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Original Article

Berberine prevents high glucose-induced cell viability inhibition and apoptosis in podocytes

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Abstract: Type 2 diabetics are predisposed to diabetic nephropathy (DN), a major pathological disorder leading to end-stage renal disease, which is the leading cause of premature death in diabetic patients. Podocytes are highly specialized and terminally differentiated glomerular cells that play a vital role in the development and progression of DN. Previous studies have demonstrated that berberine is clinically effective in alleviating type 2 diabetes. However, the effect of berberine on podocyte viability and apoptosis during high glucose exposure has not yet been fully elucidated. In this study, we showed that a high glucose environment could inhibit podocyte viability and increase apoptotic rates, with this effect alleviated by berberine. Furthermore, berberine-treated podocytes exposed to high glucose levels exhibited significantly increased nephrin and podocin, podocyte biomarkers, mRNA and protein expression relative to the high glucose exposure group.

Keywords: Diabetic nephropathy, podocyte, high glucose, berberine, viability, apoptosis

Introduction

Type 2 diabetics are predisposed to diabetic nephropathy (DN), a major pathological disorder leading to end-stage renal disease, which is the leading cause of premature death in diabetic patients [1]. Proteinuria, a clinical symptom of early DN, is closely associated with changes in podocytes due to injury [2]. Podocytes are terminally differentiated cells that are unable to regenerate and located at the outer layer of the glomerular basement membrane (GBM). These cells also play a crucial role in maintaining the structure and function of the glomerular filtration barrier [3, 4]. DN has become a major worldwide health problem, with one of the key treatment factors being to maintain the number of functionally stable podocytes. Thus, the development of novel drugs, possibly from natural products, is necessary to combat global DN-associated health problems.

The popular medicinal Chinese herb *Coptis chinensis* (Huang-Lian, a common herb in traditional Chinese medicine) has been widely used in Eastern Asia to promote health for more than one thousand years. The dried powder of *Coptis chinensis* has been used in traditional Chinese medicine to prevent or treat different diseases, including intestinal infections, particularly bacterial-associated diarrhea [5]. The major pharmacological properties of *Coptis chinensis* have been attributed to berberine (BBR), which is an isoquinoline alkaloid [6]. In 1980, the hypoglycemic clinical effect of BBR was found in China when BBR was used to treat diarrhea in diabetic patients. After that, BBR has been used as an anti-hyperglycemic agent by many physicians in China. Furthermore, accumulating evidence suggests that BBR is a potent oral hypoglycemic agent that is clinically effective in alleviating type 2 diabetes [7] and significantly decreases fasting plasma glucose (FBG), postprandial blood glucose, glycated hemoglobin, total cholesterol and low-density lipoprotein cholesterol levels [8-10]. However, the exact mechanism regarding how BBR acts in diabetes treatment is unclear. On one hand, BBR may reduce fasting blood glucose through an insulin-dependent signaling pathway, possibly through the activation of Adenosine monophos-

phate-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC). Moreover, the effect may be mediated through the up-regulation of hepatic low-density lipoprotein receptor mRNA expression, improvement of fatty acid oxidation, stimulation of glycolysis in peripheral tissue cells [11], insulin receptor expression and activation, promotion of insulin secretion, inhibition of liver gluconeogenesis [12] and promotion of intestinal glucagon-like protein-1 secretion [13]. BBR may also improve insulin sensitivity through the AMPK pathway or through insulin receptor expression induction [14-16]. On the other hand, recent studies have found that BBR improves fasting blood glucose by directly inhibiting gluconeogenesis in the liver through insulin-independent signaling [17]. Furthermore, while BBR has been shown to have a significant effect in DN patient treatment, the effect and mechanism of BBR induced podocyte protection was clear until now.

This study hypothesized that BBR could reduce podocytes viability inhibition and prevent cellular apoptosis in a high glucose (HG) environment. To examine this, podocytes were exposed to an HG environment and treated with BBR to see if cellular protection and an enhanced cell survival were achieved. These results indicate that novel mechanisms are involved in the actions of BBR and presents new potential therapeutic targets

Materials and methods

Reagent

BBR (molecular formula, $C_{20}H_{18}ClNO_4$) was purchased from Sigma-Aldrich, USA. The purity of BBR, detected by high-performance liquid chromatography, was 99%. The BBR was suspended in 0.5% sodium carboxymethylcellulose (CMC-Na, Sigma-Aldrich) prior to use. Conditionally immortalized mouse podocytes was purchased from the Cell Culture Center (Peking Union Medical College, Beijing, China). In this cell line, a temperature-sensitive SV40 large T-cell antigen (tsA58 Tag) is controlled by a γ-interferon inducible H-2Kb promoter.

