Effects of Qijin granules on high glucose-induced proliferation, apoptosis and expression of nuclear factor-kB and MCP-1 in rat glomerular mesangial cells

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Original Research Article

Abstract

Purpose: To investigate the effects of Qijin granules on high glucose-induced proliferation and apoptosis in rat glomerular mesangial cells (MC).

Methods: MC cells from rats were passaged and cultured, and randomly divided into control group (CNG), high glucose group (HGG), Western medicine group (WMG, high glucose + Benazepril + Gliquidone), and Qijin granules 1/2/3 group (high glucose + different doses of Qijin granules). Mesangial cells proliferation was measured using MTT assay. The NF-kB, MCP-1 and inflammatory factors in supernatant were determined by ELISA. Apoptosis rate and cell cycle were assessed by flow cytometry. The apoptosis-related TGF-β1/Smad signaling pathway-related protein expressions were measured by Western blot.

Results: The A-value and early apoptosis rate, apoptosis rate and S-phase percentage, and protein expressions of NF-kB, MCP-1, IL-6, IL-2, TNF-α, Bax, Cyt-C, caspase-3, TGF-β1, and p-Smad3 of MC cells in the HGG at 12 h, 24 h and 48 h were higher than those in the CNG. The above indices were lower in the WMG, and Qijin granules 1/2/3 groups than in the HGG. The Bcl-2, Smad7 protein expression level and the percentage of G1 and G2/M phase were lower in the HGG than in the CNG, and the above indeices were higher in the WMG and Qijin granules 1/2/3 group than in HGG.

Conclusion: Qijin granules can dose-dependently inhibit high glucose-induced proliferation and apoptosis in rat MC cells, block the cell cycle and reduce inflammatory responses. This may be related to the regulation of NF-kB, MCP-1 and TGF-β1/Smad signaling pathways. These findings provide theoretical and experimental basis for the clinical treatment of early diabetic nephropathy.

Keywords: Glomerular mesangial cells, Hyperglycemia, Qijin granules, Cell proliferation, Apoptosis, NF-kB

INTRODUCTION

Diabetic nephropathy (DN) is one of the common microvascular complications of diabetes mellitus, with glomerular mesangial cell (MC) proliferation, thickening of glomerular basement membrane (GBM), and accumulation of extracellular matrix as the main histological features. As the disease worsens, patients develop a series of pathological changes, such as renal interstitial...
fibrosis and glomerulosclerosis, which will eventually lead to decline in renal function and renal failure [1-4].

Studies have confirmed that a high glucose environment can cause MC cell proliferation and induce an excessive production of reactive oxygen species in MC cells, leading to severe inflammatory responses and oxidative stress, aggravating extracellular matrix deposition and the condition of DN [5-7]. Therefore, methods to reduce MC cell damage induced by high glucose and inhibit abnormal cell proliferation are particularly crucial for the prevention and treatment of early DN.

DN is defined as "edema" and "turbid urine" secondary to "Excessive Thirst" in Traditional Chinese medicine, and treatment should be based on ascending Yang and consolidating the essence, benefiting Qi and strengthening the spleen [8,9]. "Qijin Granules" is a cipher prescription by Professor Li Zhengsheng for the treatment of patients with chronic nephritis and nephrotic syndrome, with the efficacy of tonifying the deficiency and consolidating the essence, benefiting the kidney and filling the essence. A study found that Qijin granules are effective in refractory nephrotic syndrome, and facilitates the reduction of adverse reactions related to immunosuppressants and hormones [10]. However, there are few studies on the specific mechanism of the drug's efficacy on MC cells. In the previous study, we established animal models of C-BSA-induced nephritis and diuresis in rats, and analyzed the therapeutic effects of Qijin granules on chronic glomerulonephritis in terms of urine protein, blood creatinine and blood urea nitrogen, and found that Qijin granules could effectively improve kidney function. To explore whether Qijin granules can delay the progression of DN, this study created the high glucose environment in vitro and analyzed the effects of Qijin granules on the proliferation, apoptosis and expression of NF-κB and MCP-1 in MC cells induced by high glucose, aiming to provide theoretical and experimental basis for the clinical treatment of early DN.

