Diabetes, Metabolic Syndrome and Obesity

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ORIGINAL RESEARCH

Inhibition of Caspase-3/GSDME Pathway-Mediated Pyroptosis of Renal Tubular Epithelial Cells by Dagliflozin in the Pathogenesis of Diabetic Kidney Disease and Study of Its Mechanism

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Objective: Investigating the effects and mechanisms of dapagliflozin on pyroptosis of renal tubular epithelial cells under high-glucose conditions through the regulation of the Caspase-3/GSDME signaling pathway, providing experimental evidence for the clinical treatment of diabetic kidney disease.

Methods: Human renal tubular epithelial cells (HK-2) were cultured in vitro and divided into control group (5mmol/L D-glucose), high-glucose group (30mmol/L D-glucose), dagliflozin group (2.5μmol/L dagliflozin), and monoinhibitor group (20μmol/L caspase-1 inhibitor), dual inhibitor group (20μmol/L caspase-1 inhibitor + 50μmol/L caspase-3 inhibitor), and SiRNA transfection group. All groups were intervened for 48h. The cell viability was detected by cell counting kit-8 and the glucose and dagliflozin concentrations of the intervention were determined. Caspase-1, caspase-3, GSDMD, GSDME, GSDME-N, caspase-8, NF-κB were detected by Western blot. Detection of cellular pyroptosis in each group by flow cytometry.

Results: Compared with the control group, the D-glucose group showed decreased cell viability, increased cell pyroptosis, and increased levels of caspase-1, caspase-3, GSDMD, GSDME, GSDME-N, caspase-8, NF-κB, and other related proteins ($P<0.05$). Compared with the D-glucose group, the rate of cellular pyroptosis and the levels of caspase-1, caspase-3, GSDMD, GSDME, GSDME-N and other related proteins were decreased in the dagliflozin group and the dual inhibitor group ($P<0.05$). Compared with the transfected control group, the cellular pyroptosis rate and the levels of caspase-1, caspase-3, GSDMD, GSDME, GSDME-N, and other related proteins were then further reduced in the transfected group targeting SGLT2 knockdown ($P<0.05$).

Conclusion: In the proximal tubular cells of diabetic kidney disease, dagliflozin inhibited high glucose-induced pyroptosis of human HK-2, and its mechanism of action may be related to the inhibition of caspase 3/GSDME pathway signaling.

Keywords: diabetic kidney disease, renal tubular epithelial cells, dagliflozin, pyroptosis

Introduction

The prevalence of type 2 diabetes mellitus (T2DM) has been increasing in recent years, and it is now recognized that approximately 40% of patients with T2DM will develop diabetic kidney disease(DKD), which is the most significant contributor to the dramatic increase in end-stage renal disease (ESRD) worldwide. Globally, more than 3 million patients are currently being treated for ESRD and this is expected to increase to more than 5 million patients by 2035. Therefore, the search for new therapeutic agents for the treatment of diabetes mellitus to slow down the progression of DKD is a hot research topic both at home and abroad. There have been reports that vulpinic acid (VA) carnosic acid (CA) and usnic acid (UA) could be useful in the treatment of diabetic complications.[1] And Inhibition of AR (aldose reductase) is also considered a potential approach for treating diabetes-related issues.[2] Recently, it has been found that sodium-glucose co-

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transporter protein 2 inhibitor (SGLT-2i) reduces blood glucose by inhibiting proximal tubular glucose reabsorption, improving glomerular hyperfiltration, and increasing urinary glucose excretion, and also slows down the progression of renal function through various mechanisms such as lipid-lowering, blood pressure-lowering, uric acid-lowering, and reduction of body weight, sodium homeostasis and volume status, and inhibition of oxidative stress, which makes it a new and important therapeutic T2DM and DKD It has become a new important target drug for the treatment of T2DM and DKD.

Pyroptosis is a recently discovered form of lysosomal programmed cell death that can exacerbate several complications of diabetes, including diabetic kidney disease. In recent years, an increasing number of studies have shown that different modes of cell death, such as pyroptosis, apoptosis, and necrosis, are key factors in glomerular and tubular injury in the kidneys of DKD.[3] Pyroptosis is different from apoptosis and necrosis and is a lytic inflammatory form of cell death. By disrupting the integrity of the cell membrane, it causes cell rupture and death, and releases a large number of inflammatory factors to exert its biological effects. When cells undergo pyroptosis, oligomeric pores are formed in the cell membrane, leading to the release of cellular contents, and morphological changes such as cell swelling, organelle deformation, and cell membrane pore formation. Pyroptosis mainly triggers lytic cell death through two pathways: one is the canonical pyroptosis pathway mediated by Caspase-1, and the other is the non-canonical pyroptosis pathway mediated by Caspase-4/5/11. Both pathways achieve the lytic cell death process by activating GSDMD (Gasdermin D).

With the deepening of research, scholars have found that the mechanisms of pyroptosis are not limited to caspase-1 dependence but can also be triggered by the activation of other caspases, such as caspase-4, caspase-5, and caspase-11. Even when the GSDMD/Caspase-1 pathway is inhibited, pyroptosis can still occur under pathological conditions, and it is worth further studying whether there are other pyroptosis pathways. It has been reported that lipopolysaccharide (LPS) can simultaneously activate the Caspase-1/GSDMD and Caspase-3/GSDME pathways, inducing pyroptosis in synovial fibroblasts and thus promoting the pathogenesis of rheumatoid arthritis.[4] Blocking the Caspase-1/GSDMD and Caspase-3/GSDME pyroptosis pathways can rescue mice from silicosis.[5] It has also been found that the combination of apatinib and melittin can induce pyroptosis through the Caspase-1/GSDMD and Caspase-3/GSDME axes, thereby exerting synergistic antitumor effects.[6]

The caspase signaling pathway has been shown to be involved in a variety of renal diseases, including the pathogenesis of DKD.[7,8] Of the 14 known caspase family members identified to date, several actuator caspases, including caspase-3, may be involved in the ultimate degradation of intracellular proteins.[9] Activation of caspase-3 cleaves a variety of downstream substrates, leading to the morphological and biochemical changes typical of apoptotic cells, including cell shrinkage, chromatin condensation, DNA breaks, and externalization of phosphatidylserine from the inner plasma membrane. When GSDME is highly expressed, active caspase-3 cleaves the N-terminal structural domain of GSDME and executes pyroptosis by forming a non-selective pore in the membrane, changing the mode of cell death from apoptosis to pyroptosis.[10–12]