Cell culture

Conditionally immortalized mouse podocytes were cultured according to the manufacturer's protocol. To induce proliferation, cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA), 100 U/ml penicillin, 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and 10 U/ml γ-INF (PeproTech, Rocky Hill, NJ, USA). Cells were cultured in a type I collagen-coated culture flask (BD Biosciences, Bedford, MA, USA) at 95% air and 5% CO_2 at 33°C, with media replaced every day until the cell density reached ~75-80%. To induce quiescence and phenotype differentiation, cells were cultured at 37°C in RPMI-1640 medium supplemented with 10% FBS (without γ-INF supplementation) and 1-2 drops of penicillin and streptomycin, with the media replaced every other day until reaching cellular maturity after 10-14 days. In the stage, the volume of podocytes and foot processes were observed under a phase contrast microscope and confirmed differentiation. When the podocytes reached 75-85% confluency, the cells were grown in serum-free RPMI-1640 medium for 24 h to synchronize cell growth and used for subsequent experimentation.

Group and treatment

Differentiated mouse podocytes were randomly divided into four groups, Group 1: treated with normal glucose (5 mmol/L, NG), Group 2: treated with high hypertonic mannitol as an osmotic control (30 mmol/L, MA), Group 3: treated with high glucose (30 mmol/L, HG) and Group 4: treated with high glucose (30 mmol/L) plus Berberine (HG+BBR). Cells were harvested at 24 h, 48 h and 72 h following treatment for each assay.

Cell viability assay

Cellular proliferation was monitored using a MTT assay kit (Promega) according to the manufacturer's protocol. Briefly, cells (5×10^3 cells/mL) were incubated in a 96-well plate according to their group specifications in a final volume of 0.1 mL at 37°C for the appropriate incubation time (24 h, 48 h and 72 h). Ten μl of MTT reagent was added to each well and the plate incubated for 4 h at 37°C. Before completing the incubation, the absorbance was measured at 570 nm using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), with each sample assayed in triplicate.

