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Protective effects of gliclazide on high glucose and AGEs-induced damage of glomerular mesangial cells and renal tubular epithelial cells *via* inhibiting RAGE-p22phox-NF-κB pathway

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Abstract. – OBJECTIVE: Gliclazide is one of the most widely used therapeutic drugs for diabetes. As a second-generation sulfonylurea oral hypoglycemic drug, it can lower blood glucose level and delay the occurrence and development of diabetic nephropathy (DN). However, the underlying mechanism remains unclear. Therefore, the aim of this study was to explore whether gliclazide had protective effects on high glucose and advanced glycation end products (AGEs)-induced injury of human mesangial cells (HMCs) and renal tubular epithelial cells.

MATERIALS AND METHODS: HMC and renal tubular epithelial cell lines [human kidney 2 (HK-2)] were cultured in vitro. All cells were then divided into the follow groups: 1) blank control group (5.6 mmol/L glucose), 2) AGEs group [400 μg/mL AGE-bovine serum albumin (AGE-BSA)], 3) high glucose group (25 mmol/L glucose), 4) gliclazide + AGEs group (400 μg/mL AGE-BSA + 20 μmol/L gliclazide) and 5) gliclazide + high glucose group (25 mmol/L glucose + 20 μmol/L gliclazide). Cell counting kit-8 (CCK-8) assay was adopted to determine cell viability. Flow cytometry was used to detect cell apoptosis. The levels of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were measured as well. Furthermore, the mRNA expressions of receptor for AGE (RAGE), p22phox and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were measured *via* fluorescence quantitative Real-time polymerase chain reaction (qRT-PCR).

RESULTS: Compared with control group, significantly accelerated apoptosis of HMCs and HK-2, increased MDA level, decreased SOD and GSH-Px levels, and up-regulated mRNA expressions of RAGE, p22phox and NF-κB were observed in HMCs and HK-2 of high glucose group and AGEs group. Meanwhile, there were obviously alleviated apoptosis of HMCs and HK-2, decreased MDA level, increased SOD and GSH-Px levels, as well as down-regulated mRNA expressions of RAGE, p22phox and NF-κB in HMCs and HK-2 of gliclazide group compared with high glucose and AGEs group. Furthermore, significant correlations were found between the mRNA expression of RAGE and the apoptosis rate of HMCs and HK-2 (HMCs: r=0.701, *p*=0.004 and HK-2: r=0.633, p=0.011).

CONCLUSIONS: Gliclazide has protective effects on high glucose and AGEs-induced damage of glomerular mesangial cells and renal tubular epithelial cells *via* inhibiting RAGE-NADPH oxidase-NF-kB pathway.

Key Words:
Gliclazide, Oxidative stress, AGEs, Diabetic kidney disease.

Introduction

As one of the primary micro-vascular complications of diabetes, diabetic nephropathy (DN) is a leading cause of end-stage renal disease and death of diabetic patients[1]. The occurrence of diabetes is accompanied by increased production of oxygen free radicals and decreased ability of antioxidant defense. Broken balance exerts crucial effects on the occurrence and progression of diabetic complications[2]. Glomerular hypertrophy, extracellular matrix accumulation, thickened basement membrane, glomerular sclerosis and a train of other renal pathological changes are the main manifestations of renal damage[3].

Advanced glycation end products (AGEs) are a type of covalent compounds produced by the

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oxidation reaction of glucose and protein lipid under non-enzymatic conditions. Previous studies[4,5] have shown that it exerts a key role in the occurrence and development of DN. AGEs are primarily formed in *in-vivo* proteins with long half-life, such as collagen, crystalline lens, β2 microglobulin and hemoglobin. Meanwhile, its level in the body increases slowly with age. High blood glucose level in diabetic patients expedites the formation of AGEs. AGEs can bind to *in-vivo* receptor for AGE (RAGE) and activate oxidative stress, further accelerating the deposition of AGEs *in vivo*. This is a positive feedback process[6]. In the occurrence of DN, the proliferation of human mesangial cells (HMCs) is reduced, and the production of fibronectin goes up. Meanwhile, mesangial matrix hyperplasia happens with the deposition of AGEs on matrix proteins[7]. Other than damage to mesangial cells, DN, as a tubular disease, can also destroy renal tubular cells. This may eventually lead to tubulointerstitial fibrosis[8].