SGLT2 inhibitors decreased the accumulation of nuclear NF-κB p65 in high glucose-treated HK-2 cells,[13] and it has been reported that the increased expression of NF-κB can lead to an increase in the production of pro-inflammatory cytokines, cause the activation of caspase-8, and then induce cellular pyrokinesis through the caspase-3/GSDME pathway, which was investigated and progressed in in vitro cellular and in vivo experiments in rats. It has been reported that the combination of ticagrelor and dapagliflozin has an additive effect on improving DKD in T2DM mice. Compared with diabetic controls, dapagliflozin can also improve histopathological examination, inflammatory and apoptotic markers in a dose-dependent manner, which suggests that SGLT2i may have nephroprotective effects.[14] In addition, in fructose-streptozotocin-induced diabetic rats, dapagliflozin can improve early markers of DKD.[15] Dapagliflozin can reduce the levels of ROS, IL-8, TGF-β in renal tubular epithelial cells and exert a protective effect against high glucose-induced apoptosis, indicating that SGLT2i can improve renal tubular oxidative stress, inflammation and fibrosis.[16] There are reports that SGLT2i can protect the kidneys by reducing ROS levels mediated by hyperglycemia and regulating autophagic flux.[17] However, there is a scarcity of data on the effects and mechanisms of SGLT2i on pyroptosis of renal tubular epithelial cells under high-glucose conditions.

In this study, we investigated whether the caspase-3/GSDME pathway induced cellular pyroptosis in renal tubular epithelial cells under high glucose status, and clarified the pathway mode of action and the regulatory role of dagliflozin,

with the aim of confirming that dagliflozin can inhibit the caspase-3/GSDME signaling pathway to reduce cellular pyroptosis and protect HK-2 cells.

Materials and Methods

Reagents and Antibodies

Z-DEVD-FMK(caspase-3 inhibitor HY-12466) and Z-YVAD-FMK(caspase-1 inhibitorHY-P1009)were obtained from MedChemExpress. GSDME-N(ab215191) antibodies was obtained from Abcam(Cambridge, UK); caspase-3(14220), caspase-1(3866), caspase-8(4790), NF-κBp65(4764), GSDME(19453), GSDMD(39754) antibodies were obtained from Cell Signaling Technology, Inc. β-actin (BL005B) antibodies from Biosharp; anti-rabbit IgG (H + L) HRP (W401B) from Biosharp. Apoptosis kit(BB-4101)was purchased from Shanghai Bebo Biotechnology Co., Ltd. Lipofectamine 6000(C0526) was purchased from Beyotime Biotech Inc. Small interfering (si)RNA was purchased from Beijing Tsingke Biotech Co., Ltd. dapagliflozin was purchased from AstraZeneca(Cambridge, UK). All cell culture reagents were obtained from Wuhan Pricella Biotechnology Co., Ltd.

Cell Culture

Proximal tubule epithelial HK2 cells (CL-0109)were purchased from Pricella Cells were cultured in DMEM at 37 °C and 5% CO2, supplemented with 10% FBS (164210, Wuhan Pricella Biotechnology Co., Ltd.) and 100 units/mL penicillin-streptomycin (PB180120, Wuhan Pricella Biotechnology Co., Ltd)and cultured in DMEM (PM150220, Wuhan Pricella Biotechnology Co., Ltd). When 70–80% confluent, the cells were cultured with high glucose stimulation in 30 mm glucose containing medium (HG) for 48 h. Monoinhibitor, Dual Inhibitor incubation for 48 h, and the SGLT2 inhibitor dapagliflozin was used at a final concentration of 2.5μM for 48h.

Western Blotting

Proteins of HK2 cells were prepared using RIPA buffer (P0013B Beyotime Biotechnology, China) supplemented with protease inhibitor and phosphatase inhibitor (1:100 dilution). After centrifugation (12,000 rpm at 4 °C for 15 min), supernatants were collected, and protein concentration was measured by the BCA protein assay kit(P0010S, Beyotime Biotechnology, China). Protein samples from cell were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a PVDF membrane (Millipore, USA). After blocking with Protein Free Rapid Blocking Buffer(PS108 Epizyme Biotech, China) in Tris-buffered saline solution containing 0.05%Tween 20 for 1 h at room temperature and overnight incubation with specific primary antibodies (1:1000) at 4 °C, the membranes were rinsed with TBST buffer (0.05% Tween 20, 0.2 mm Tris, and 137 mm NaCl) and exposed to IgG-HRP-conjugated secondary antibody (1:12000) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescent substrate (Millipore, USA). The bands were quantified densitometrically analyzed by Image J software 6.0 (National Institutes of Health, Bethesda, MD, USA) and normalized to the β-actin level.

Transfection of Small Interfering RNAs (siRNAs)

HK-2 cells were cultured in six-well plates, and when 70–80% confluent, the cells were transfected with siRNA targeting SGLT2 (#289404, 200 nM), or a negative control siRNA (AM4635, siCON) with Lipofectamine 6000 (C0526-1.5mL, Beyotime Biotechnology, China). The cells were incubated for 6 h with Opti-MEM(11058021, Thermo Fisher Scientific, San Jose, CA, USA), and then the medium was replaced with DMEM with HG (30 mm) for 48 h. Real-time fluorescence quantitative PCR to detect the relative expression level of SGLT2. SGLT2-hum-siRNA and NC-SiRNA were purchased from Beijing Tsingke Biotech Co., Ltd. Sequences for SGLT2-hum-siRNA were as follows:

sense, 5′-CGGGUCUCUUCGACAAAUATT-3′;

antisense,5′-UAUUUGUCGAAGAGACCCGTT-3′.

Detection of Apoptosis by Flow Cytometry

According to the experimental groups, each group of HK-2 cell suspension was inoculated into 6-well plates at a density of 5×104 cells per well, and each group of cells was set up with 3 replicate wells, and the inoculated HK-2 cells were

cultured at 37 °C with 5% CO2 for 48 h. The cells of each group were collected and digested with 0.25% trypsin, and then washed with pre-cooled phosphate buffer for three times. The cells were digested with 0.25% trypsin and then centrifuged at 300×g for 5 min. The precipitate was collected and washed three times with pre-cooled phosphate buffer. The cells were resuspended with 1X Annexin V conjugate at a concentration of approximately 1×106 cells/mL. 5 μL of Annexin V-FITC solution was added to each group of cells and mixed well, and then 5 μL of propidium iodide was added for 20 min, and then stained with PI for 5 min at 4 °C, and then the cells were examined for pyrogenic death rate by flow cytometer.

Statistical Analysis

All data were presented as mean ± SEM (standard error of mean) with n representing the number of different experiments. Image J Acquisition and Analysis Software were used to analyze the Western blotting results. The statistical significance of the differences between two groups was obtained by unpaired *t* tests. Differences among three or more groups were analyzed by one-way ANOVA or two-way ANOVA, followed by Fisher's LSD test. Statistical analysis was carried out by GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). P values less than 0.05 were considered to be statistically significant.

