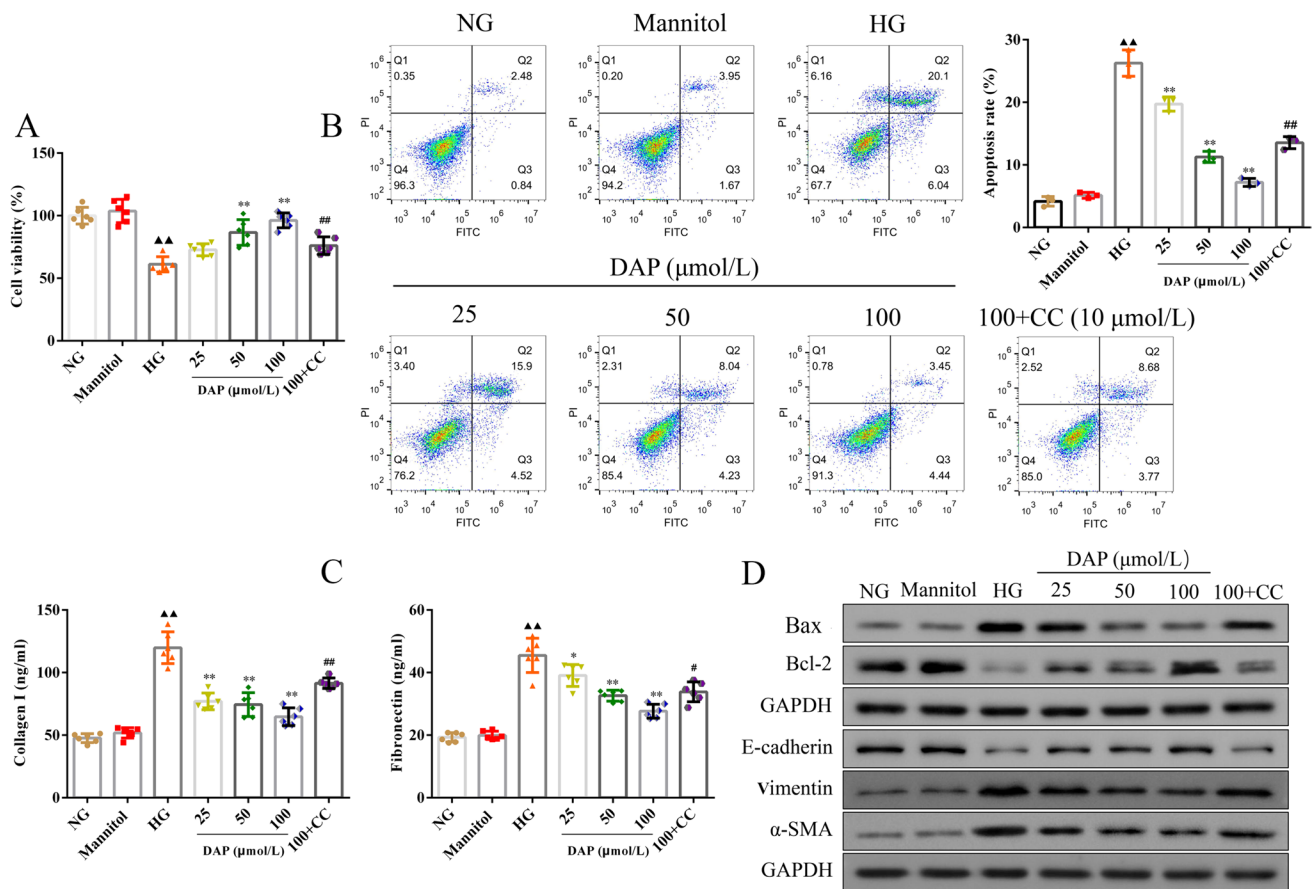


Fig. 7 Effect of DAP on cell viability, apoptosis, and ECM deposition in HG-mediated HK-2 cells. **A** Cell viability was assessed by CCK-8 assay ($n=6$ per group). **B** The apoptotic rate of HG-mediated HK-2 cells with DAP treatment was determined by flow cytometry using the Annexin-V FITC/PI staining. **C** The collagen I content