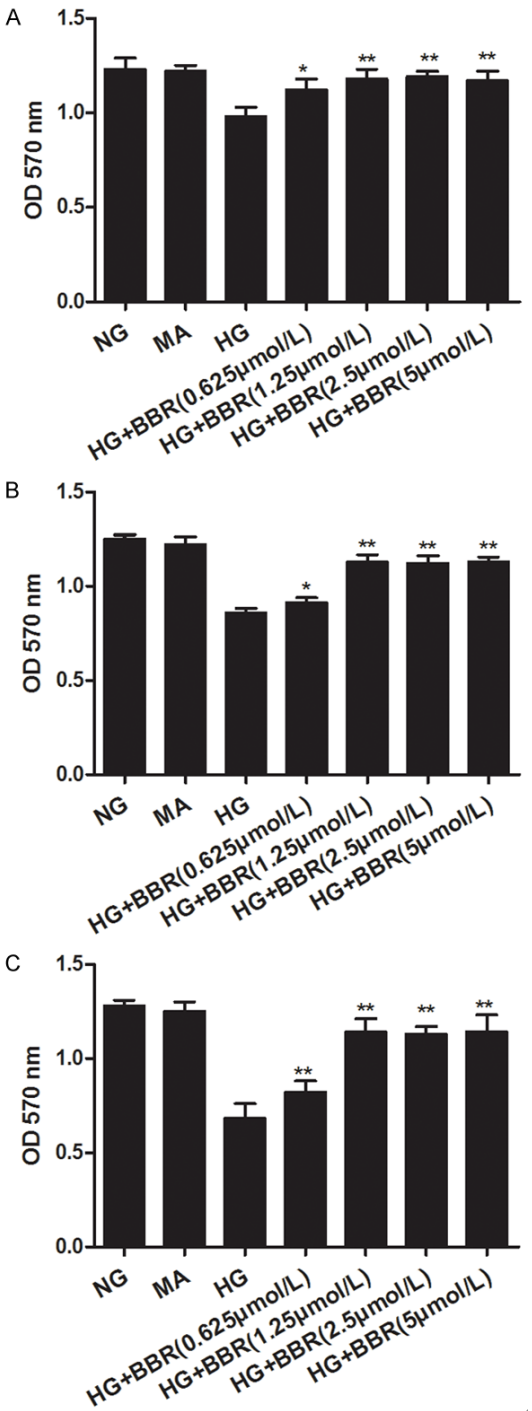

Figure 1. Effect of BBR on podocyte viability. Viability was determined by MTT assay at different time points (24 h, 48 h and 72 h). Samples were examined in triplicate and data are shown as a mean ± SD. A. Cells harvested 24 h post-treatment; B. Cells harvested 48 h post-treatment; C. Cells harvested 72 h post-treatment; **P* < 0.05, ***P* < 0.01, experimental group *vs.* HG group. NG: normal glucose; MA: Hypertonic mannitol; HG: high glucose; BBR: Berberine.

Cell apoptosis assay

We washed 5×10^5 cells twice with PBS, centrifuged them at 2,000 rpm for 5 min, and the cells were then suspended in 500 μL binding buffer from a KGA107 kit (KeyGene, Nanjing, China). Next, 5 μl of Annexin V-fluorescein isothiocyanate (FITC) was added to the sample and mixed well, followed by 5 μl of propidium iodide. After 10 min at room temperature in the dark, these cells were analyzed immediately by flow cytometry.

Quantitative real-time PCR

Podocyte marker gene mRNA expression levels, including nephrin and podocin, were detected by quantitative real-time PCR (qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Specific oligonucletide primers for mouse nephrin and podocin were designed as follows, mouse nephrin forward primer: 5'-TCTTCAAATGCACAGCCACCA-3' and reverse primer: 5'-CAAAGCCAGGTTTCCACTCCA-3', mouse podocin forward primer: 5'-AAGGACAGATATGGGCACTGTCA-3' and reverse primer: 5'-CCAGGAGCA CCTAAGCTATGGAA-3' and mouse β-actin forward primer: 5'-GGAGATTAC TGCCCTGGCTCCTA-3' and reverse primer: 5'-GACTCATCGTACTCCTGCTT GCTG-3'. cDNA synthesis was performed according the manufacturer's protocol (Promega). The qPCR reaction mixture contained 12.5 μl of 2× SYBR green PCR mix (Fermetas), 0.3 μM of forward/reverse primers, and 1 μl of cDNA template, up to a final volume of 25 μl with distilled water. Cycling parameters were set as follows: initial activation step at 95°C for 5 min and then 35 cycles of amplification, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 15 s. Relative expression levels were calculated using β-actin as an internal control, with all samples amplified in triplicate.

Western blotting analysis

Podocyte cells (2×10^6) were washed twice with ice-cold PBS, lysed RIPA lysis buffer containing protease and phosphatase inhibitors for 40 min on ice, and centrifuged at 12,000 *g* for 15 min at 4°C. Supernatants were collected and resolved via 10% SDS-PAGE and transferred onto the PVDF membrane (PALL, USA). Mem-