Results

Excessive Cellular Pyroptosis of HK-2 Cells in vitro Under High Glucose Status

Detection of Cellular Pyroptosis Levels in Cell Models at the Cellular Level

Flow cytometry assay was performed using Annexin V-FITC/PI kit staining for flow cytometry detection. Cellular pyroptosis occurred with perforation of the cell membrane and an increase in the double-positive cells of AV(+)/PI(+), rather than the Annexin V single-positive (lower right quadrant) as demonstrated by early apoptosis, which was consistent with the phenomenon of pyroptosis. The results of this experiment showed that the percentage of cells in the double-positive area of AV(+)/PI(+) was significantly increased in the high-glucose group compared with the normal group ($P<0.01$), and the proportion of pyroptosis cells was significantly increased.(Figure 1)

Detection of Cellular Pyroptosis Levels in Cell Models at the Molecular Level

WB results showed that the expression of pyrogenesis markers GSDMD, GSDME, GSDME-N, caspase-1, caspase-3, caspase-8, and NF-κB proteins were increased in the hyperglycemic cell model (Figure 2) Semi-quantitative analysis of

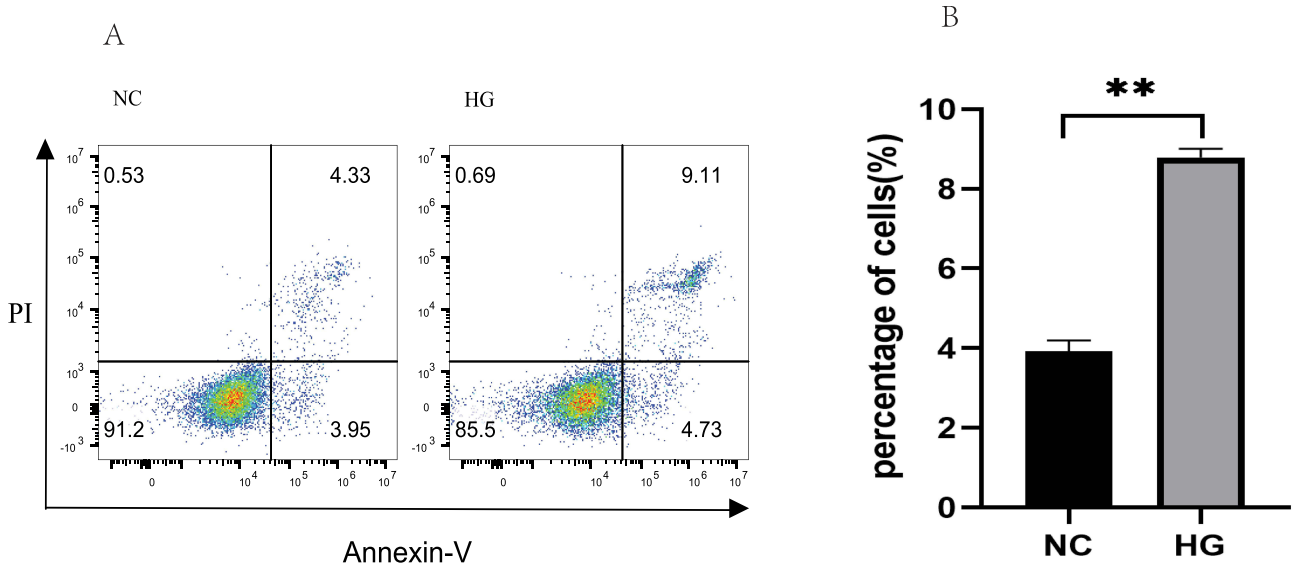

Figure 1 (**A**) Flow cytometry to detect pyroptosis levels in cell models of high glucose and normal groups respectively.n=3. (**B**) Statistical analysis results of percentage of double positive cells for AV(+)/PI(+).n=3. **$P<0.01$.
Abbreviations: NC, negative control; HG, high glucose.