Gliclazide is a second-generation sulfonylurea oral hypoglycemic drug. Due to its unique nitrogen heterocyclic structure compared with other sulfonylurea drugs, it can lower blood glucose level and scavenge oxygen free radicals. Existing studies[9,10] have revealed that compared with glibenclamide, gliclazide can improve oxidative stress in patients with type 2 diabetes mellitus independent of its hypoglycemic effect. A series of other studies have demonstrated that gliclazide can also inhibit the apoptosis of normal and tumor cells[11], as well as scavenge oxygen free radicals[12]. ADVANCE study has verified that gliclazide based therapy in patients with type 2 diabetes may delay the onset and progression of kidney disease[13]. However, ACCORD and VATD research (choose glimepiride) shows no other benefits[14,15]. In addition to hypoglycemic effect, whether gliclazide has direct renal protective effect is worth looking forward to. In this experiment, HMCs and HK-2 were cultured with high glucose and AGEs. Gliclazide was used to investigate whether gliclazide had protective effects on high glucose and AGEs-induced injure of human mesangial cells (HMCs) and renal tubular epithelial cells (HK-2).

Materials and Methods

Cells and Reagents

HMCs (ScienCell, Carlsbad, CA, USA), renal tubular epithelial cell lines [human kidney 2 (HK-2)] (Cell Bank of Chinese Academy of Sciences, Shanghai, China), Reverse transcription kit (TaKaRa, Otsu, Shiga, Japan), Polymerase chain reaction (PCR) kit (TaKaRa, Otsu, Shiga, Japan), Dulbecco's modified Eagle's medium (DMEM) (HyClone, South Logan, UT, USA), fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), PCR primers (Shanghai Sangon Biotech Co., Ltd., Shanghai, China), Gliclazide (Servier, Suresnes, France), Flow-type Annexin-fluorescein (FITC)/propidium iodide (PI) double staining kit and Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan).

Cell Culture

HMCs and HK-2 were digested with 0.25% trypsin for 1 min under aseptic conditions. Digestion was terminated when the lower part was dispersed into a net-like form. The cells were added with medium containing 10% heat-inactivated FBS, repeatedly and gently beat, evenly inoculated and cultured *in vitro*. HK-2 cells ($1.5\text{-}2.0\times10^5$/mL) were cultured in DMEM/F12 supplemented with 10% heat-inactivated FBS in an incubator at 37°C with 5% CO_2. Meanwhile, HMC cells ($1.5\text{-}2.0\times10^5$/mL) were cultured in DMEM supplemented with 10% heat-inactivated FBS in an incubator at 37°C with 5% CO_2. When the cells grew to the sub-fusion state, they were cultured in serum-free medium for 24 h for synchronization and subsequent experiments.

Preparation of AGEs

50 g bovine serum albumin (BSA), 0.5 moL glucose and 10 mL penicillin (100 UI/mL) streptomycin (100 μg/mL) double antibiotic were dissolved in 0.2 M phosphate-buffered saline (PBS) (pH 7.4). Next, the mixture was incubated at 37°C for 3 months away from light to form AGEs-BSA. Under the same conditions, non-glycosylated BSA was prepared as control. After the formation of AGEs, dialysis was conducted using 0.2 M PBS (pH 7.4) to remove redundant glucose. After filtering *via* a 0.22 μm filter membrane, vacuum treatment was carried out using a freeze dryer for 16 h. Subsequently, lyophilized powder was prepared and stored at -20°C for use.

Experimental Grouping

All cells were divided into 5 groups, including: 1) normal control group (NG group): 5.6 mmol/L glucose with no other stimulants added, 2) high

glucose group (HG group): 25 mmol/L glucose, 3) AGEs group: 400 μg/mL, 4) high glucose+gliclazide group (HG+gliclazide group): 25 mmol/L glucose and 20 μmol/L gliclazide, and 5) AGEs+gliclazide group: 400 μg/mL AGEs and 20 μmol/L gliclazide.