and Fibronectin content in the culture supernatant were detected by ELISA. **D** The protein levels of Bax, Bcl-2, E-cadherin, vimentin, and α -SMA were measured using Western blotting. $\blacktriangle\blacktriangle P<0.01$ vs. NG and mannitol group. $*P<0.05$, and $**P<0.01$ vs. HG group. $\#P<0.05$, and $\#\#P<0.01$ vs. DAP (100 $\mu\text{mol/L}$) group

podocytes under diabetic conditions [37]. Kume et al. demonstrated that mTOR signaling was highly activated in podocytes from diabetic kidneys of both humans and animals and that both pharmacological and genetic inactivation of mTOR ameliorated podocyte dysfunction in DKD [38]. Similarly, the results of the present study showed that the p-AMPK/AMPK ratio was significantly decreased and the p-mTOR/mTOR ratio was significantly increased in the model group of rats compared with the control group. Compared with the model group, the p-AMPK/AMPK ratio was significantly higher and the p-mTOR/mTOR ratio was significantly lower in the DAP-treated groups. Collectively, these results preliminarily suggest that DAP could inhibit renal fibrosis in DN rats by regulating cellular autophagy mediated by the AMPK/mTOR signaling pathway.

Furthermore, the cell viability of HG-induced HK-2 cells was also strengthened by the treatment of DAP, as demonstrated by CCK-8 assays. Flow cytometry showed that DAP treatment significantly reduced apoptosis of

HG-induced HK-2 cells in this study. Besides, Yang and Xu et al. found that DAP effectively mitigated advanced glycation end product-induced podocyte injury or HG-induced HK-2 cells through upregulation of autophagy by targeting AMPK/mTOR pathway [39, 40]. To further clarify the role of cellular autophagy mediated by the AMPK/mTOR signaling pathway in DAP treatment of diabetic kidney injury. In this experiment, we administered the AMPK inhibitor (Compound C, CC) to HG-mediated HK-2 cells in the DAP-treated group, and the results showed that the AMPK inhibitor was able to reverse the effect of DAP in HG-mediated cellular damaging, which was mainly manifested as a decrease in the p-AMPK/AMPK ratio, an increase in the p-mTOR/mTOR ratio, LC3-II/LC3-I ratio, ECM deposition and apoptosis. Therefore, these results also confirm that our results are consistent with the literature reports, indicating that DAP can play a therapeutic role in DN by regulating the AMPK/mTOR signaling pathway.