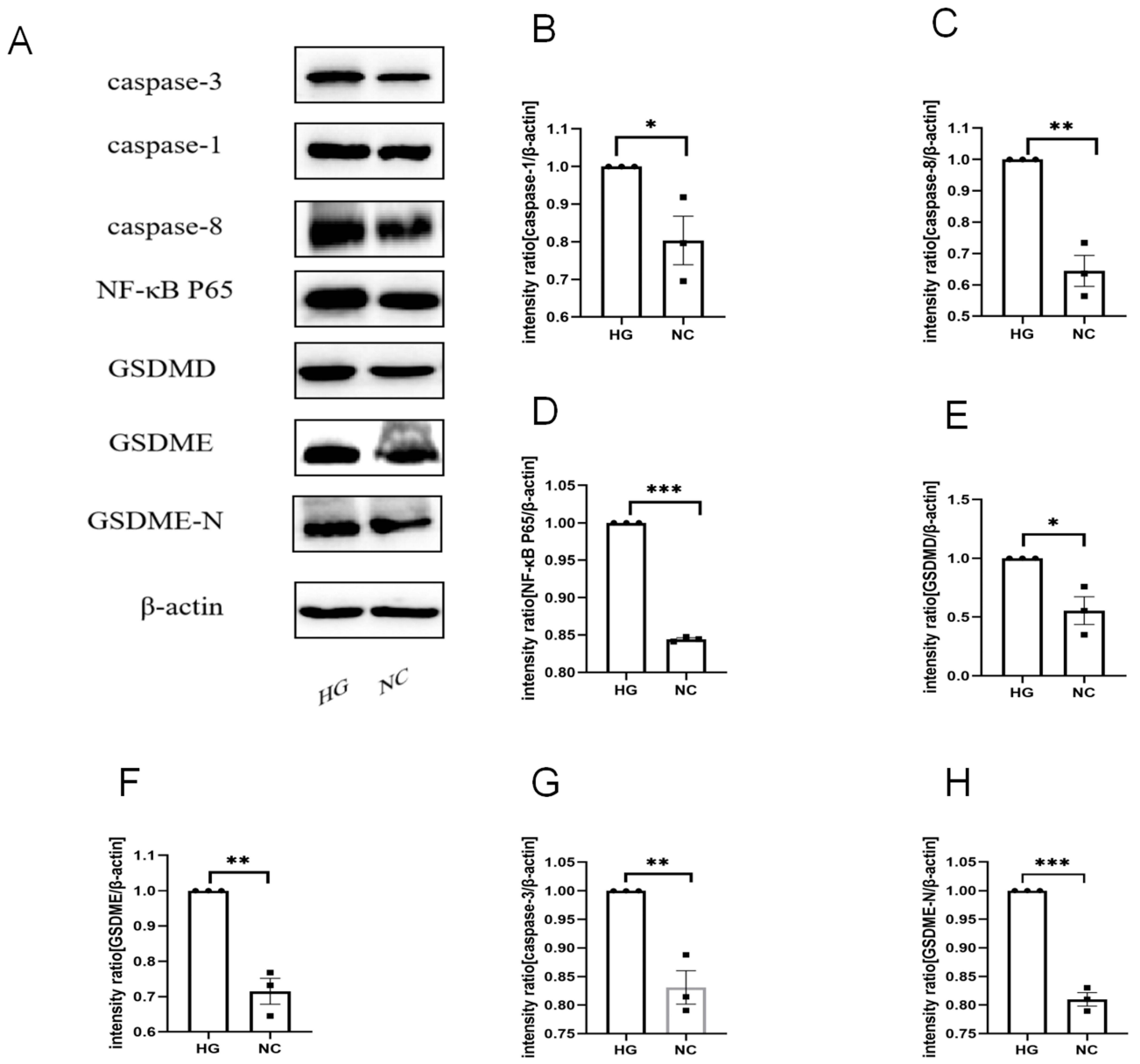

Figure 2 (**A**) Western Blot was applied to detect the expression of GSDMD, GSDME, GSDME-N, caspase-1, caspase-3, caspase-8, NF-κB pyroptosis proteins in the high glucose group and control group, respectively.n=3.; (**B**–**H**) relative quantitative analysis of the expression of caspase-1, caspase-8, NF- κB, GSDMD, GSDME, caspase-3, GSDME-N relative quantitative analysis.n=3. **P*<0.05,***P*<0.01, ****P*<0.001.
Abbreviations: NC, negative control; HG, high glucose.

the above proteins was statistically significant at $P<0.05$. In summary, the high glucose cell model showed a significant increase in the level of cellular pyroptosis compared with normal cells.

The Caspase-3/GSDME Pathway, a Representative of the Non-Classical Pyroptosis Pathway, Leads to Excessive Pyroptosis of Renal HK-2 Cells in the State of Diabetic Nephropathy

HK-2 cells were cultured in vitro with caspase-1 inhibition, and pyroptosis still appeared after inhibition of classical caspase-1/GSDMD, confirming that pyroptosis in HK-2 cells is related to the caspase-3/GSDME pathway; activation of caspase-3 mediates cleavage of GSDME, which is responsible for the formation of the plasma membrane pore, and thus

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induces pyroptosis; GSDME-induced pyroptosis plays a critical role in diabetic nephropathy. GSDME-induced cell death plays a key role in diabetic nephropathy.

Detection of Cellular Pyroptosis Levels in Cell Models at the Cellular Level

Flow cytometry assay was performed using Annexin V-FITC/PI kit staining for flow cytometry, cellular pyroptosis occurred with perforation of the cell membrane and an increase in double-positive cells of AV(+)/PI(+). The results of this experiment showed that the percentage of cells in the double-positive area of AV(+)/PI(+) was significantly increased in the high-glucose group compared with the normal group ($P<0.001$), and the percentage of apoptotic cells was significantly increased. Compared with the high-glucose group, the percentage of cells in the double-positive area decreased after application of dual inhibitors of caspase-1 and caspase-3 ($P<0.05$) (Figure 3), and the rate of apoptotic cells decreased. The above results suggest that the caspase-3/GSDME pathway can lead to excessive cellular pyroptosis of renal HK-2 cells.

Detection of Cellular Pyroptosis Levels in Cell Models at the Molecular Level

Western blot results showed that the protein expression of GSDMD, GSDME, GSDME-N, caspase-1 and caspase-3, markers of pyroptosis, was significantly higher than that of the normal group at high glucose, and after the application of caspase-1 inhibitor, the protein expression of caspase-1 and GSDMD were decreased, and that of GSDME, GSDME-N, GSDME, GSDME-N, and caspase-3 protein expression did not change significantly. GSDME, GSDME-N, caspase-3, caspase-1, GSDMD protein expression decreased after application of dual inhibitors (caspase-1 inhibitor and caspase-3 inhibitor) and dagliflozin intervention, respectively(Figure 4). Semi-quantitative analysis of the above proteins was statistically significant at $P<0.05$.

In conclusion, HK-2 cell pyroptosis in high glucose state is related to caspase-3/GSDME pathway. Dagliflozin was tentatively considered to exert a renal protective effect by inhibiting caspase-3/GSDME pathway-mediated renal tubular epithelial cell pyroptosis. Caspase-3 activation mediated the cleavage of GSDME, which is responsible for plasma membrane pore formation, and thus induced pyroptosis.

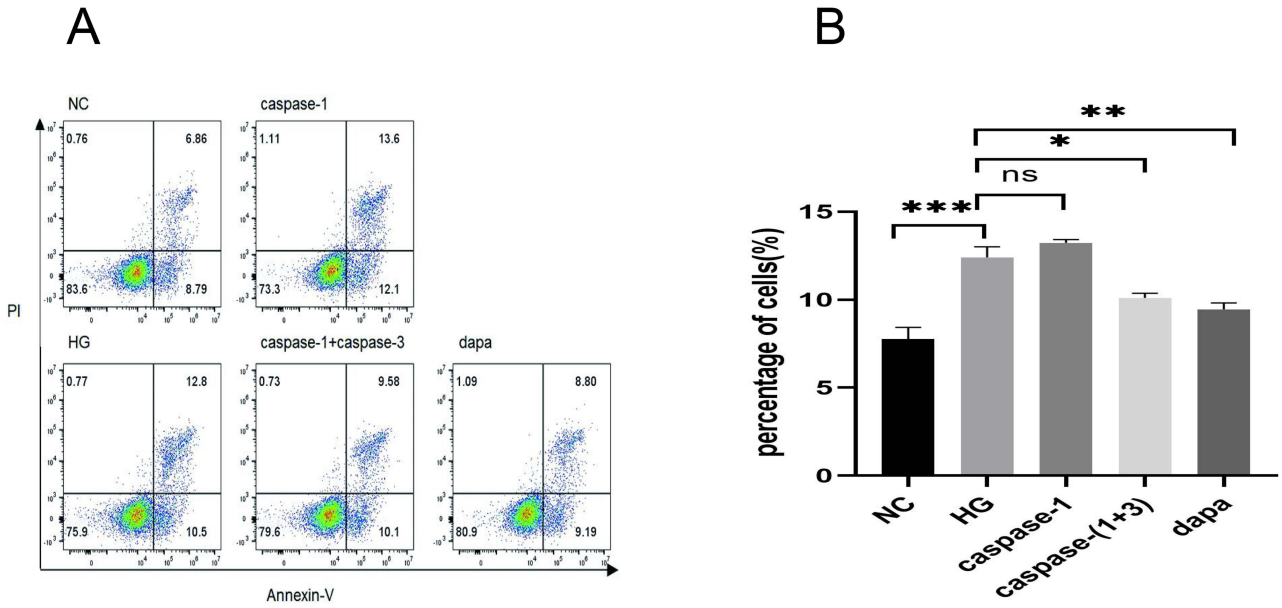

Figure 3 (**A**) Flow cytometry to detect the level of cellular pyroptosis in the cell models of high glucose group and control group, caspase-1 mono-inhibitor group, caspase-1 +caspase-3 dual-inhibitor group, and dagliflozin group, respectively.n=3. (**B**)Statistical analysis of the percentage of double positive cells for AV(+)/PI(+).n=3. ns $P>0.05$, *$P<0.05$,**$P<0.01$, ***$P<0.001$.