Detection of Gene Expression via qRT-PCR

HMCs and HK-2 were first inoculated into 6-well plates at a density of 10^5/well and cultured for 24 h. The medium was replaced according to the above grouping for intervention. After that, the cells were further cultured for 24 h *in vitro*. Total mRNA in cells was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). PCR reaction system was: 2 μL complementary deoxyribose nucleic acid (cDNA), 0.8 μL of each upstream and downstream primers, 6.4 μL distilled water and 10 μL SYBR Green RT-PCR Master Mix (20 μL in total). Specific PCR conditions were as follows: pre-denaturation at 95°C for 30 s, melting at 95°C for 5 s, annealing at 58°C for 5 s and extension at 72°C for 5 s, for a total of 40 cycles. Primer sequences used in this study were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH): sense 5'-AGGGCTGCTTTTAACTCTGGT-3', antisense 5'-AGGGCTGCTTTTAACTCTGGT-3'. RAGE: sense 5'-CAGGACCAGGGAACCTACAG-3', antisense 5'- ACAAGATGACCCCAATGAGC-3'. NF-κB: sense 5'-AGGTCGTAGAGAAGAGCGAGAG-3', antisense 5'-TTGTGAATGACCTCAACAGCTT-3'. p22phox: sense 5'-CTTTGGTGCCTACTCCATTGT-3', antisense 5'-ACGGCCCGAACATAGTAATTC-3'.

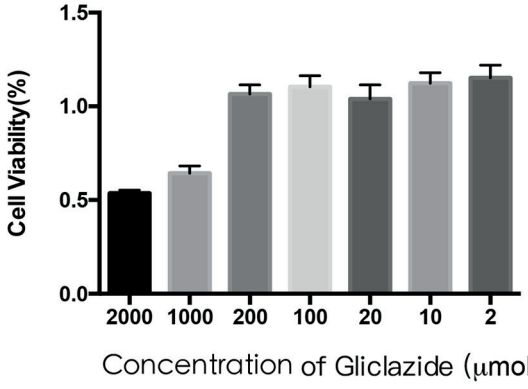

Figure 1. Effects of different concentrations of gliclazide on cytotoxicity. HMC cells in the growth phase were first inoculated into 96-well plates. CCK-8 was added at 24 h after the addition of drugs, and OD value of each well was measured. When the concentration of gliclazide was below 200 μmol/L, there was no cytotoxicity.

Detection of MDA Content and the Activity of SOD and GSH-Px via Kits

HMCs and HK-2 were inoculated into 6-well plates at a density of 10^5/well, followed by culture for 24 h. Subsequently, cell supernatant was collected, and MDA content and the activity of SOD and GSH-Px were determined.

Detection of Cell Apoptosis via Flow Cytometry

HMCs and HK-2 were inoculated into 6-well plates at a density of 10^5/well and cultured for 24 h. After digestion with ethylenediaminetetraacetic acid (EDTA)-free trypsin, cell suspension was collected. After washing twice with PBS, the suspension was centrifuged at 1000 r/min for 5 min. Subsequently, 500 μL binding buffer, suspension cells and 5 μL VI were added and mixed well. After that, 5 μL PI was added and gently mixed. Finally, the reaction was performed at room temperature for 10 min in dark.

Detection of Cell Proliferation Activity via Cell Counting Kit-8 (CCK-8)

HMCs in logarithmic growth phase were first inoculated into 96-well plates at a density of 10,000 cells per 100 μL, followed by culture for 24 h. 10 μL CCK-8 (Dojindo, Kumamoto, Japan) was added to each well, followed by incubation for 2 hours in dark. Optical density (OD) value of each well at 450 nm was measured using a microplate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was adopted for all statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Independent two-sample *t*-test was conducted for intergroup comparison. $p<0.05$ was considered statistically significant.

