Renoprotection of dapagliflozin in human renal proximal tubular cells via the inhibition of the high mobility group box 1-receptor for advanced glycation end products-nuclear factor-κB signaling pathway

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Abstract. Sodium-glucose co-transporter 2 (SGLT2) inhibitors are recently developed oral hypoglycemic agents, which act on renal proximal tubules by reducing the reabsorption of glucose and increasing the excretion of glucose in the urine. However, the mechanism underlying renoprotection has not been fully elucidated. Previous studies have indicated that the expression of high mobility group box 1 (HMGB1) increased in patients with kidney disease, and may result in renal damage through the activation of nuclear factor-κB (NF-κB) and an increase in receptor for advanced glycation end products (RAGE) expression. The aim of the present study was to evaluate the effects of the SGLT-2 inhibitor dapagliflozin on cultured human proximal tubular epithelial cells (HK-2). HK-2 cells were grown under high glucose conditions for 48 h in the presence or absence of dapagliflozin. The markers of oxidative stress, inflammation and fibrillation levels were then detected by reverse transcription-quantitative polymerase chain reaction and western blotting. Hyperglycemia increased the mRNA expression and protein levels of malondialdehyde (MDA), superoxide dismutase (SOD), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), fibronectin (FN), collagenase type 1 (COL-1), HMGB1, RAGE and NF-κB, and the effects could be reversed by dapagliflozin in a concentration-dependent manner. The results of the present study suggested that HMGB1 increased the expression and secretion of markers of inflammation, oxidative stress and fibrillation, including MDA, SOD, MCP-1, ICAM-1, FN and COL-1, in diabetic nephropathy. However, dapagliflozin significantly reduced the levels of inflammatory markers and postponed the progression of renal injury. It was therefore suggested this may be mediated through the inhibition of HMGB1-RAGE-NF-κB signaling pathway.

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Introduction

Diabetic nephropathy is one of the most serious microvascular complications of diabetes and is the main cause of end-stage renal disease (ESRD). Strict control of glucose and blood pressure may delay the progression of diabetic nephropathy; however, many patients still progress to ESRD (1). The patho-physiological mechanism(s) of diabetic nephropathy is complex and has not been fully elucidated. Hyperglycemia status, the activation of the renin-angiotensin-aldosterone system (RAAS), oxidative stress, inflammation and dysregulation of various angiogenic factors have been shown to be involved. In high glucose conditions, advanced glycation end products (AGEs) and receptor for advanced glycation end products (RAGE) accelerated diabetic nephropathy via a complex reaction. In addition, the other endogenous ligand for RAGE, high mobility group box 1 (HMGB1) protein, also proved to play a role in diabetic nephropathy. HMGB1 protein is a member of the high mobility group nuclear protein family, and is one of the most evolutionarily conserved proteins (2). Normally, HMGB1 is expressed in the cell nuclei; occasionally, it is released from cells through passive release, which occurs as a result of cellular necrosis in most eukaryotic cells (3). Extracellular HMGB1 is involved in several inflammatory diseases, such as septic shock, systemic lupus erythematosus, autoimmune hepatitis, rheumatoid arthritis and so on (4). Growing evidence shows that HMGB1 is associated with diabetic nephropathy and is highly expressed in both the cytoplasmic and nuclear compartments of renal glomerular cells and tubular epithelial cells of diabetic animal models; however, the exact mechanism is still unknown.

As an internal ligand for RAGE, HMGB1 has been shown to interact with RAGE and to activate various intracellular signal transduction processes, including

inflammation, proliferation, apoptosis, autophagy, and migration (5). Kim *et al* (3) first proved that HMGB1 is extensively expressed in renal tissues of diabetic rats, and HMGB1 is released from renal glomerular cells and renal tubular epithelial cells, and it participates in the occurrence of diabetic nephropathy by signaling through RAGE. RAGE expression and nuclear factor (NF)-κB activity were also elevated in diabetic rats where the binding of NF-κB to the RAGE promoter was increased. In addition, they also found hyperglycemia-induced HMGB1 increased NF-κB activity. Moreover, NF-κB activation is a major intracellular signaling pathway of RAGE. Extracellular HMGB1 is able to induce a signaling cascade that activates NF-κB, leading to the synthesis of proinflammatory cytokines. Hyperglycemia-induced HMGB1 release may induce renal injury in diabetic rats, and the pathogenic role of HMGB1 might be dependent on RAGE through activation of NF-κB.