Abbreviations: NC, negative control; HG, high glucose; caspase-1, caspase-1 mono-inhibitor; caspase-(1+3), dual inhibitor of caspase-1 and caspase-3.

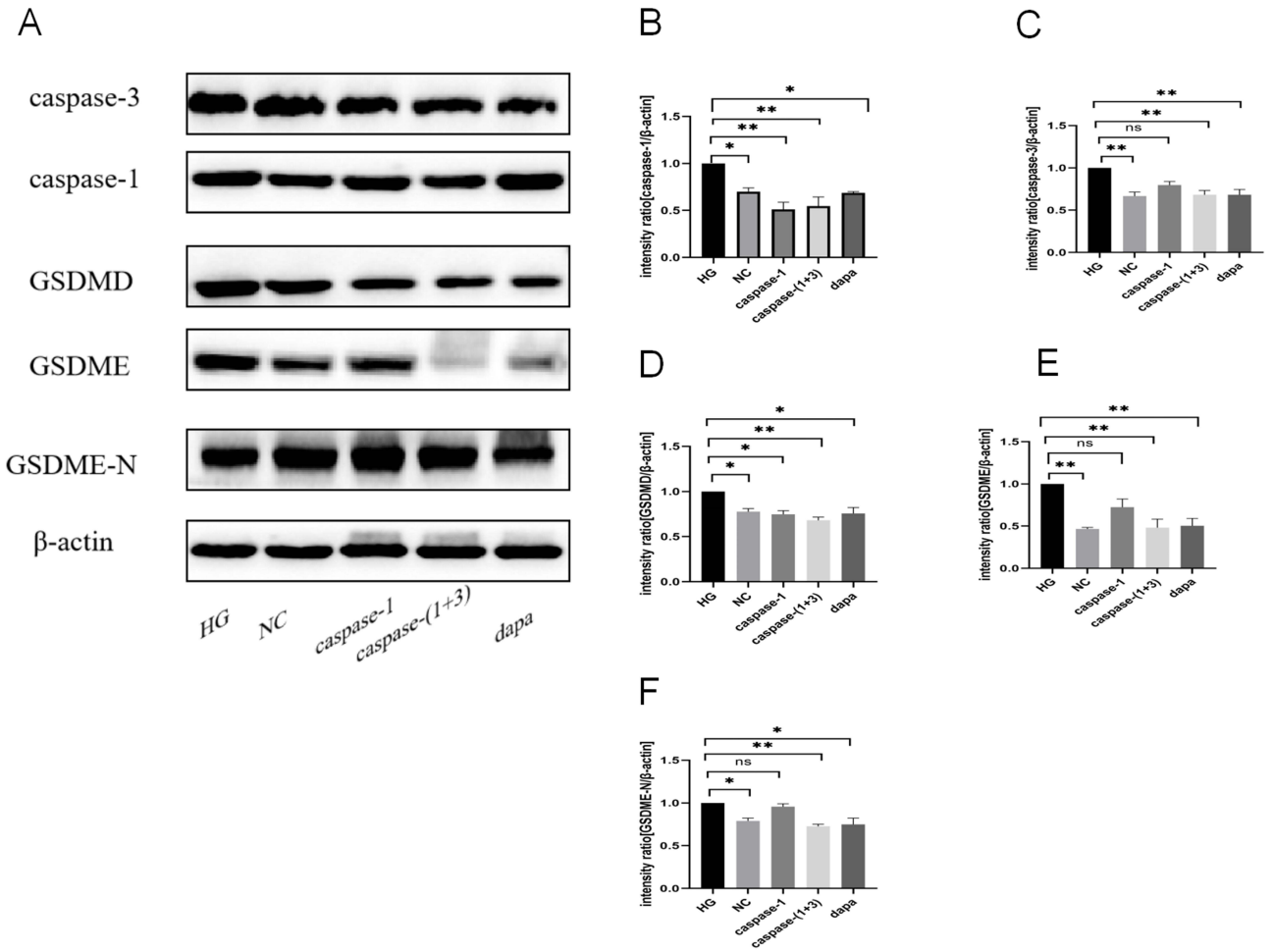

Figure 4 (**A**) Western Blot was applied to detect the expression of GSDMD, GSDME, caspase-1, caspase-3, GSDME-N, and other pyroptotic proteins in the high-glucose group and the control group, the caspase-1 mono-inhibitor group, the caspase-1+caspase-3 dual-inhibitor group, and the dagliflozin group, respectively.n=3; (**B-F**) Relative quantitative analysis of caspase-1, caspase-3, GSDMD, GSDME, and GSDME-N.n=3. ns $P>0.05$,*$P<0.05$,**$P<0.01$.
Abbreviations: NC, negative control; HG, high glucose; caspase-1, caspase-1 mono-inhibitor; caspase-(1+3), dual inhibitor of caspase-1 and caspase-3; dapa, dapagliflozin.

Dagliflozin Slows Renal Disease Progression by Reducing HK-2 Cell Pyroptosis in Diabetic Nephropathy in vitro Cultures Through Inhibition of the Caspase-3/GSDME Pathway

Detection of Cellular Pyroptosis Levels in Cell Models at the Cellular Level

Flow cytometry assay was performed using Annexin V-FITC/PI kit staining for flow cytometry detection, cellular pyroptosis occurred with perforation of the cell membrane and an increase in double-positive cells of AV(+)/PI(+). The results of this experiment showed that the percentage of cells in the double-positive area of AV(+)/PI(+) was significantly reduced in the transfected group compared with the high-glucose group ($P<0.01$), and the percentage of apoptotic cells was significantly reduced. Compared with the control transfection group, the percentage of cells in the double-positive region decreased after knockdown of SGLT2 ($P< 0.05$) (Figure 5), and the rate of apoptotic cells decreased.