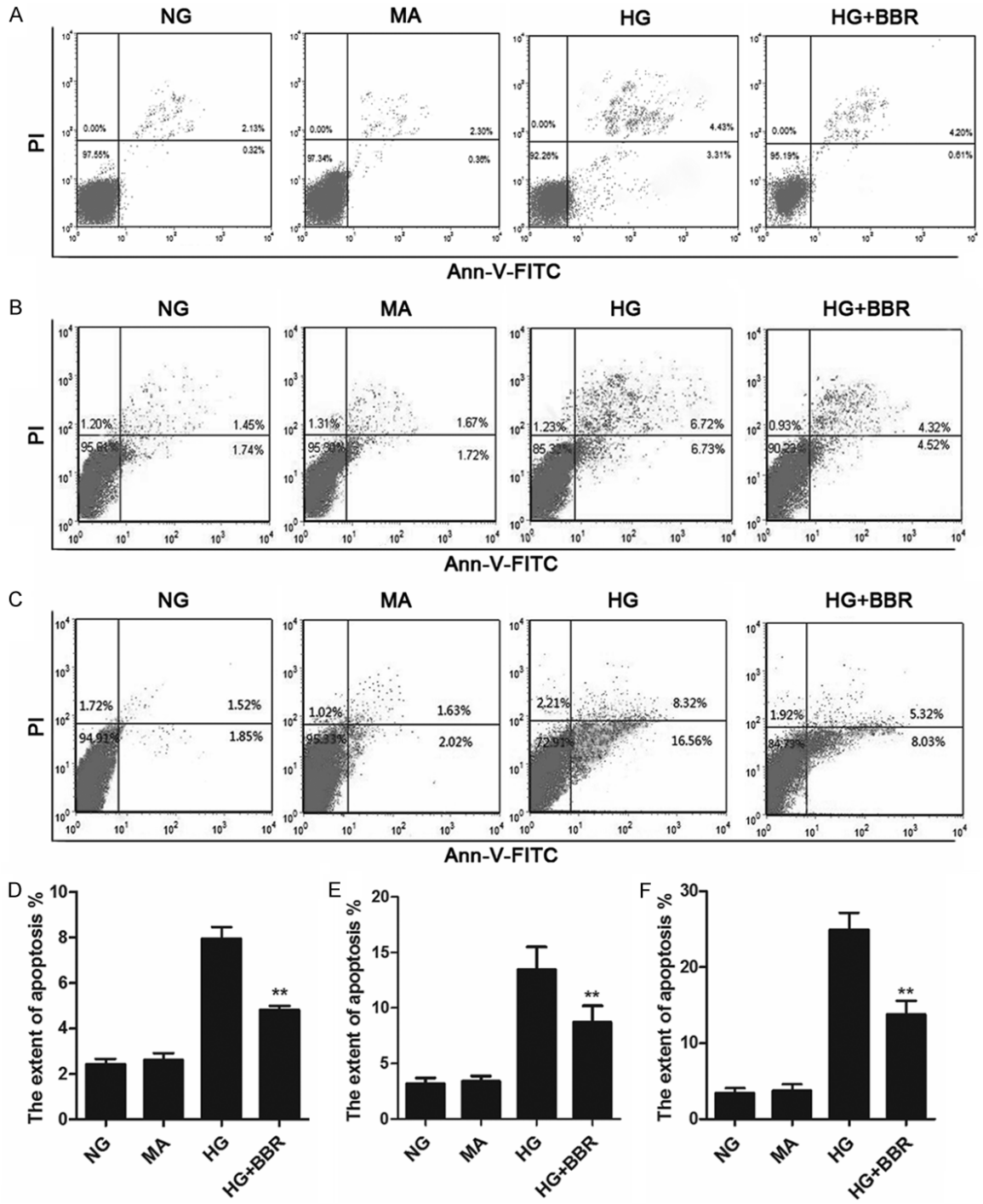

Figure 2. Effect of BBR on podocyte cellular apoptosis. Podocytes were treated for 24 h, 48 h and 72 h and examined via flow cytometry with Annexin FITC/PI staining. A. Podocytes 24 h post-treatment; B. Podocytes 48 h post-treatment; C. Podocytes 72 h post-treatment; D. The extent of apoptosis among different experimental groups 24 h post-treatment; E. The extent of apoptosis among different experimental groups 48 h post-treatment; and F. The extent of apoptosis among different experimental groups 72 h post-treatment. Data are presented as a mean ± SD. $^{**}P < 0.01$, HG *vs.* HG+BBR. NG: Normal glucose; MA: Hypertonic mannitol; HG: High glucose; BBR: Berberine.

branes were blocked for 1.5 h at 37°C with 5% non-fat milk and then incubated with mouse anti-nephrin (1:4,000, Abcam), mouse anti-podocin (1:4,000, Abcam) and mouse anti-β-