The kidney plays an important role in controlling glucose homeostasis via gluconeogenesis and the reabsorption of filtered glucose in the proximal tubules (6). Nearly 180 g of glucose is filtered in the glomerular filtrate every day. The kidney absorbs ~90% of this glucose through SGLT-2, which is located on the apical side of proximal tubule cells and transports sodium and glucose concurrently (7). In patients with type 2 diabetes, SGLT-2 proteins can be upregulated, causing an increase in the renal threshold for reabsorption of glucose, and worsening hyperglycemia (8). Increased glucose reabsorption results in renal hyperfiltration; this is an important cause of renal damage in diabetic nephropathy. The principal of pathological changes in diabetic nephropathy include glomerular and tubulointerstitial lesions, while the latter is more closely related to renal function decline. Reducing glucose transport in human proximal tubular cells may lower the inflammatory response and fibrosis of kidney tubules (7). Sodium-glucose co-transporter 2 (SGLT2) inhibitors are new recently developed oral hypoglycemic agents that target the kidney and block the reabsorption of glucose to achieve glycemic control. In addition, the hypoglycemic effect is independent of insulin. Emerging data indicates that SGLT2 inhibitors have a protective effect on the kidney beyond the glucose-lowering effects. Furthermore, SGLT-2 can achieve hypotension and weight loss due to the drugs' diuretic effect, all which are independent of its hypoglycemic effects (9).

Here, we propose the hypothesis that HMGB1 may bind RAGE to activate NF-κB and lead to various intracellular mechanisms, finally resulting in renal injury. Therefore, activation of the HMGB1-RAGE-NF-κB signaling pathway could worsen diabetic nephropathy; however, SGLT-2 inhibitors may block this signaling pathway to achieve renoprotection. To test this hypothesis, we designed a study that used dapagliflozin, a new SGLT-2 inhibitor to treat HK-2 cells that were exposed to high glucose to explore the effect of SGLT-2 inhibitors on diabetic nephropathy.

Materials and methods

Cell culture and treatment. The human proximal tubular cell line (HK-2) was bought from the Shanghai Institute for Biological Sciences (Shanghai, China) and was cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F-12 medium; GE Healthcare Life Sciences; Logan, UT, USA). Cells were cultured in DMEM/F12 with 25 and 5 mmol/l glucose, respectively, and supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin plus 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were incubated at 37˚C, with 5% CO_2. Dapagliflozin (Selleck Chemicals, Houston, TX, USA) was added to the culture medium (10 or 100 μM final concentration) for 48 h to reconfirm the promotion of oxidative stress makers and inflammatory cytokines.

RNA isolate and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from HK-2 cells using TRIzol regent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, reverse transcribed into cDNA using the Prime Script® RT reagent kit (Takara Bio, Inc., Otsu, Japan). RT-qPCR was performed using the SYBR Premix Ex Taq TM kit and the ABI prism 7000 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. Relative levels of gene expression were determined using the housekeeping gene GAPDH. RT-qPCR data were normalized to the expression of GAPDH, and relative expressions were calculated using the $2^{-\Delta\Delta Cq}$ method (10). The primer sequences are presented in Table I.

Antibodies and western blot analysis. HK-2 cells were collected and lysed in Radio Immunoprecipitation Assay lysis buffer and protease inhibitor mixture (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The supernatant of HK-2 cells was harvested and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA), then separated by SDS-PAGE and blotted to polyvinylidene fluoride membranes (PVDF; Bio-Rad Laboratories, Inc., Shanghai, China). The blots were blocked with 5% skimmed milk, followed by incubation with antibodies against malondialdehyde (MDA), superoxide dismutase (SOD), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA), fibronectin (FN), collagenase type 1 (COL-1) and HMGB1, RAGE NF-κB and GAPDH (all Abcam, Cambridge, MA, USA) antibody, horseradish peroxidase (HRP) labeled sheep anti-rabbit or sheep anti-mouse were bought from Santa Cruz Biotechnology, Inc. Blots were visualized using the enhanced chemiluminescent (ECL) detection system. Blots were then incubated with species-specific horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.), and visualized using Scion 146 Image software (Scion Corporation, Frederick, MD, USA).