Results

Effects of Different Concentrations of Gliclazide on Cytotoxicity

During synchronization of cells for 24 h, 2000 μmol/L, 1000 μmol/L, 200 μmol/L, 100 μmol/L, 20 μmol/L, 10 μmol/L and 2 μmol/L gliclazide were added, respectively. When the concentration of gliclazide was under 200 μmol/L, no signifi-

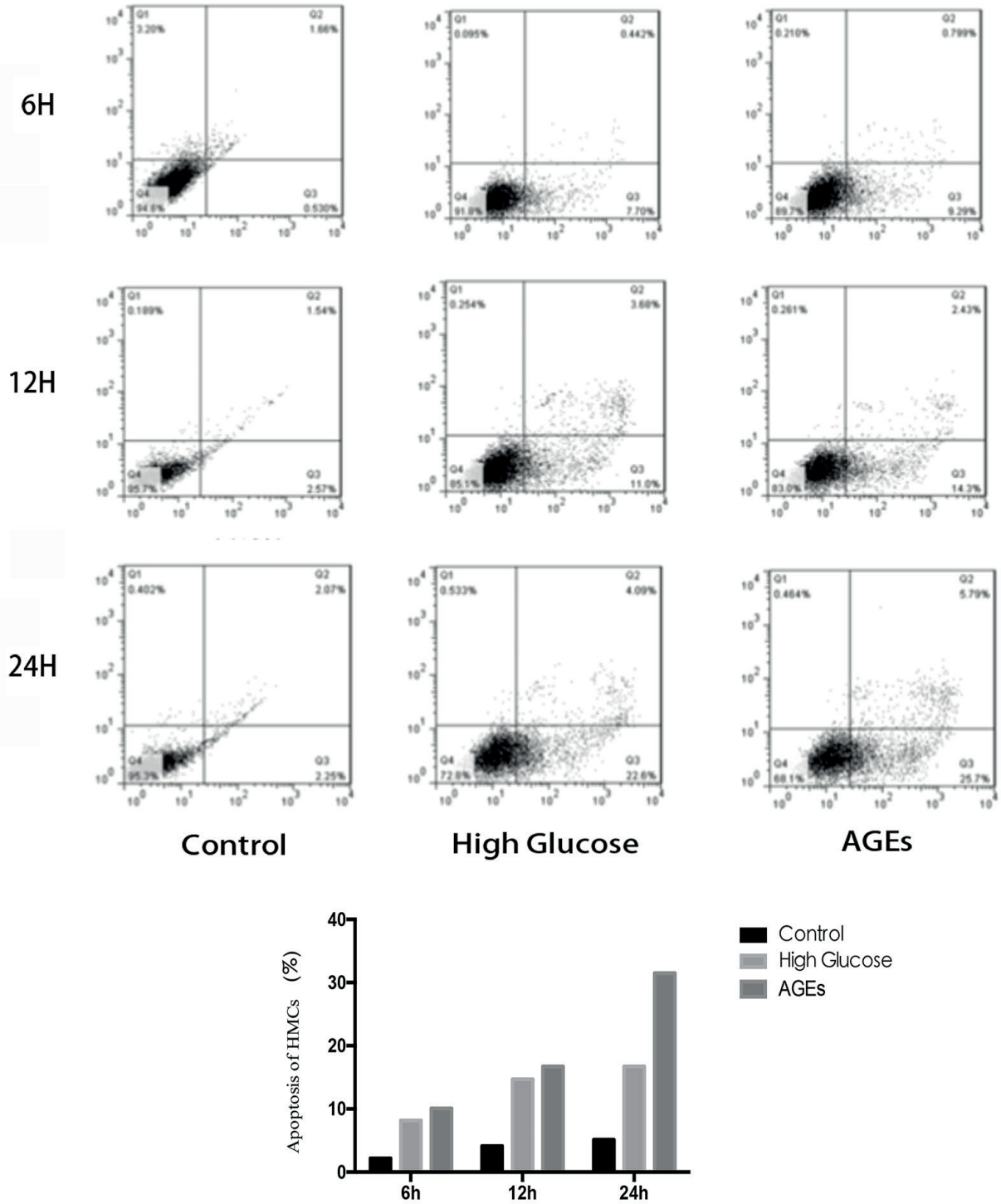

Figure 2. Effects of high glucose and AGEs on the apoptosis of HMCs at different time of action.

cant differences were observed in cell viability among treatment groups (Figure 1). Slivinska et al[11] have reported that 20 μmol/L gliclazide shows the strongest anti-apoptosis effects. Therefore, 20 μmol/L gliclazide was chosen in this study.

