Huaiqihuang may protect from proteinuria by resisting MPC5 podocyte damage via targeting p-ERK/CHOP pathway

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ABSTRACT

The purpose of this study was to investigate the potential effects of Huaiqihuang (HQH) granule, a Chinese herbal medicine, in treating proteinuria and to reveal its possible mechanism. MPC5 podocytes were cultured *in vitro* at 37°C and induced with tunicamycin (TM). The TM-induced cells were treated with HQH at different concentrations. The cell proliferation was detected using the MTT assay. The optimal effective dose of HQH for MPC5 cells was determined by the MTT assay and LDH assay respectively. The influences of HQH on the proteinuria-related protein expression and the signaling pathway associated protein expression were also detected using quantitative reverse transcription PCR and Western blotting analysis. The results showed that the MPC5 cell model was successfully constructed *in vitro*. The HQH application could improve the harmful effects induced by TM on the MPC5 cells, including promoted cell proliferation and suppressed cell apoptosis. Furthermore, the protein expression, including podocin, nephrin, and synaptopodin was down-regulated by the TM treatment in the MPC5 cells. On contrary, the expression of these proteins was up-regulated after the HQH application. Also, the effect of TM on integrin α3 and integrin β1 expressions was also reversed by the HQH treatment. Moreover, the HQH application decreased the expression of p-ERK and DNA-damage-inducible transcript 3 (DDIT3 or CHOP) in the MPC5 cells, which was opposite to the effect observed in the cells treated with TM. Taken together, our study suggest that HQH application may protect podocytes from TM damage by suppressing the p-ERK/CHOP signaling pathway.

KEYWORDS: Proteinuria; podocytes; Huaiqihuang; protective role; p-ERK/CHOP pathway
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INTRODUCTION

Proteinuria is a typical symptom of chronic kidney disease, which is caused by the dysfunction of glomerulus filtration membrane [1]. The glomerulus filtration membrane consists of endothelial cells, basement membrane, and podocytes [2]. Previous studies have demonstrated that the pathological changes in podocytes remain to be the major cause of proteinuria [3,4]. So far, the major treatment methods for proteinuria are medications, including adrenal cortical hormones and immune inhibitors [5,6]. However, some of these medications are prohibitive and they may produce large side effects such as high blood pressure, slow growth, diabetes, and femoral head necrosis [7,8]. Hence, it is necessary to develop useful and inexpensive medications for the proteinuria control.

Huaiqihuang (HQH) granules, a kind of Chinese herbal medicine, are composed of tree ear fungus, medlar, and Huang Jing [9]. The mechanisms of HQH in treating diseases are complex, and include the activation of macrophages, neutrophils, or natural killer cells, promotion of the proliferation and differentiation of T cells, and improvement in humoral immunity [10]. HQH is mainly applied when treating childhood diseases, including infantile respiratory infections and primary nephrotic syndrome [11]. In addition, a recent study has reported the application of HQH in treating adult primary IgA nephrotic syndrome [12]. Previous evidence shows that a possible mechanism of proteinuria is complex, and may be linked to glomerular podocyte protection, inflammation associated with podocyte damage, cell adhesion, and space conformation of cytoskeletal proteins [2,13].

Increasing evidence shows that the application of HQH in glomerular-related and immune inhibitor-induced

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autoimmune diseases demonstrate good effects [11,14]. Recently, other studies reported that HQH functions as a protector in nephrotic syndrome patients by reducing proteinuria and shortening the course of the disease [15,16]. Additionally, Liu et al. reported that HQH reduces proteinuria by enhancing nephrin expression and regulating the necrosis factor κB signaling pathway in adriamycin-induced nephropathy [17].

Extracellular signal-regulated kinases (ERK), also known as mitogen-activated protein kinases (MAPK), act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, and development [18]. DNA damage-inducible transcript 3 (DDIT3), also known as C/EBP homologous protein (CHOP), functions as a dominant-negative inhibitor by forming heterodimers with other C/EBP members, and preventing their DNA biding activity [19]. Previous studies showed that the activation of ERK or CHOP (p-ERK/CHOP) may result in the damage of podocytes [20].