Statistical analysis. SPSS version 20.0 statistical software (IBM Corp., Armonk, NY, USA) was used for all data normality tests. All values were expressed as the mean ± standard deviation. One-way analysis of variance with Tukey's post hoc test was performed for statistical analysis of differences between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Target	Primer (5'-3')
MDA	Forward, TTGCCTGGGTTTTACCCTGC
	Reverse, AAGGCTTCCCACAGTTTCTGG
SOD	Forward,GGTGGGCCAAAGGATGAAGAG
	Reverse, CCACAAGCCAAACGACTTCC
MCP-1	Forward, CAGCCAGATGCAATCAATGCC
	Reverse, TGGAATCCTGAACCCACTTCT
ICAM-1	Forward, ATGCCCAGACATCTGTGTCC
	Reverse, GGGGTCTCTATGCCCAACAA
Collagen I	Forward, CCTGCTGGGATATTAGCTCCA
	Reverse, CAGCGGTAGGTGTCGAAGC
Fibronectin	Forward, CGGTGGCTGTCAGTCAAAG
	Reverse, AAACCTCGGCTTCCTCCATAA
GAPDH	Forward, CGGAGTCAACGGATTTGGTCG TAT
	Reverse, AGCCTTCTCCATGGTGGTGAA GAC

MDA, malondialdehyde; SOD, superoxide dismutase; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1.

Results

Dapagliflozin inhibits oxidative stress induced by high glucose in HK-2 cells. As shown in Fig. 1, mRNA and protein levels of the oxidative stress makers MDA and SOD were higher in high glucose conditions compared to the control group. This effect was inhibited by dapagliflozin; however, the expression of MDA and SOD was lowest with 100 μM dapagliflozin, suggesting that the inhibitory effect is positively correlated with the concentration of dapagliflozin.

Dapagliflozin inhibits inflammation induced by high glucose in HK-2 cells. Inflammation mediated by high glucose plays an important role in the development of diabetic nephropathy. As shown in Fig. 2, we detected the level of the inflammatory factors MCP-1 and ICAM-1 in cultured HK-2 cells by RT-qPCR and western blot. The mRNA and protein levels of MCP-1 and ICAM-1 were increased in high glucose conditions, and could be reduced by dapagliflozin. The effect of inhibition was more noticeable with the high concentration of dapagliflozin.

Dapagliflozin inhibits fibrosis induced by high glucose in HK-2 cells. Tubulointerstitial fibrosis correlates more closely with deterioration of renal function than histological changes in the glomerulus. Interstitial fibrosis accompanies proximal tubular cell basement membrane thickening, hyperplasia and hypertrophy and ultimately correlates with the demise of renal function. As shown in Fig. 3, We evaluated FN and Col 1, as indicators of fibrosis, and found that high glucose increased the level of FN and Col 1 protein and mRNA compared to the control group. However, the expression of FN and Col 1 in HK-2 cells treated with dapagliflozin was significantly decreased and lowest in the high concentration dapagliflozin group.

Dapagliflozin inhibits the activation of the HMGB1-RAGE-NF-κB signal pathway induced by high glucose in HK-2 cells. As an indicator of inflammation, HMGB1 is proven to be involved in the development of diabetic nephropathy though multiple intracellular reactions, and RAGE and NF-κB were key to these processes.

As shown in Fig. 4, the protein expression of HMGB1, RAGE and NF-κB was significantly increased in HK-2 cells treated with high glucose, while they were decreased in dapagliflozin groups, and lowest in the high concentration group.

Discussion

SGLT-2 inhibitors are the latest class of hypoglycemic agents and have a unique mechanism of lowering glucose independent of insulin. SGLT-2 inhibitors are used for treating type 2 diabetes at present, and numerous studies have demonstrated that SGLT-2 inhibitors can decrease the level of serum glucose, HbA1c, and hypertension, and increase weight loss. However, the effect and mechanism of SGLT-2 inhibitors on diabetic nephropathy is not fully understood.

Oxidative stress, inflammation, and fibrosis mediated by high glucose is directly responsible for pathological changes in diabetic nephropathy. Together, this results in the classic structural and functional alterations of diabetic kidney disease with alterations in glomerular permeability, glomerular hyperfiltration, glomerular basement membrane thickening, mesangial matrix synthesis, and ultimately the development of glomerulosclerosis and interstitial fibrosis (11).