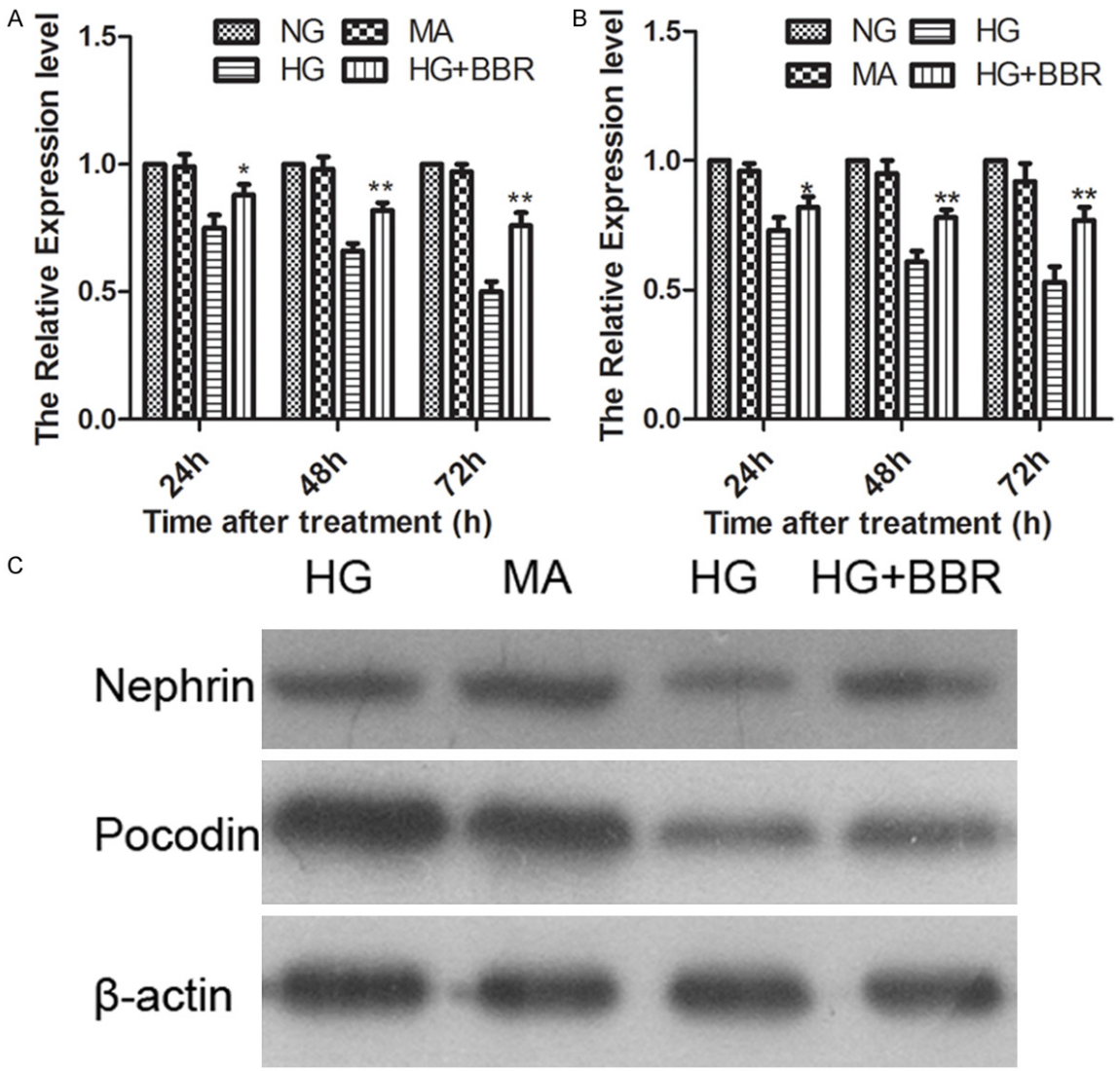

Figure 3. Effect of BBR on nephrin and podocin expression. Podocytes were treated for 24 h, 48 h and 72 h and analyzed via qPCR and western blot analysis. A. Relative nephrin mRNA expression levels via qPCR detection; B. Relative podocin mRNA expression levels via qPCR detected. C. Protein expression level of nephrin and podocin detected via western blot. Data are presented as a mean ± SD. $^{**}P < 0.01$, HG *vs.* HG+BBR. NG: Normal glucose; MA: Hypertonic mannitol; HG: High glucose; BBR: Berberine.

actin (1:400, Santa Cruz) in 5% non-fat milk for 1 h at 37°C. After washing in TBS with 0.5% Tween 20 (TBST), the membrane was incubated with HRP-conjugated secondary antibody at room temperature for 40 min. This incubation was followed by a TBST wash and the membranes were assayed with enhanced chemiluminescence (ECL) and recorded on X-ray films.