Although various papers have demonstrated the protective role of HQH in proteinuria and glomerular-related diseases, these mechanisms are still unclear.

In this study, we cultured podocytes *in vitro*, induced the proteinuria model using tunicamycin (TM), and analyzed the potential effects of HQH on the biological processes of MPC5 podocytes. Comprehensive experimental methods were used to detect the effects of HQH on the MPC5 podocyte cell proliferation, apoptosis, and associated protein expression. This study was aimed to investigate the possible molecular mechanism of HQH in treating proteinuria. Our study may provide theoretical basis for the application of combined traditional Chinese and Western medicine in clinical treatment of proteinuria.

MATERIALS AND METHODS

Podocyte culture *in vitro*

The cell culture plates were pretreated with 50 μg/ml of type I collagen for 1h at 37°C. After that, the MPC5 podocytes were cultured in RPMI 1640 medium supplemented with 10 U/ml of γ-interferon and 10% fetal bovine serum (FBS) (Sigma, USA) at 33°C in an atmosphere of 5% CO_2. After 24h of incubation, the cultured cells were washed with sterile PBS buffer, two time each, then 1 ml of Trypsin-EDTA (0.25%: 0.02%) was added into the cells for tens of seconds at room temperature. When the cells were characterized with increased intercellular space and retracting round cell bodies, 2 ml of fresh medium was added into the cells. After that, cells were centrifuged at 1000 rpm for 5 min before the cell collection. The collected cells were diluted (1:2) with the fresh medium for the generation preparation.

Model construction and HQH treatment

The MPC5 podocytes were transferred onto 96-well plates with RPMI 1640 containing 1% FBS. After 24h of incubation, the cells were separated into 6 groups, followed by the subsequent treatment [21,22]: control group (cells treated without TM), experimental group 1 (cells treated with 0.5 μmol/l of TM), experimental group 2 (cells treated with 1 μmol/l of TM), experimental group 3 (cells treated with 5 μmol/l of TM), experimental group 4 (cells treated with 10 μmol/l of TM), and experimental group 5 (cells treated with 50 μmol/l of TM). The cells in each group were cultured for 24h and 48h before the cell collection.

The HQH (Batch number: 20070302, Qidong GAITIANLI co., LTD, China) was dissolved in purified water to prepare the mixture with the concentration of 200 mg/ml. The mixtures were then added to the cultured cells to adjust the appropriate concentration for the following assay. For HQH treatment, MTT and LDH toxicity assays were used as previously described [23, 24], to determine the optimal concentration when applying HQH to MPC5 cells. Briefly, the cells were washed with PBS buffer, two time each. Then, 20 μl of MTT was added into the cells and they were incubated for 4h at 37°C. After that, 150 μl of dimethylsulfoxide (DMSO) was mixed with the cells for 10 min. The absorbance of the cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan). In addition, for LDH toxicity assay [25], the necrotic cells were collected and 10 μl of the cells was mixed with NAD I (v: v, 5:1). After that, 60 μl of the cell mixtures was treated with 2,4-DNPH at 37°C for 15 min. Then, 150 μl of stop buffer was used to stop the reaction. The percentage of LDH release was calculated using the form: the LDH activity unit in medium/(the LDH activity unit in medium + the LDH activity unit in cell lysis solution) ×100.

Quantitative reverse transcription PCR (qRT-PCR)

The total RNA extraction from the MPC5 cells, collected at 48 h, was performed using TRIzol Reagent (Invitrogen, USA), as previously described [26], and RNase-free DNase I (Promega Biotech, USA). The concentration and purity of the isolated RNA were measured with SMA 400 UV-VIS (Merinton, Shanghai, China). The purified RNA (density of 0.5 μg/μl), dissolved in nuclease-free water, was used for cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen). The expression was detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-PCR Kit (Takara, China). Phosphoglyceraldehyde dehydrogenase (GAPDH) was used as the internal control. Primers used for the amplification are shown in Table 1.