EXPERIMENTAL

Materials

Thirty-six male Sprague Dawley (SD) rats, weighing (180 ± 20) g, 6 - 8 weeks old, were purchased from Beijing Experimental Animal Research Center provided (Animal Use License No. SYXXK (Beijing, China) 2018-0013). Other materials used include Rat MC cell line (HBZY-1) (China Center For Type Culture Collection (Wuhan University), Wuhan, China); D-glucose (Shanghai Baoman Biotechnology Co. Ltd, Shanghai Other materials used include China); Qijin granules (Astragalus, Euphorbiae HumifusaeHerba, Rehmannia glutinosa, Cornus officinalis, Codonopsis pilosula, Codonopsis pilosula, Wolfiporia extensa, Alisma plantago-aquatica); Benazepril (Beijing Novartis Pharmaceutical Co. Ltd, Beijing, China); Glipizide (Beijing Wanhui Shuanghe Pharmaceutical Co., Ltd., Beijing, China); Fetal bovine serum (Invitrogen GIBCO, Carlsbad, CA, USA); Penicillin mixture, Trypsin digest (Beijing Ita Biotechnology Co. Ltd., Beijing, China). The following were also used: tetramethylazolyl salt (Shanghai Yanjin Biotechnology Co. Ltd., Shanghai, China); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazonium bromide) cell proliferation assay kit (Shanghai Fanshu Biotechnology Co. Ltd., Shanghai, China); High-sugar Dulbecco’s modified eagle medium (DMEM) medium (Beijing Bialyro Technology Co. Ltd, Beijing, China); enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Elite Biotechnology Co., Ltd., Wuhan, China); bicinchoninic acid (BCA) kit, radioimmunoprecipitation assay (RIPA) lysate (Shanghai Biolab, Shanghai, China); Mouse anti-rat Bax, Cyt-C, Caspase-3, TGF-β1, p-Smad3, Smad7 monoclonal antibodies (CST Inc, Danvers, MA, USA). Every procedure was approved by the Animal Care and Use Committee of the Third People's Hospital of Guizhou Province.

Equipment

QuickSpeed 3200 Mini High Speed Centrifuge (Mona (Suzhou) Biotechnology Co., Ltd., Suzhou, China); FACS calibur flow cytometry analyzer (Becton Dickinson, Franklin Lakes, NJ, USA); CO2 incubator (Heraeus, Hanau, Germany); SW-CJ-2D ultra clean bench (Suzhou Purification, Suzhou, China); inverted microscope (Shanghai Yuyan Scientific Instruments Co. (Suzhou Purification, Suzhou, China); Ultra-low temperature freezer (Revco, Waltham, MA, USA); 96-well cell culture plate (Corning, Corning, NY, USA); Gel Doc EZ automatic gel imaging analysis system (BIO-RAD, Hercules, CA, USA).

Preparation of drug-containing serum

Thirty-six male SD rats were randomly divided into 6 groups (n = 6 for each group) after 1 week of casual feeding. Qijin Granules were administered by gavage 10, 20 and 40 g/(kg-d) in form of infusion powder (6.67 g raw drug / 1 g
paste powder) in Qijin granules groups 1, 2 and 3, respectively, and western medicine was administered by gavage with 5 mg/(kg-d) of Benadryl + 18 mg/(kg-d) of Glipizide, and equal volume of saline was given to the control group (CNG).

The drug was administered for 7 days. After 2 h of final dosing, the blood was collected from the carotid artery, left at room temperature for 30 min and then centrifuged at 3500 r/min to separate the serum. The complement was inactivated with water bath at 56 °C for 30 min, and decontaminated and filtered for freezing and storage.

**Cell culture**

HBZY-1 was routinely cultured in DMEM containing 10 % fetal bovine serum, 100 mg/L streptomycin, and 100 U/mL penicillin in an incubator (37 °C, 5 % CO₂), and the medium was changed every 2 - 3 days. The cells were digested and passaged using trypsin containing 0.03% EDTA.

**Cell grouping in serum of Qijin granules**

Twelve replicate wells were set up in each group. The cells and supernatant were collected for testing after 48 h of routine incubation in a 37 °C, 5 % CO₂ cell incubator.

**3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT)**

The cells were treated with the corresponding drugs. After incubation for 48 h, 20 μL of 0.5 % MTT solution was added to each well, followed by incubation for 4 h. The supernatant was discarded and 150 μL of dimethyl sulfoxide (DMSO) was added to each well, shaken for 10 min, and the absorbance value (A) at 490 nm was measured using an enzyme marker in triple.

**Flow cytometry**

Cells were treated for 72 h, digested using trypsin, rinsed twice in phosphate buffered saline (PBS), fixed in 75 % ethanol, washed again in PBS, resuspended in 1 mL of DNA-staining solution containing 1 mg/mL RNAase and 0.02 mg/mL PI, developed at room temperature and protected from light.