Statistical analysis

Statistical analysis was performed using the SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare the differences between HG, NG, MA and HG+BBR; LSD analysis was used to compare the differences between HG and HG+BBR. All tests performed two-sided. $P < 0.05$ was considered to be statistically significant.

Results

The effect of BBR on podocyte viability

To examine the effect of BBR on podocytes viability, podocytes were cultured for 24 h, 48 h and 72 h following treatment and evaluated

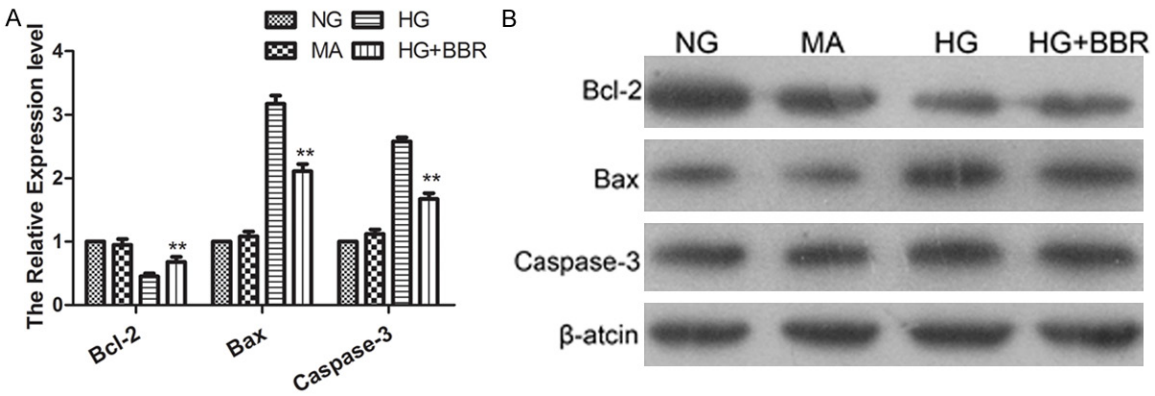

Figure 4. Effect of BBR on apoptosis-related gene expression. Podocytes were treated for 24 h, 48 h and 72 h and examined via qPCR and western blot analysis. A. Relative Bax, Bcl-2 and Caspase-3 mRNA expression levels detected via qPCR and B. Protein expression level detected via western blot. Data are presented as a mean ± SD. $^{**}P < 0.01$, HG *vs.* HG+BBR. NG: Normal glucose; MA. Hypertonic mannitol; HG: High glucose; BBR: Berberine.

with a MTT assay. As expected, mannitol had no effect on podocyte viability. When compared to the HG group, a protective effect was seen 24 h post-treatment in the HG+BBR groups at a concentration of 0.625 μmol/L ($P < 0.05$) and at 1.25 μmol/L, 2.5 μmol/L and 5 μmol/L ($P < 0.01$, **Figure 1A**). The results 48 h post-treatment showed a similar trend to the 24 h results (**Figure 1B**), while cultures examined 72 h post-treatment showed a higher protective effect across all concentrations ($P < 0.01$, **Figure 1C**). These results indicated that a BBR concentration of 1.25 μmol/L provided the optimal protective activity and thus this concentration was use for subsequent experiments.

The effect of BBR on podocyte apoptosis

To verify whether BBR affects apoptosis, podocytes were treated according to each group and analyzed via flow cytometry. The results demonstrated that BBR treatment could reduce the percentage of cellular apoptosis relative to the HG group (**Figure 2**), with a higher apoptotic percentage seen in the HG group (7.93 ± 0.74%) and a lower percentage in the HG +BBR group (4.82 ± 0.35%) 24 h post-treatment (**Figure 2A**, **2D**). Similar trends were seen 48 h and 72 h post-treatment, with higher percentages seen in the HG group (13.48 ± 2.04%, **Figure 2B**; 24.91 ± 2.26%, **Figure 2E**) and lower percentages seen in the HG+BBR group (8.74 ± 1.47%, **Figure 2C**; 13.75 ± 1.83%, **Figure 2F**). Additionally, the total percent of apoptotic podocytes in the NG and MA groups were consistent over time.