Western blotting

Cells cultured at 48h in each group were treated with RIPA assay (radioimmunoprecipitation; Sangon Biotech, China) lysate containing PMSF (phenylmethanesulfonyl fluoride; Sigma), and then were centrifuged at 12 000 rpm for 10 min at 4°C. The supernatant was collected for the measurement of protein concentration using the BCA Protein Assay Kit (Pierce, Rochford, IL). For Western blotting [27], 40 μg of proteins per cell lane was subjected to 12% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), then the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The PVDF membranes were blocked in Tris-Buffered Saline Tween (TBST) containing 5% non-fat milk for 1h at room temperature. Then the membranes were incubated with rabbit anti-human antibodies (Cullin-5, Bip, p-ERK, ERK, and CHOP, 1:100 dilution, Invitrogen) overnight at 4°C. After that, the membranes were incubated with horseradish peroxidase-labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1h. Finally, the PVDF membranes were washed 3 times with 1× TBST buffer for 10 min each. The signals were detected after the incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, β-actin was used as the internal control.

Statistical analysis

All the experiments were conducted three times independently. The data are expressed as mean ± standard deviation (SD). The data were analyzed using the SPSS13.0 software and the statistical analysis between two groups was calculated using the Student's *t* test. The value of $p < 0.05$ was considered as statistically significant.

RESULTS

The TM-induced MPC5 endoplasmic reticulum stress model construction

TABLE 1. Primers used for targeted genes in this study

Target	Primer	Sequence (5'-3')
GAPDH	Sense	GGGTGGAGCCAAACGGGTC
	Antisense	GGAGTTGCTGTTGAAGTCGCA
SYNPO	Sense	GCCAGGGACCAGCCAGATA
	Antisense	AGGAGCCCAGGCCTTCTCT
ITGA3	Sense	GCCAAGCTAATGAGACCATC
	Antisense	TCAGCACGAGTGCTCGAGACTTG
ITGB1	Sense	AAC GAGGTCATGGTTCATGTTGTG
	Antisense	GCATTCAGTGTTGTGGGATTTGC
ERK	Sense	AAAGAATTCGCCCGTTCATTCCTTTGCT
	Antisense	AAAGCGGCCGCTTCTTCATCGCCTCTTCC
DDIT3	Sense	GAGTTGGAGGCGTGGTATGA
	Antisense	CCTTGGTGGCGATTGGTGAA

ERK: Extracellular signal-regulated kinase

The rat MPC5 cells were cultured at 33°C and 37°C to induce the cell proliferation and differentiation respectively for the endoplasmic reticulum stress model construction (Figure 1A). The Western blotting showed that the expression of Cullin-5, a cell proliferation indicator, was higher in the MPC5 cells treated at 33°C compared to the cells treated at 37°C (Figure 1B). Additionally, the immunofluorescence results showed that the MPC5 cells were successfully constructed (Figure 1C). Furthermore, the cell viability assay revealed that the optimal concentration of TM for the MPC5 cells was around 1-10 μmol/l (Figure 1D).

Effects of HQH on the TM-treated MPC5 cells

The influence of HQH on the TM-induced MPC5 cells was analyzed based on the MTT and LDH assays. The results showed that the optimal concentration of HQH for MPC5 cell viability was 0.2 mg/ml and the optimal concentration of HQH for MPC5 cell toxicity was 2 mg/ml (Figure 2A and 2B). The number of apoptotic and necrotic MPC5 cells was increased when the TM concentration was increased (Figure 2C). In addition, we observed that the protective effect of HQH on the MPC5 cells was more apparent with longer HQH application. Figure 2D shows that when the MPC5 cells were pretreated with HQH for 6h and another 30 h, the damage induced by TM on the MPC5 cells was less severe.

Effects of HQH on proteinuria-related protein expression

We further analyzed the influence of HQH on proteinuria-related protein expression, including the cytoskeletal proteins, adhesion molecules, and foot process-related proteins (Figure 3). The results showed that the protein expression of synaptopodin, podocin, and nephrin was decreased with increased TM concentrations (from 0.5 to 5 μmol/l) (Figure 3A and 3B). Interestingly, integrin-α3 and integrin-β1 levels were increased by 0.5 μmol/l of TM, while they were decreased by 5 μmol/l of TM (Figure 3C). Furthermore, the Cullin-5 and Bip were down-regulated by the TM treatment in the MPC5 cells, but their levels were increased after the HQH application for 6h (Figure 3D).