Detection of Cellular Pyroptosis Levels in Cell Models at the Molecular Level

After knocking down SGLT2 expression in HK-2 cells by SiRNA transfection, Western blot was used to detect the level of cellular pyroptosis, and the results showed that the knockdown group showed a decrease in the expression of GSDME, caspase-3, caspase-1, and GSDMD proteins when compared with the control group. GSDME, GSDME-N, caspase-3, caspase-1, and GSDMD protein expression were significantly decreased in the knockdown group compared with the high glucose group (Figure 6). Semi-quantitative analysis of the above proteins was statistically significant at $P<0.05$. The mRNA expression level of knockdown SGLT2 group was detected by quantitative PCR. The above results indicated that

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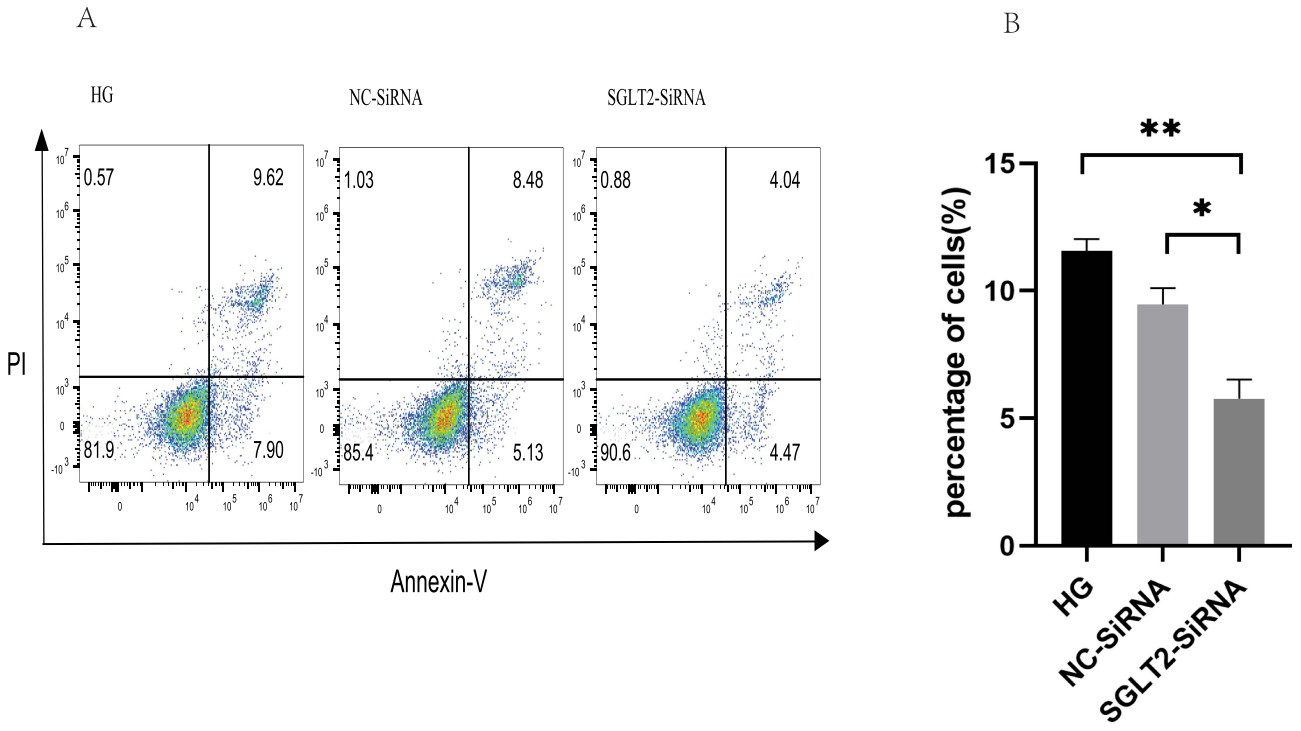

Figure 5 (**A**) Flow cytometry to detect the level of cellular pyroptosis in the cell model of high glucose group with NC-SiRNA and SGLT2-SiRNA group respectively n=3. (**B**)Statistical analysis results of the percentage of double positive cells for AV(+)/PI(+) n=3. *P<0.05,**P<0.01.
Abbreviations: HG, high glucose; NC-SiRNA, control siRNA; SGLT2-SiRNA, SGLT2 siRNA.

knocking down SGLT2 could inhibit renal tubular epithelial cell scorched death and play a protective role against diabetic nephropathy.

In conclusion, dagliflozin may exert a protective effect against renal injury by inhibiting the activation of the caspase-3/GSDME pathway.

Discussion

In recent years, although the development of clinical treatment for DKD has made great strides, the pathogenesis of DKD is complex, and there is currently a lack of effective treatments that can inhibit its progression and its exacerbation of renal failure still requires urgent attention. So far, angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor II antagonists (ARBs) are the only drugs that can reduce the risk of progression of chronic kidney disease. However, neither drug has been shown to reduce the risk of all-cause mortality. In order to effectively control and delay renal disease progression, it is important to consider the multifactorial mechanisms that lead to the progression of nephrogenic kidney disease. In the early stages of DKD, the importance of renal tubular lesions is increasingly recognized. Therefore, an in-depth exploration of the pathogenesis of DKD and the role of renal tubular lesions will help identify new therapeutic strategies to slow disease progression.

Renal tubular epithelial cells (TECs) are the most common cell type in renal tubules and perform various regulatory functions under different pathophysiological conditions. When the kidneys are exposed to a high glucose environment, TECs are the initial site of injury and are susceptible to metabolic disturbances that induce oxidative stress and secretion of multiple cytokines,[18,19] leading to interstitial inflammation and renal fibrosis. Considering the initial site of injury, the renal tubules are considered not only as target tissues suffering from injury, but also as drivers of induced renal disease.

In recent years, the role of Caspase-3/GSDME in promoting kidney diseases has been identified. Caspase-3 can specifically cleave GSDME, and the resulting GSDME-N fragment can penetrate the cell membrane, thereby inducing pyroptosis. Zhang et al confirmed that Caspase-3-mediated GSDME can induce pyroptosis in breast cancer cells.[20] Another study found that knocking down GSDME can inhibit the function of the Caspase-3/GSDME pathway, thereby inhibiting tubular cell pyroptosis and improving renal function.[21] These studies provide clues for exploring other pathways that regulate pyroptosis beyond the Caspase-1/GSDMD pathway.