Protective Effects of Gliclazide on High Glucose and AGEs Induced Cell Apoptosis

High glucose and AGEs induced cell apoptosis increased significantly in a time-dependent manner. Compared with NG group, there was signif-

Table I. Effects of gliclazide on GSH-Px, SOD and MDA in HMCs caused by high glucose/AGEs.

	Control	High-glucose	High-glucose +Gliclazide	AGEs	AGEs+Gliclazide
GSH-Px (μmol/ gprot)	65.89±4.66	53.64±3.15**	70.36±6.06$^{\#\#}$	56.04±4.79*	69.77±3.00^{++}
SOD (U/ mgprot)	42.89±4.13	31.04±5.83*	43.51±7.65$^{\#}$	25.87±4.00**	33.94±5.01
MDA (mmol/L)	2.18±0.35	3.06±0.50*	2.21±0.34$^{\#}$	3.22±0.46*	2.40±0.58^{+}

*p<0.05 and **p<0.01 *vs.* control group, ^{+}p<0.05 and ^{++}p<0.01 *vs.* AGEs group, and $^{\#}p$<0.05 and $^{\#\#}p$<0.01 *vs.* high glucose group.

icant increased apoptosis of HMCs and HK-2 in HG and AGEs group. However, the apoptosis of HMCs and HK-2 in gliclazide-treated group decreased significantly when compared with high glucose and AGEs group (Figures 2-3).

Effects of Gliclazide on MDA Levels, Activity of SOD and GSH-Px in HMCs and HK-2

High glucose and AGEs significantly decreased the activity of SOD and GSH-Px and increased MDA content in HMCs and HK-2. However, gliclazide treatment significantly increased the activity of SOD and GSH-Px and decreased MDA content. Elevated level of MDA, which was a product of lipid peroxide, could be used as a marker of oxidative stress. SOD primarily scavenges superoxide anions, and GSH-Px can scavenge H_2O_2 and MDA. Reduced activity of intracellular SOD and GSH-Px indicates that the ability to remove reactive oxygen species (ROS) and MDA is reduced in the body[16]. The above results suggested that gliclazide could improve oxidative stress in the body triggered by high glucose and AGEs (Table I and II).

Effects of Gliclazide on the mRNA Expressions of RAGE, p22phox and NF-κB

AGEs significantly increased the mRNA expressions of RAGE, p22phox and NF-κB. However, gliclazide could significantly reduce the expressions of the above genes. Similarly, high glucose remarkably increased the mRNA expressions of p22phox and NF-κB, while gliclazide reduced their expressions. Significant correlations were observed between the mRNA expression of RAGE and the apoptosis rate of HMCs and HK-2 (HMCs: r=0.701, p=0.004 and HK-2: r=0.633, p=0.011). These findings indicated that the activation of RAGE was inseparable from cell apoptosis (Figure 4).

Discussion

As one of the key organs for drug metabolism, kidney influences drug excretion in the occurrence of DN. Meanwhile, DN patients are a high-risk group of hypoglycemia[17]. This leads to difficulties in the clinical treatment of DN[18]. Blood glucose control exerts certain effects on delaying the occurrence and development of diabetic complications. High glucose can cause damage to the body through activating polyol pathway, increasing the production of AGEs, activating PKC pathway and increasing the activity of aminohexose pathway[19]. Currently, sulfonylureas are frequently used as oral hypoglycemic drugs in clinical practice. According to the ADVANCE study, gliclazide-based treatment in patients with type 2 diabetes can delay the onset and progression of kidney disease[13]. However, ACCORD and VATD studies (using glimepiride) shows no similar ben-

Table II. Effects of gliclazide on GSH-Px, SOD and MDA in renal tubular epithelial cells treated with high glucose/high AGEs.

	Control	High-glucose	High-glucose +Gliclazide	AGEs	AGEs+Gliclazide
GSH-Px (μmol/gprot)	75.01±4.39	55.31±3.60**	69.44±3.53$^{\#\#}$	58.93±1.22**	70.96±3.22^{++}
SOD (U/ mgprot)	46.58±3.82	26.56±2.41	32.08±2.62$^{\#}$	27.47±5.06**	34.51±3.90^{+}
MDA (mmol/L)	2.12±0.45	3.24±0.43**	2.37±0.27$^{\#}$	3.07±0.42*	2.61±0.57^{+}

*p<0.05 and **p<0.01 *vs.* control group, ^{+}p<0.05 and ^{++}p<0.01 *vs.* AGEs group, and $^{\#}p$<0.05 and $^{\#\#}p$<0.01 *vs.* high glucose group.