The apoptosis rate was determined using FACS calibur flow cytometry analyzer, and the percentage of cells in each phase of cycle was analyzed using Cell Quest in triple.

**Enzyme-linked immunosorbent assay (ELISA)**

The cell concentration was adjusted to 5 × 10⁵/mL and inoculated in 6-well plates for 24 h, and the corresponding drug intervention was given. Each group was set with 4 duplicated wells. The corresponding culture plates were taken out after 48 h of culture, and the supernatant of each group was collected. The levels of NF-κB, MCP-1, IL-6, IL-2, TNF-α were determined according to ELISA kit instructions in triple.

**Western blot assay**

Cells were washed three times with pre-chilled PBS rinse. The total protein was extracted with RIPA lysate. The protein quantification was performed by BCA method, followed by SDS-polypropylene gel electrophoresis, membrane transfer, and blocking with skim milk powder. After treatment with primary antibodies (Bax, Cyt-C, Caspase-3, TGF-β1, p-Smad3, Smad7, GAPDH antibodies, 1:1000), the membrane was incubated overnight at 4°C at room temperature. The secondary antibody (1:5000) was added, followed by 1 h of incubating shakers. The membrane was washed with TBST and developed with the ECL luminescence with GAPDH as the internal reference. The grayscale values were calculated using Image J software in triple.

**Statistical analysis**

Statistical Package for the Social Sciences (SPSS) 24.0 statistical software (IBM, Armonk, NY, USA) was used for statistical analysis. Measurement data conforming to normal distribution are expressed as mean ± SD, one-way analysis of variance (ANOVA) was used for comparison among multiple groups, followed by post hoc LSD-t test. P < 0.05 was considered statistically significant.

**RESULTS**

**Effect of Qijin granules on the proliferative activity of MC cells**

The A values of MC cells at 12, 24, and 48 h were higher in the HGG than in the CNG, and were lower in the WMG, Qijin granules 1/2/3 groups than in the HGG, with the lowest A value in Qijin granules 3 group (p < 0.05), indicating that high glucose could induce excessive proliferation of MC cells, while Qijin granules inhibited the proliferation ability of cells in a dose-dependent manner (Figure 1).
Effect of Qijin granules on the proliferation of MC cells

Qijin granules inhibit cell proliferation capacity in a dose-dependent manner. Compared with the control group, **P < 0.01, ***P < 0.001; compared with the high glucose group, #P < 0.05, ##P < 0.01, ###P < 0.001.

Effect of Qijin granules on the apoptosis of MC cells

The early apoptosis rate and apoptosis rate of MC cells in the HGG were higher than those in the CNG (P < 0.05) and were lower in the WMG, Qijin granules 1/2/3 groups than in the HGG, with lowest level in Qijin granules 3 group (P < 0.05), indicating that high glucose could promote apoptosis of MC cells, while Qijin granules could reverse high glucose-induced MC apoptosis in a dose-dependent manner (Figure 2).

Effect of Qijin granules on the expressions of NF-κB and MCP-1 in MC cells

NF-κB and MCP-1 levels in supernatants were higher in the HGG than in the CNG (P < 0.05), and were lower in the WMG, Qijin granules 1/2/3 groups than in the HGG, with the lowest level in Qijin granules 3 group (P < 0.05), showing that high glucose could aggravate the inflammatory response while Qijin granules could down-regulate NF-κB and MCP-1 expression in a dose-dependent manner (Figure 3).

Effect of Qijin granules on the expression of apoptosis-related proteins in MC cells

Bax, Cyt-C, and Caspase-3 levels were higher and Bcl-2 was lower in the HGG than in the CNG (P < 0.05). Bax, Cyt-C, and caspase-3 were lower and Bcl-2 was lower in WMG, Qijin granules 1/2/3 groups than in the HGG, and the improvement was most obvious in Qijin granules 3 group (P < 0.05), indicating that high glucose could inhibit the expression of apoptosis-related proteins in MC cells, while Qijin granules could up-regulate Bcl-2 expression and down-regulate Bax, Cyt-C, Caspase-3 expression in a dose-dependent manner (Figure 4).
Effect of Qijin granules on cell cycle

The percentage of G1 and G2/M phase of MC cells in the HGG was lower than that in CNG, and the percentage of S phase was higher than that in the CNG (P < 0.05). The percentage of G1 and G2/M phase of MC cells in the WMG, Qijin granules 1/2/3 groups were higher while the proportion of S phase was lower than that in the HGG, and the improvement was most obvious in Qijin granules 3 group (P < 0.05), suggesting that the ratio of G1 phase decreased and S phase increased in the high glucose environment, and apoptosis was reduced, while Qijin granules could promote S phase block and induce apoptosis in a dose-dependent manner (Figure 5).