HMGB1 expression is upregulated in high glucose conditions; the expression of RAGE, a potential receptor for HMGB1, and NF-κB activity also play important roles in diabetic nephropathy. In addition, diabetes enhances the binding of NF-κB to the RAGE promoter. HMGB1 has been proposed as a potential causative factor of renal damage (12). RAGE is found on podocytes and tubular epithelial cells, while HMGB1 acts directly on RAGE and participates in the induction of diabetic nephropathy. NF-κB is an important transcription factor involved in the regulation of inflammation, immune responses, cell survival and proliferation (13). In addition, it is reported that HMGB1 increases NF-κB activity (3), and NF-κB activation is a main intracellular signaling pathway of RAGE (14). Moreover, NF-κB signaling pathways are essential for the destruction of tubular epithelial cells (15). In the present study, we used dapagliflozin, a novel selective SGLT-2 inhibitor to treat HK-2 cells that were exposed to high glucose. We then detected the gene and protein expression of MDA, SOD, MCP-1, ICAM-1, FN and COL-1 to observe the renoprotective effect of the SGLT-2 inhibitor. The involvement of RAGE and the NF-κB signaling pathway was also studied.

Lowering of glucose levels can slow nephropathy progression but takes at least 10 years to achieve clinically relevant outcomes (16). However, the hypoglycemic effect of SGLT-2 inhibitors is independent of insulin and directly acts

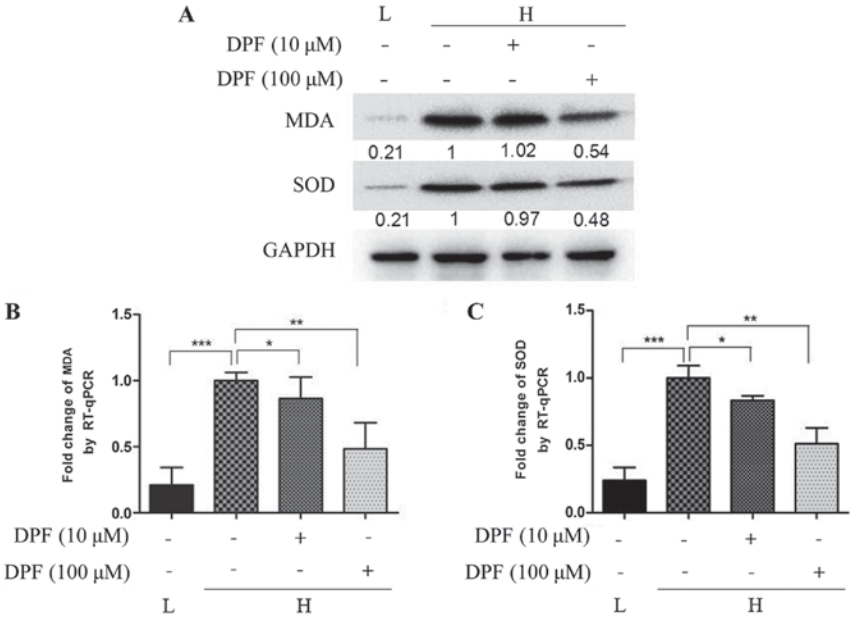

Figure 1. Effects of DPF on MDA and SOD protein and mRNA expression in HK-2 cells. HK-2 cells were treated with or without high glucose (33.3 mM) in the presence or absence of 10 or 100 μM DPF for 48 h. (A) Western blotting and (B and C) RT-qPCR analyses were performed for MDA and SOD in each group. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001, as indicated. DPF, dapagliflozin; MDA, malondialdehyde; SOD, superoxide dismutase; L, low glucose; H, high glucose; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