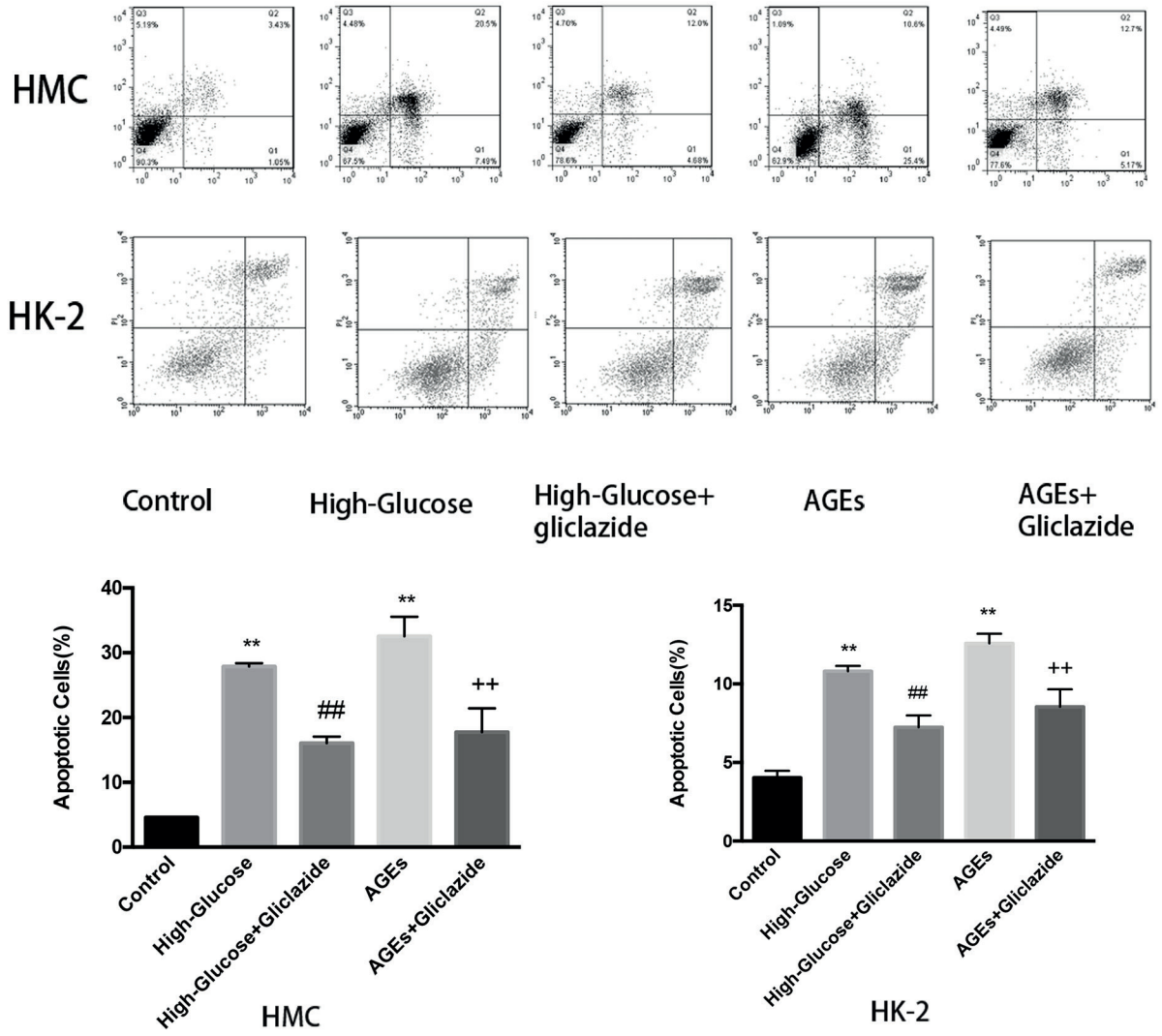

Figure 3. Gliclazide could improve the increased apoptosis of HMCs and renal tubular epithelial cells induced by high glucose/AGEs. **A,** Flow figure of the apoptosis of HMCs; **B,** Statistics of the apoptosis rate of HMCs; **C,** Flow figure of the apoptosis of renal tubular epithelial cells; **D,** Statistics of the apoptosis rate of renal tubular epithelial cells (**$^{**}p$<0.01 *vs.* low glucose group, ^{++}p<0.05 *vs.* AGEs group, and $^{\#\#}p$<0.05 *vs.* high glucose group).

eficial effect[14,15]. In addition to hypoglycemic effect, the protective effect of gliclazide on kidneys may be independent of its hypoglycemic effect. In the present study, *in-vitro* experiments indicated that gliclazide could reduce the apoptosis of renal cells caused by high glucose and AGEs.

AGEs are products of non-enzymatic glycation of long-term high glucose and *in-vivo* proteins in diabetic patients. Meanwhile, AGEs-RAGE interaction plays a key role in the occurrence and development of diabetic chronic complications. Incubation of HMC with AGEs *in vitro* leads to increased extracellular matrix, indicating that the damage of AGEs can be independent of high glucose[20]. Different from high glucose self-oxidation or oxidized cell membrane lipids to produce free radicals, AGEs can activate NADPH oxidase through acting with RAGE on the cell surface. This may further trigger oxidative stress in cells[21]. RAGE is a specific signal transduction receptor for AGEs, which is widely expressed *in vivo*. Under normal physiological conditions, RAGE is lowly expressed. However, its expression level increases and is continuously up-regulated under DM and stress conditions[15]. AGEs-RAGE axis-induced ROS can further increase the expression