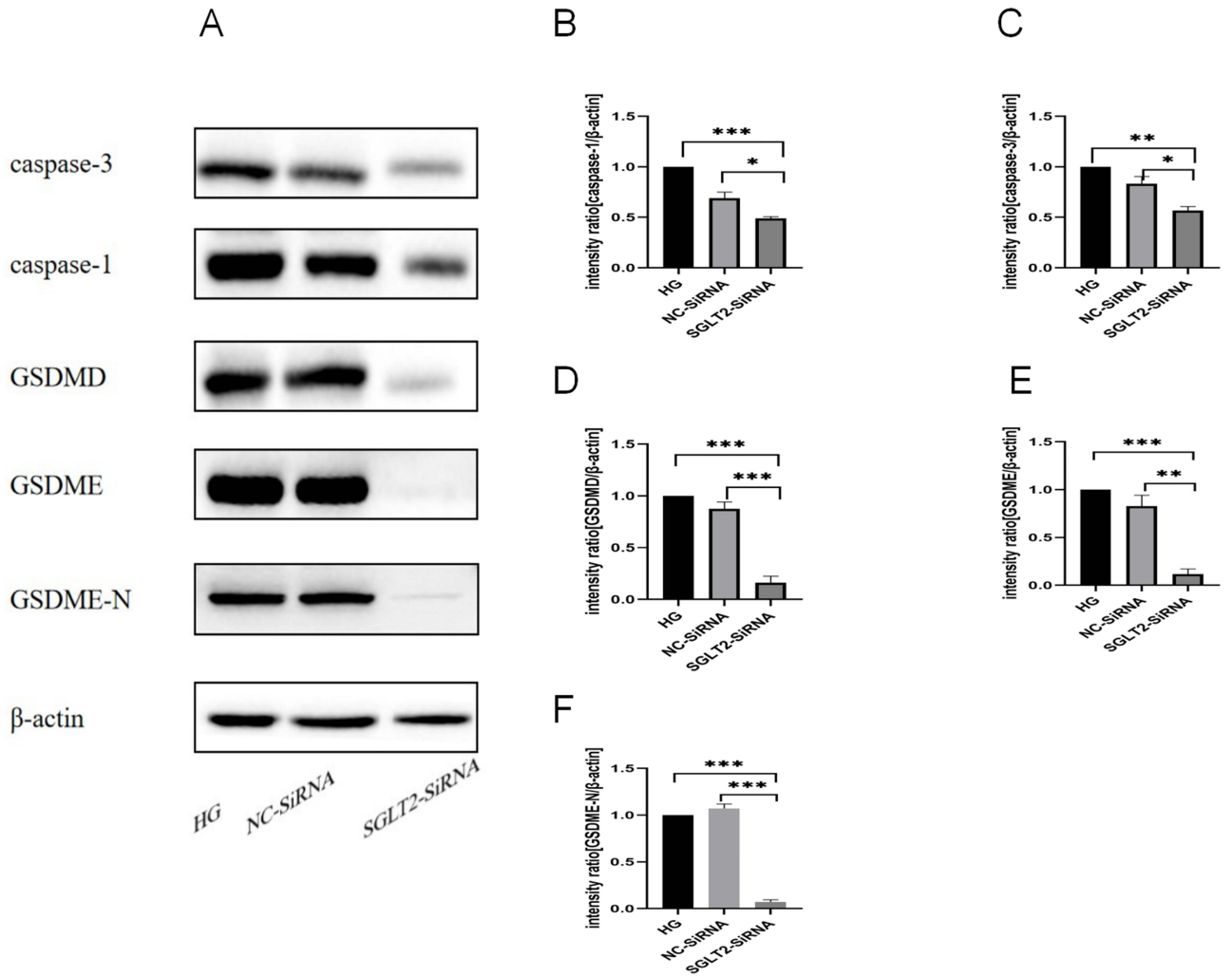

Figure 6 (**A**) Western Blot was applied to detect the expression of cellular pyroptosis proteins such as GSDMD, GSDME, caspase-1, caspase-3 in the high-glucose group versus the NC-SiRNA and SGLT2-SiRNA groups, respectively n=3; (**B**–**F**) caspase-1, caspase-3, and GSDMD, GSDME, GSDME-N relative quantitative analysis.n=3. **P*<0.05, ***P*<0.01, ****P*<0.001.

Abbreviations: HG, high glucose; NC-SiRNA, control siRNA; SGLT2-SiRNA, SGLT2-SiRNA.

The characteristic feature of pyroptosis is the disruption of cell membrane integrity, leading to the release of cellular contents. In this study, the occurrence rate of pyroptosis was assessed using an Annexin V-FITC/PI kit. When cell membrane stability is compromised, leading to the leakage of intracellular materials, the number of double-positive cells marked as AV (+) /PI (+) will significantly increase. By detecting the proportion of double-positive cells, the occurrence rate of pyroptosis can be quantified. Flow cytometry found that a significant increase in the percentage of double-positive cells, that is, a marked increase in the proportion of pyroptotic cells. Western Blot results showed that compared with the normal group, the expression levels of pyroptosis markers GSDMD, GSDME, GSDME-N, Caspase-1, Caspase-3, Caspase-8, and NF-κB proteins were increased in the high-glucose cell model. Combining the above test results, it can be concluded that cells will be damaged under high-glucose conditions.

Z-YVAD-FMK has excellent cell-penetrating ability and is a specific Caspase-1 inhibitor that is currently widely used in the scientific research field. Flow cytometry found that after the application of the Caspase-1 single inhibitor, there was no significant decrease in the rate of pyroptosis. Western Blot results showed that in the high-glucose state, the expression levels of pyroptosis markers GSDMD, GSDME, GSDME-N, Caspase-1, and Caspase-3 were significantly higher than those in the normal group. After the application of the Caspase-1 inhibitor, the expression levels of Caspase-1 and GSDMD were significantly reduced compared to the high-glucose group, while the expression levels of GSDME, GSDME-N, and Caspase-3 were not significantly decreased compared to the high-glucose group. This indicates that pyroptosis still occurred after the application of the Caspase-1