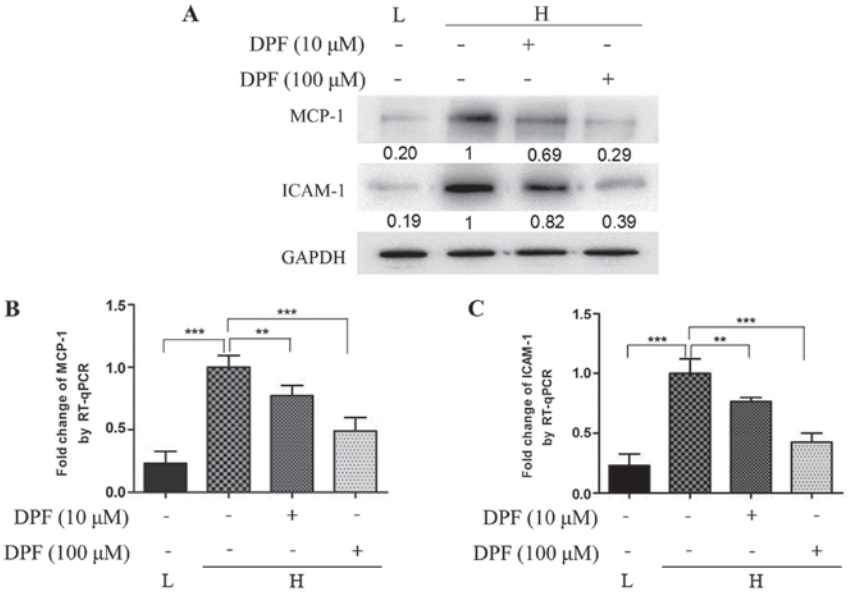

Figure 2. Effects of DPF on MCP-1 and ICAM-1 protein and mRNA expression in HK-2 cells. HK-2 cells were treated with or without high glucose (33.3 mM) in the presence or absence of 10 or 100 μM dapagliflozin for 48 h. (A) Western blotting and RT-qPCR analyses were performed for (B) MCP-1 and (C) ICAM-1 in each group. Data are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001, as indicated. DPF, dapagliflozin; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; L, low glucose; H, high glucose; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

on renal proximal tubules. The SGLT2 inhibitor has also been associated with a 10-15% reduction in plasma uric acid levels as a result of enhanced glycosuria, leading to the secretion of uric acid in exchange for glucose reabsorption through the GLUT9 transporter (17), and the effect of lowering serum uric acid by SGLT2 inhibition might ameliorate endothelial dysfunction, hypertension, and microvascular injury in diabetes (18,19).

Moreover, SGLT2 inhibitors were effective in decreasing the albuminuria in patients with type 2 diabetes and hypertension through RAAS inhibition after adjusting for changes in HbA1C, blood pressure, body weight, and estimated glomerular filtration rate (GFR) (20). Investigations into the extra effects of SGLT2 inhibitors beyond glucose reduction have been established, and the mechanisms underlying the renoprotection induced by SGLT2 inhibitors are gradually becoming the focus of research. Oxidative stress is the initial stage of diabetic nephropathy and activates a variety of pathological pathways in virtually all types of kidney cells (21).

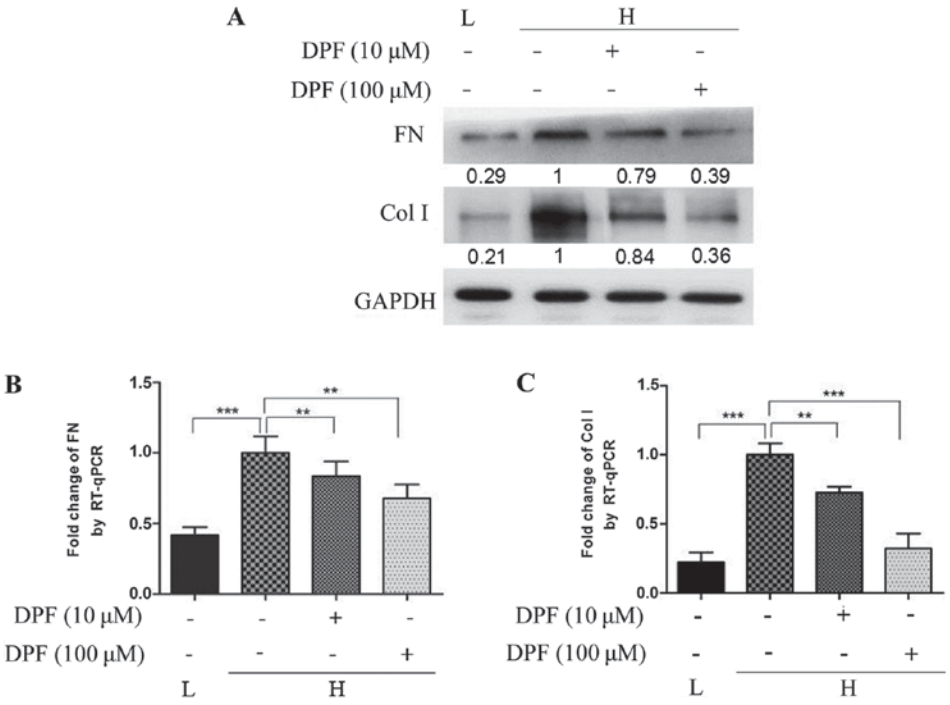

Figure 3. Effects of DPF on FN and Col 1 protein and mRNA expression in HK-2 cells. HK-2 cells were treated with or without high glucose (33.3 mM) in the presence or absence of 10 or 100 μM DPF for 48 h. (A) Western blotting and RT-qPCR analyses were performed for (B) FN and (C) Col 1 in each group. Data are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001, as indicated. DPF, dapagliflozin; FN, fibronectin; Col 1, collagenase type 1; L, low glucose; H, high glucose; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