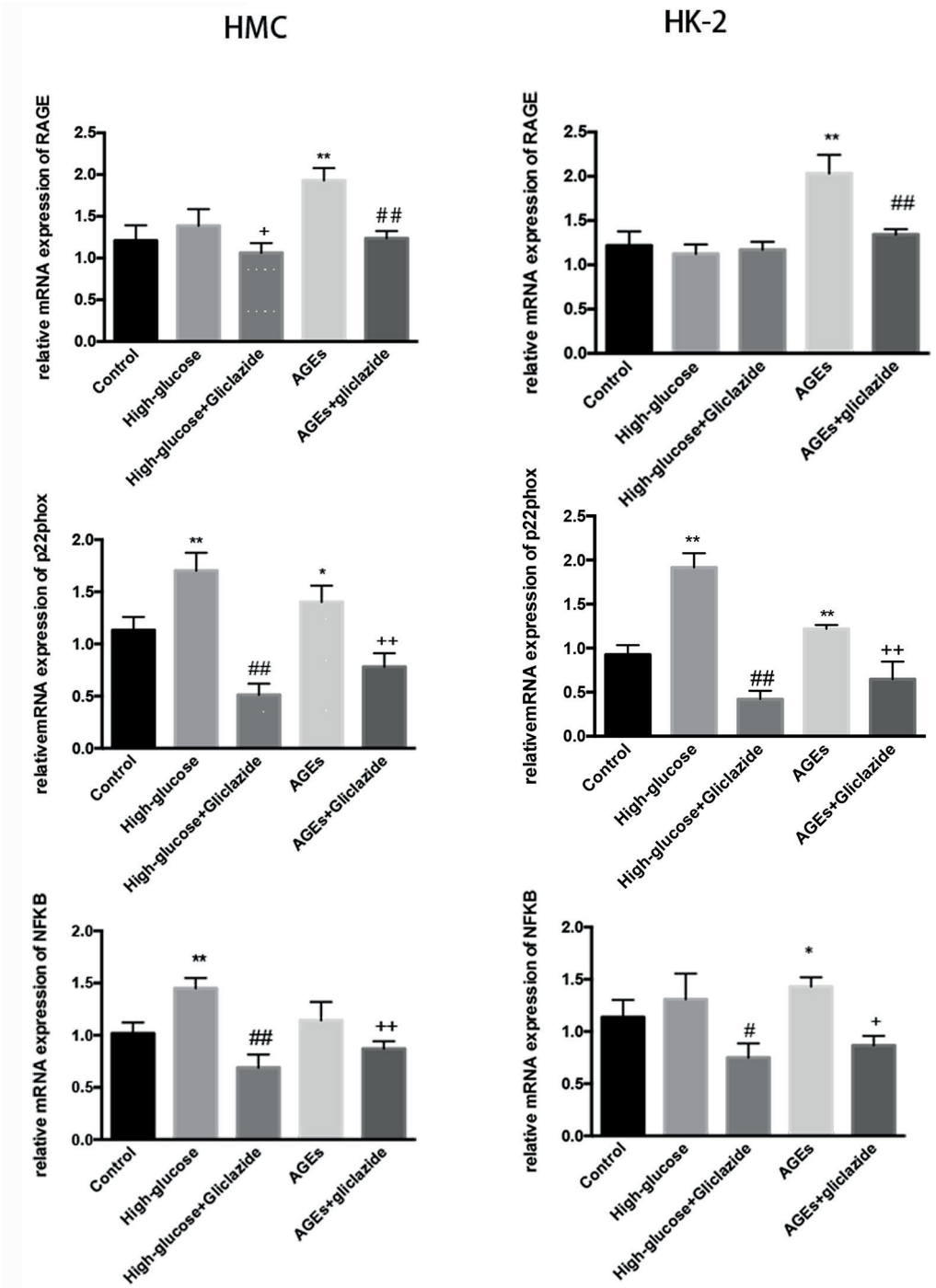

Figure 4. A, Effects of gliclazide on the expression of RAGE mRNA in HMCs treated with high glucose/high AGEs; **B,** Effects of gliclazide on the expression of p22phox mRNA in HMCs treated with high glucose/high AGEs; **C,** Effects of gliclazide on the expression of NF-κB mRNA in HMCs treated with high glucose/high AGEs; **D,** Effects of gliclazide on the expression of RAGE mRNA in renal tubular epithelial cells treated with high glucose/high AGEs; **E,** Effects of gliclazide on the expression of p22phox mRNA in renal tubular epithelial cells treated with high glucose/high AGEs; **F,** Effects of gliclazide on the expression of NF-κB mRNA in renal tubular epithelial cells treated with high glucose/high AGEs ($^{**}p<0.01$ *vs.* low glucose group, $^{++}p<0.05$ *vs.* AGEs group, and $^{\#\#}p<0.05$ *vs.* high glucose group).

of RAGE. This positive feedback has been believed to promote and enhance the pathogenicity of the AGEs-RAGE axis[22]. In the present study, gliclazide could directly reduce the expression of RAGE and NADPH oxidase subtype p22phox triggered by AGEs, reduce MDA level and elevate

activity of SOD and GSH-Px in HMCs and HK-2 *in vitro*. These findings exerted an indirect effect to alleviate the positive feedback of AGEs-RAGE through reducing the formation of ROS.

Activating the AGEs-RAGE axis can multiply promote the production of intracellular ROS and activate NF-κB, thereby triggering inflammatory responses[23]. As a core link in the inflammatory pathway, NF-κB activates other relevant signaling pathways, such as p21Ras and p38 MAPK. A series of inflammations is a crucial way for the occurrence and development of chronic complications[24]. In the present study, there were significant increased apoptosis of HMCs and HK-2 and significant expressions of RAGE and NADPH oxidase subtype p22phox. Moreover, a significant correlation was observed between the expression of RAGE and the apoptosis rate of HMCs and HK-2. Gliclazide could reduce the expressions of NADPH oxidase and NF-κB and apoptosis of HMCs and HK-2 triggered by high glucose and AGEs. The results suggested that gliclazide could withstand the damage of renal cells caused by AGEs and high glucose through inhibiting the RAGE-p22phox-NF-κB pathway and improving intracellular oxidative stress.

Conclusions

Gliclazide has protective effects on high glucose and AGEs-induced damage of glomerular mesangial cells and renal tubular epithelial cells *via* inhibiting RAGE-NADPH oxidase-NF-κB pathway.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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