The effect of BBR on nephrin and podocin expression

Nephrin and podocin expression were examined by qPCR and western blot analysis (**Figure 3**). Relative to the HG group, the expression of nephrin (**Figure 3A**) and podocin (**Figure 3B**) in the HG+BBR group had significantly increased after culturing for 24 h, 48 h and 72 h, with these findings confirmed via western blot (**Figure 3C**). Furthermore, when compared to the NG group, the expression of nephrin and podocin in the MA group had no significant differenced across all time points.

The effect of BBR on apoptosis-related gene expression

Apoptotic-associated genes Bcl-2, Bax and caspase-3 were examined via qPCR and western bolt analysis (**Figure 4**). While the MA group was not significantly different from the HG group, a significant difference was noted between the HG and HG+BBR groups. Relative to the HG group, Bcl-2 increased and Bax and caspase-3 decreased in the HG+BBR group (**Figure 4A**), with these findings confirmed via western blot (**Figure 4B**).

Discussion

In DN, podocyte pathological changes mainly include lesions, apoptosis, and GBM detachment, with the remaining podocytes undergoing compensatory hypertrophy to cover the area of the basement membrane and broad-

ened the foot process. This leads to an increased GBM permeability and emergence of overt proteinuria, thus causing the clinic appearance of glomerulosclerosis and progressive renal dysfunction. With advances in DN research, the influence of podocyte lesions on prognosis has been found to be an important factor. In this study, we successfully constructed a high glucose-induced podocyte model to investigate the possible mechanism of BBR renoprotection. Here we demonstrated that compare with normal glucose and high mannitol induced samples, that HG exposure reduced podocyte viability and increased the percentage of apoptotic podocytes. Further, these findings suggest that hyperglycemia can lead to podocyte apoptosis, while hyperosmolay cannot.

BBR is a naturally occurring antidiabetic drug, with previous studies demonstrating that BBR plays a key role in regulating the cellular processes induced by high glucose [18-20]. For example, BBR can attenuate high glucose-induced proliferation and extracellular matrix accumulation in mesangial cells. Additionally, BBR exerts renoprotection during a high-fat diet and in streptozotocin-induced DN rats by modulating EP protein expression in the EP-G protein-cAMP signaling pathway [21]. Moreover, BBR could ameliorate renal injury in diabetic C57BL/6 mice through the suppression of the SphK-S1P signaling pathway [22]. These results amply demonstrate that BBR can play a key role in the treatment of DN. Podocytes play a critical role in maintaining glomerular permselectivity and podocyte damage is a key factor aggravating DN [23, 24]. In this study, we demonstrated that BBR (1.25 μmol/L) could significantly reduce high glucose-induced cell viability inhibition and cellular apoptosis in podocytes. This work provides new insight into the mechanism whereby BBR regulates renoprotection in DN mice and thus has important implications in DN therapeutic development.

Podocytes are attached to the basement membrane through sparse foot processes, with the cracks between adjacent foot processes connected by a slit diaphragm (SD). The SD acts as the main macromolecule filtration barrier and is composed of nephrin and podocin among others. Nephrin and podocin are uniquely expressed in podocytes and their down-regulation is associated with heavy proteinuria [25, 26]. In DN models, expression changes in nephrin were associated with changes in podocin protein and mRNA expression levels. The results in this study revealed that BBR could up-regulate nephrin and podocin mRNA and protein expression levels during HG treatment. Similarly to our results, nephrin and podocin protein expression was found to be significantly increased following BBR treatment in rats with diabetes induced by a high-fat diet or streptozotocin [27]. Additionally, the present study showed that BBR could decrease pro-apoptotic gene expression, including Bcl-2 and caspase-3, and increase the expression of apoptotic suppressor genes such as Bax. Furthermore, BBR was shown to reduce apoptic rates in the presence of HG treatment via flow cytometer. These findings suggest that the BBR protective effect in the presence of HG may be related to the inhibition of glucose-induced apoptosis that in turn up-regulates the expressions of nephrin and podocin.

In conclusion, BBR can promote a protective effect against HG-induced cell viability inhibition and cellular apoptosis, with this protective mechanisms possibly related to the up-regulation of nephrin and podocin. However, the signaling pathways modulating nephrin and podocin expression and their relation to apoptosis-related genes are still vague and will require further examination.

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Disclosure of conflict of interest

None.

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