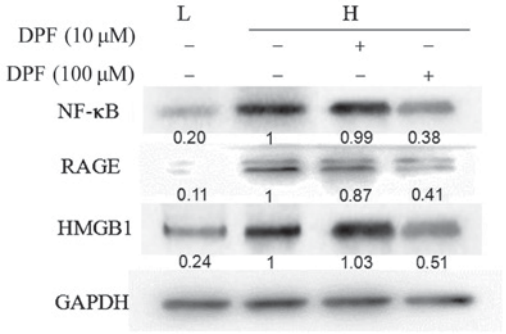

Figure 4. Effects of DPF on HMGB1, RAGE and NF-κB protein expression in HK-2 cells. HK-2 cells were treated with or without high glucose (33.3 mM) in the presence or absence of 10 or 100 μM DPF for 48 h. Western blot analysis was performed for HMGB1, RAGE and NF-κB protein expression in each group. DPF, dapagliflozin; HMGB1, high mobility group box 1; RAGE, receptor for advanced glycation end products; NF-κB, nuclear factor-κB; L, low glucose; H, high glucose.

In our study, we found an increase in MDA and SOD production in HK-2 cells in the high glucose group compared to the control group. Dapagliflozin inhibited the production of MDA and SOD induced by hyperglycaemia, indicating that dapagliflozin could prevent the oxidative stress process *in vitro*, and this effect is concentration-dependent since the levels of MDA and SOD were lowest in the high concentration dapagliflozin (100 μM) group. The development of diabetic nephropathy is associated with significant inflammatory cell infiltration and increasing plasma levels of inflammatory cytokines. These cytokines act in a paracrine or autocrine manner and induce a variety of effects on different renal structures, playing a significant role in the development and progression of several renal disorders (22). MCP-1 and ICAM-1 are recognized as proinflammatory cytokines that participate in cytokine-associated signaling pathways, and engage in the pathogenesis of diabetic nephropathy through different actions, including intrarenal hemodynamic alterations, modifications of the renal structure, abnormalities in the expression of diverse molecules, cellular necrosis and apoptosis, modification in the permeability of glomerular endothelium, and increment in the production of ROS (23). Our data showed that MCP-1 and ICAM-1 were highly expressed in the high glucose group but expression could be decreased by dapagliflozin, which suggests that the SGLT-2 inhibitor may achieve its renal protection through inhibiting the inflammatory process. Similar results were reported by Panchapakesan *et al* (9). Numerous studies have shown that oxidative stress and inflammation promote renal tubule and interstitial fibrosis, resulting in irreversible renal damage, eventually leading to end-stage nephropathy. In our study, we examined the expression of FN and COL1 which act as makers of renal fibrosis, and we observed that dapagliflozin reduced the level of FN and COL1 induced by hyperglycaemia in a dose-dependent manner. These results suggest that dapagliflozin ameliorates the renal injury of diabetic nephropathy by reducing oxidative stress, inflammation and fibrosis in diabetic kidneys. However, the exact mechanism of renoprotection is unknown. In addition, we also observed the changes of HMGB1, RAGE and NF-κB in the presence or absence of dapagliflozin. HMGB1, RAGE and NF-κB were highly expressed in the high glucose group and were inhibited by dapagliflozin. We propose the hypothesis that hyperglycaemia promotes the progression of oxidative stress, inflammation and fibrosis, and results in the occurrence of diabetic nephropathy via activating the HMGB1-RAGE-NF-κB

signaling pathway, and this effect could be prevented by SGLT-2 inhibition.

In conclusion, dapagliflozin has renoprotective effects through its anti-oxidative stress and anti-inflammatory action, and our results suggest the effects might be achieved via inhibition of the HMGB1-RAGE- NF-κB signaling pathway. However, further studies are also needed.

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Availability of data and materials

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

Authors' contributions

DY conceived the study, designed the experiments and wrote the manuscript. SW performed the experiments and analyzed the data. MY performed the experiments and organized the figures. WL designed the experiments, contributed to the writing of the manuscript and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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