The possible mechanism of HQH in treating proteinuria

The effects of HQH on the protein expression related to p-ERK/CHOP signaling pathway were analyzed using Western blotting and qRT-PCR (Figure 4). The results showed that there was no significant difference in the protein and mRNA levels of ERK in the MPC5 cells before or after the TM treatment, as well as after treatment with combined TM and HQH (Figure 4A and 4D). Nevertheless, the mRNA and protein expression of p-ERK and CHOP were all significantly increased by the TM treatment, in a dose independent

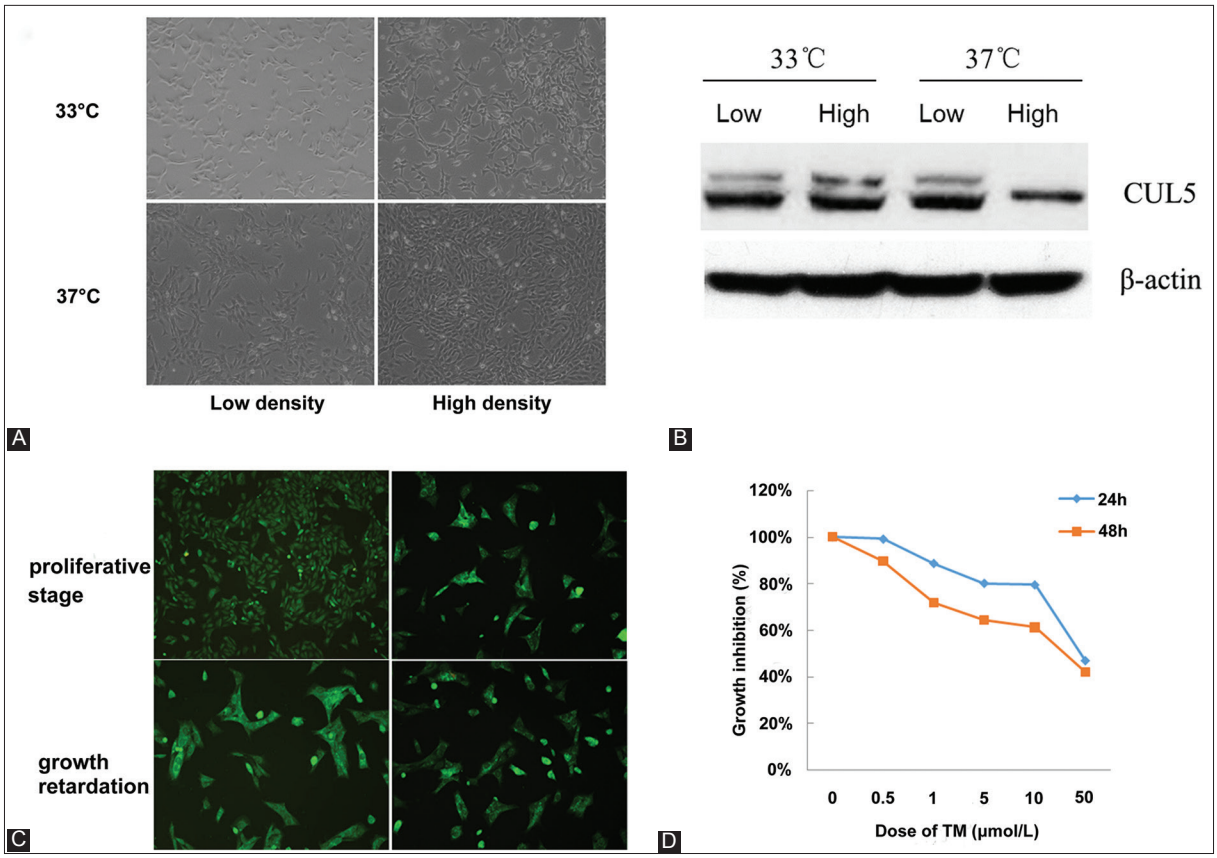

FIGURE 1. The tunicamycin (TM)-induced MPC5 model construction. A: The MPC5 cell proliferation was increased by a high dose of TM at 33°C, while the cell differentiation was induced by a high dose of TM at 37°C; B: Western blotting showed that the expression of Cullin-5, a cell proliferation indicator, was decreased by a high dose of TM compared to low dose TM treatment in the MPC5 cells; C: The immunofluorescence assay showed that the MPC5 model cells were successfully constructed; D: The cell viability assay revealed that the optimal concentration of TM for MPC5 cells was around 1-10 μmol/l.