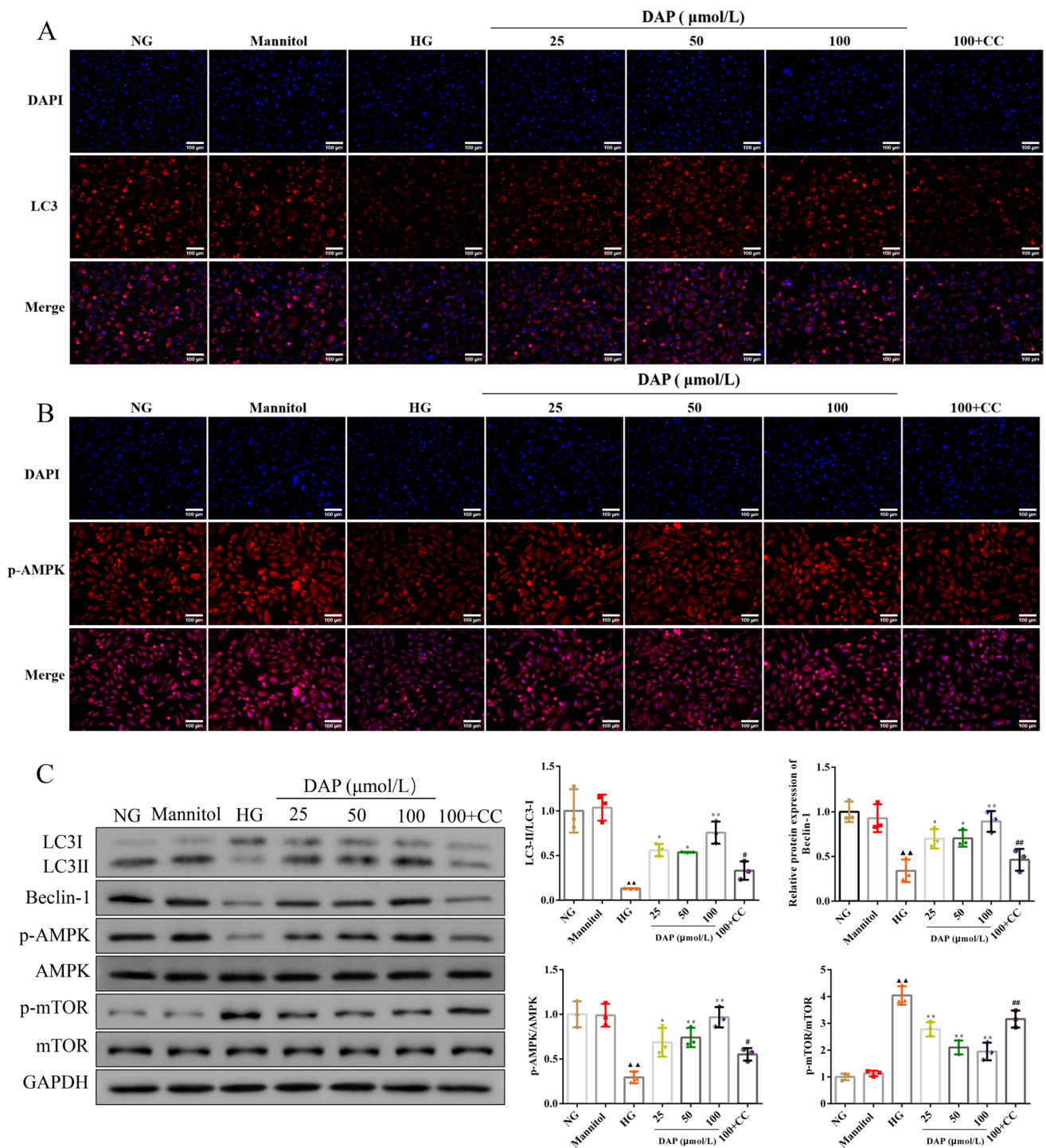


Fig. 8 Effect of DAP on autophagy and AMPK/mTOR pathway-associated targets in HG-mediated HK-2 cells. **A** Immunofluorescence staining was used to detect LC3 expression and **B** phosphorylation of AMPK in HG-mediated HK-2 cells. **C** Western blots were conducted

to determine LC3, Beclin-1, p-AMPK, AMPK, p-mTOR, and mTOR expression in the HG-mediated HK-2 cells. $\blacktriangle\blacktriangle P < 0.01$ vs. NG and mannitol group. $*P < 0.05$, and $**P < 0.01$ vs. HG group. $\#P < 0.05$, and $\#\#P < 0.01$ vs. DAP (100 $\mu\text{mol/L}$) group

Conclusions

In summary, DAP was able to alleviate DN renal tissue injury, and its mechanism may be related to the autophagy of

renal tissues mediated through the regulation of the AMPK/mTOR signaling pathway, which might provide more objective theoretical support for the use of SGLT-2 inhibitors in the clinical treatment of DN.

Author contributions Yu-Yan Ye and Yun Chen conceived and designed the study. Yu-Yan Ye, Yun Chen, Jing Yang, Jie Wu, and Peng Wang performed experimental analysis. Yu-Yan Ye supervised all the research and acquired the fund. Yu-Yan Ye wrote the first draft of the manuscript. Yu-Yan Ye and Yun Chen critically revised the manuscript. Yun Chen improved the language the manuscript. All authors have significantly participated in the manuscript preparation and approved the final version of the manuscript.

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Data availability The data used to support the findings of this study are available from the corresponding author upon request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All experiments were approved by the Experimental Animal Ethical Committee of Zhejiang Eyong Pharmaceutical Research and Development Center and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Informed consent Not applicable.

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