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inhibitor, suggesting that pyroptosis may not have been completely inhibited and that there may be other signaling pathways involved in addition to the Caspase-1/GSDMD pathway. Caspase-3 is a common key protein in both apoptosis and pyroptosis pathways. The activation of Caspase-3 can cleave a variety of downstream substrates, leading to the typical morphological and biochemical changes of apoptotic cells, including cell shrinkage, chromatin condensation, DFF40/Caspase-activated DNase (CAD) activation, and the externalization of phosphatidylserine from the inner layer of the plasma membrane. Caspase-3-dependent cleavage of GSDME is involved in pore formation in the membrane and cell lysis, which can further exacerbate tubular injury and inflammation. In the presence of activated Caspase-3, the cell death mechanism is regulated by the expression level of GSDME. When GSDME is highly expressed, active Caspase-3 will cleave it and release the N-terminal domain of GSDME to the N-terminal domain, which can execute pyroptosis by forming non-selective pores in the membrane, shifting the mode of cell death from apoptosis to pyroptosis. Conversely, when GSDME is lowly expressed, it will lead to the classical death mechanism of cells, that is, apoptosis.[22,23] Z-DEVD-FMK is a specific inhibitor of Caspase-3. By inhibiting the activation of Caspase-3, it can achieve anti-pyroptosis functions. In this study, after 48 hours of high-glucose intervention in HK-2 cells, dual treatment with Z-YVAD-FMK and Z-DEVD-FMK was applied, that is, dual inhibitors (Caspase-1 inhibitor and Caspase-3 inhibitor) were used. Western Blot showed that not only the expression levels of the two pyroptosis-related proteins Caspase-1 and GSDMD decreased, but also the expression levels of GSDME, GSDME-N, and Caspase-3 proteins were significantly reduced. Flow cytometry found that after the application of dual inhibitors of Caspase-1 and Caspase-3, the percentage of double-positive cells in the region significantly decreased. After inhibiting both pathways, pyroptosis was significantly improved, showing a protective effect on cells under high-glucose conditions. The above experimental results indicate that after inhibiting the classical Caspase-1/GSDMD pathway, pyroptosis still occurs, which may be related to the Caspase-3/GSDME pathway.

SGLT2 is expressed in renal tubular cells. The potential mechanisms underlying the renoprotective effects of SGLT2 inhibitors may be multifaceted. Hou et al reported that in renal tubular epithelial cells of diabetic kidneys, CD36 promotes the activation of the NLRP3 inflammasome through the mitochondrial reactive oxygen species (mtROS) pathway.[24] Multiple studies have shown that high glucose-induced pyroptosis is a mechanism of podocyte death.[25] SGLT2i has emerged as a promising class of drugs, with evidence suggesting that they may improve renal status in diabetic patients. Recent studies have confirmed that SGLT2 inhibitors have renal protective effects independent of their glucose-lowering actions.[26] In vitro experiments have shown that in CD-1 diabetic mouse models, SGLT2 inhibitors not only significantly improve blood glucose levels but also ameliorate renal function decline and renal tubular epithelial cell injury.[27] SGLT2 inhibitors enhance cellular remodeling in renal tubular epithelial cells by increasing autophagy levels, as evidenced by upregulated LC3II and Beclin expression and decreased p62 expression.[28] A Japanese study has indicated that SGLT-2 is involved in the process of ROS production induced by high glucose, which induces an increase in advanced glycation end products (AGEs) through the receptor for advanced glycation end products (RAGE) pathway, leading to renal tubular cell apoptosis and validating the involvement of SGLT-2 in the progression of diabetic kidney disease.[29]

Dagliflozin, the first SGLT2 inhibitor approved for the treatment of T2DM, attenuated tubular apoptosis and injury in DKD. It has been shown that dagliflozin can reduce high glucose-induced intracellular ROS, IL-8, TGF-β levels in renal tubular epithelial cells and exert a protective effect against high glucose-triggered apoptosis, suggesting that dagliflozin may ameliorate tubular oxidative stress, inflammation and fibrosis.[30] However, reports elucidating the mechanisms by which SGLT2 inhibitors regulate pyroptosis and related non-canonical signaling pathways are scarce.

In cell experiments, it is necessary to monitor the cytotoxicity of high concentrations of dapagliflozin. CCK-8 assay results showed that concentrations of dapagliflozin exceeding 5 μM can increase cell death. Therefore, in this study, a concentration of dapagliflozin between 2.5 and 5 μM was deemed safe and effective. Flow cytometry results indicated that the percentage of double-positive cells in the dapagliflozin group was significantly reduced, suggesting a marked decrease in the proportion of pyroptotic cells. Western Blot results revealed that compared with the high-glucose group, the protein expression levels of pyroptosis markers GSDMD, GSDME, GSDME-N, Caspase-1, and Caspase-3 were decreased in the cell model treated with dapagliflozin. These experimental results were consistent with those of the dual-inhibitor group, indicating that dapagliflozin could inhibit high-glucose-induced pyroptosis and exert a protective effect on cells under high-glucose conditions. Its mechanism of action may be related to the Caspase-3/GSDME signaling pathway. To further verify the reliability of these results, SGLT2 gene knockdown technology was employed for experimental validation. In this experiment, after treatment with small interfering RNA (siRNA) targeting SGLT2, the expression of tubular SGLT2 and glucose uptake by cells were

completely blocked. Under high-glucose (HG) conditions, cells were transfected with SGLT2-siRNA. Compared with cells transfected with NC-siRNA, Western Blot showed that the expression levels of pyroptosis-related proteins GSDMD, GSDME, Caspase-1, and Caspase-3 were significantly reduced in the SGLT2-siRNA group, suggesting that SGLT2 knockdown could inhibit the expression of pyroptosis proteins. Flow cytometry indicated that after SGLT2 knockdown, the rate of pyroptosis was significantly decreased. This suggests that pyroptosis was markedly improved after SGLT2 knockdown. Therefore, we speculate that SGLT2 knockdown can inhibit pyroptosis, and the results are consistent with those of the dapagliflozin intervention group. Both can counteract the pyroptotic effects of high glucose on HK-2 cells, and this may be related to the Caspase-3/GSDME pathway. Thus, we speculate that pyroptosis may be an important factor in tubular injury, playing a crucial role in the progression of DKD. In addition to the canonical pyroptosis pathway Caspase-1/GSDMD that we often mention, the signaling pathway involved may also include the Caspase-3/GSDME pathway.

In summary, the experimental results show that after intervention with dapagliflozin, the pyroptosis rate of HK-2 cells induced by high glucose was significantly reduced, cell viability was improved, and the expression levels of pyroptosis-related proteins GSDME, GSDME-N, GSDMD, Caspase-3, and Caspase-1 were decreased. These findings clearly demonstrate that dapagliflozin has an alleviating effect on high-glucose-induced pyroptosis in HK-2 cells. The treatment targeting Caspase-3/GSDME signaling pathway-dependent pyroptosis has the potential for treating DKD. Although this study has elucidated the possible mechanisms of dapagliflozin in renal protection for DKD treatment, since this is only an in vitro study, these results need to be further validated in future animal models of DKD.

Conclusion

1. Excessive cellular pyroptosis of HK-2 cells in vitro under high glucose status.

2. Presence of caspase-3/GSDME pathway causes excessive cellular pyroptosis of renal HK-2 cells in the state of DKD.

3. Dagliflozin reduces DKD HK-2 cell pyroptosis and delays renal disease progression by inhibiting the caspase-3/GSDME pathway.

Ethics and Consent Statements

Informed consent was obtained from all authors.

Approval of the research protocol: Item No.2022AH051532.

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Disclosure

The authors declare no conflicts of interest in this work.

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