![Figure 5](image)

**Figure 5:** Effect of Qijin granules on MC cell cycle. (A-C) show that Qijin granules promote S-phase block and induce apoptosis in a dose-dependent manner. Compared with the control group. ***P < 0.001; compared #p < 0.05, ##p < 0.01, ###p < 0.001, with the high glucose group

Effect of Qijin granules on the expression of TGF-β1/Smad signaling pathway-related proteins in MC cells

The protein expressions of TGF-β1 and p-Smad3 protein were higher and Smad7 was lower in the HGG than in the CNG (P < 0.05). The expression of TGF-β1 and p-Smad3 protein was lower while Smad7 protein was higher in the WMG, Qijin granules 1/2/3 groups than in the HGG. The most significant improvement was observed in the Qijin granules 3 group (P < 0.05), indicating that the TGF-β1/Smad signaling pathway was regulated in the high glucose environment, and Qijin granules could regulate the expression of TGF-β1/Smad signaling pathway-related proteins (Figure 6).

![Figure 6](image)

**Figure 6:** Effect of Qijin granules on the expression of TGF-β1/Smad signaling pathway-related proteins in MC cells. Qijin granules regulate TGF-β1/Smad signaling pathway-related protein expression in a dose-dependent manner. Compared with the control group. ***P < 0.001; compared #p < 0.05, ##p < 0.01, ###p < 0.001 with the high glucose group

Effect of Qijin granules on inflammatory factors in MC cells

The expressions of IL-6, IL-2, and TNF-α in the supernatant of MC cells in the HGG were higher than those in the CNG (P < 0.05). They were lower in the Qijin granules 1/2/3 groups than in the HGG, with the lowest level in Qijin granules 3 group (P < 0.05), indicating that high glucose could intensify the inflammatory response of MC cells, while Qijin granules could inhibit the expression of inflammatory factors in a dose-dependent manner (Figure 7).

![Figure 7](image)

**Figure 7:** Effect of Qijin granules on the expression of inflammatory factors in MC cells. Figure 7 show that Qijin granules inhibited the expression of inflammatory factors IL-6 (A), IL-2 (B) and TNF-α (C) in a dose-dependent manner. Compared with the control group. ***P < 0.001; compared #p < 0.05, ##p < 0.01, ###p < 0.001, compared with the high glucose group

DISCUSSION

As a type of intrinsic renal cells necessary to maintain glomerular function and structure, MC cells regulated many physiological processes such as matrix metabolism, secretion of cytokine, and glomerular blood flow [11]. The etiology of DN has not been fully elucidated, and it is believed that the release of many cytokines and reactive oxygen species from MC cells were induced by high glucose resulting from disorders of glucolipid metabolism, which subsequently causes oxidative stress, rheological changes and inflammatory responses in glomeruli, resulting in cell proliferation and division, leading to the
aggregation of extracellular matrix in MC cells and reducing renal function [12].

NF-κB is an important nuclear transcription factor in the nucleus that regulates the activation and expression of many genes, and participates in the signal transmission of cellular inflammatory response [13]. It was discovered that NF-κB was involved in the process of renal injury in DN via induction of inflammatory response and production of vascular endothelial growth factor [14]. MCP-1, a chemotactic protein secreted by renal tubular epithelial cells, can aggravate structural damage to renal tissue and promote glomerulosclerosis through inflammatory mechanisms.

Khan et al [15] found that high glucose upregulated MCP-1 expression in MC cells and that MCP-1 expression levels were positively correlated with proteinuria levels. In this study, we created a hyperglycemic environment in vivo during DN and induced the secretion of NF-κB, MCP-1, inflammatory factors and apoptosis-related proteins in MC cells with 25 mmol/L glucose, and found that A value, apoptosis rate, the expression of NF-κB, MCP-1, IL-6, IL-2, TNF-α, Bax, Cyt-C and Caspase-3 were higher in the HGG compared with the CNG, showing that the high glucose environment could activate the NF-κB pathway, leading to a cellular inflammatory response in MC cells and inhibiting cell proliferation and apoptosis, and the model was successfully established. Intervention with different doses of Qijin granules revealed that Qijin granules dose-dependently inhibited high glucose-induced proliferation and apoptosis, and down-regulated the expression of NF-κB, MCP-1 and inflammatory factor.

The TGF-β1/Smad signaling pathway can be involved in transdifferentiation of epithelial glomerular cells and glomerulosclerosis, and Smad3 and Smad7 can act as regulators of the extracellular matrix under pathological conditions [16, 17]. Zhang et al [18] found that high glucose induces apoptosis and proliferation of MC cells via TGF-β1/Smad signaling pathway. In the present study, we found that TGF-β1 and p-Smad3 protein expression was upregulated, Smad7 protein was decreased, and Smad3 protein phosphorylation was increased in MC cells in high glucose environment, consistent with the features of early pathological changes and similar to the findings of the above studies. After co-culture of serum containing Qijin granules with high glucose, it was found that small, medium and large doses of Qijin granules could inhibit Smad3 protein phosphorylation and TGF-β1 expression, and down-regulate Smad7 protein expression in MC cells induced by high glucose.

Dai et al [19] believed that DN was consumptive thirst involving kidney, which is similar to "edema", "cloudy urine", and "fullness", which are secondary to "deficiency of vital energy and loss of harmony between Yin and Yang". Therefore, this disease originates from the spleen and kidneys, and should be treated by nourishing the spleen and kidneys, and then consolidating the vital energy and enhancing the Yang.

The Qijn granules used in this study are composed of Chinese herbs, among which Astragalus and Euphorbiae HumifusaeHerba can relieve swelling, invigorate the spleen and promote Yang; Rehmannia glutinosa and Cornus officinalis replenishes essence and marrow, nourishes the kidney and strengthens essence and invigorates the spleen and kidney together with astragalus; Codonopsis pilosula invigorates qi and the spleen, promotes diuresis and expels dampness; Wolfiporia extensa dehumidifies and invigorates the spleen, while Alisma plantago-aquatica clears away heat, and reduces turbidity and lipid levels. The joint use of the herbs can benefit the spleen, dispersing and relieving symptoms [20]. Modern pharmacological studies have shown that Astragalus is a diuretic, scavenging free radicals, regulating immune function, promoting albumin synthesis and reducing protein urinary excretion. Astragaloside is the main active ingredient of Astragalus, which reduces oxidative stress and inflammatory response by regulating NF-κB levels, thus protecting foot cells and inhibiting apoptosis [21].

The total flavonoids in Euphorbiae Humifusae herba can enhance the immune function and scavenger free radicals. Codonopsis Pilosula Extract can promote angiogenesis and inhibited apoptosis, and animal studies have found that Codonopsis pilosula extract reduced the levels of malondialdehyde (MDA) and inflammatory factors such as urea nitrogen (BUN), creatinine (CRE) and TNF-α in renal tissues [22]. Rehmannia glutinosa slows down renal interstitial fibrosis. Dong et al [23] established a diabetic model and found that catalpol in Rehmannia glutinosa reduced angiotensin II and TGF-β1 levels, downregulated renal cortical TGF-β1 gene expression, lowered extracellular matrix deposition, inhibited epithelial-mesenchymal transition, and thus slowed down renal interstitial fibrosis. Atractylodes macrocephala promoted serum albumin synthesis, metabolism, and improved the circulation. Cornus officinalis reduced urinary protein and attenuate MC cell damage induced by chronic kidney disease.
**Alisma plantago-aquatica** lowered blood lipids levels, anti-oxidative stress, and promote immunomodulation. Feng et al [24] found that Alisma plantago-aquatica extract has dual effects on renal function, i.e., anti-diuretic effects at high doses and pro-diuretic effects at low doses.

**CONCLUSION**

Qijin granules dose-dependently inhibits the proliferation and apoptosis of MC cells induced by high glucose, blocks cell cycle and reduces inflammatory response in rats. This may be related to the regulation of NF-κB, MCP-1 and TGF-β1/Smad signaling pathways. Thus, these findings provide a theoretical and experimental basis for the management of early DN.

**DECLARATIONS**

**Acknowledgement**

This work was supported by the Guiyang Science and Technology Project (grant no. Zhuke contract [2019] - 9-4-16) and Internal Medicine Research Project (grant no. GZEYK [2020]14).

**Conflict of interest**

No conflict of interest is associated with this work.

**Contribution of authors**

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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