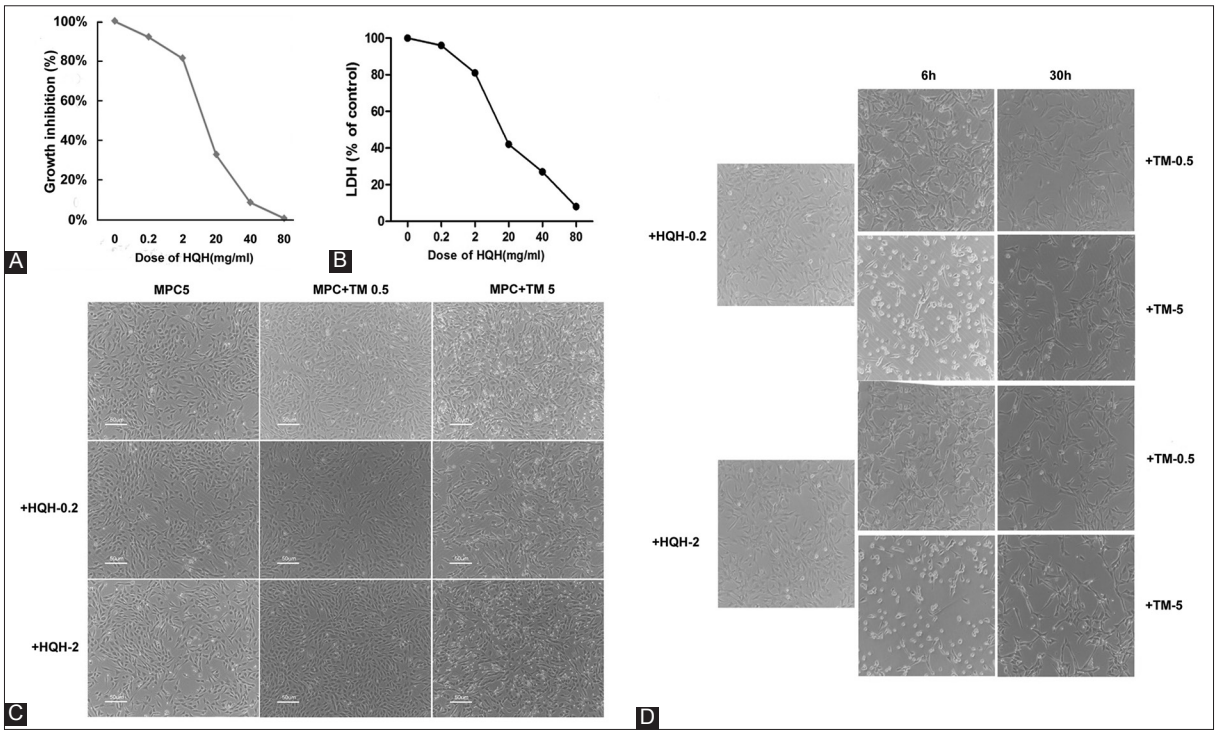

FIGURE 2. The effects of Huaiqihuang (HQH) on tunicamycin (TM)-induced MPC5 cells. A-B: The optimal concentration of HQH for MPC5 cell viability was 0.2 mg/ml and the optimal concentration of HQH for MPC5 cell toxicity was 2 mg/ml; C: Increasing the TM concentration (from 0.5 to 5 μmol/l), the number of necrotic and apoptotic cells was increased; D: The HQH treatment suppressed the effects of TM on the MPC5 cell necrosis and apoptosis.

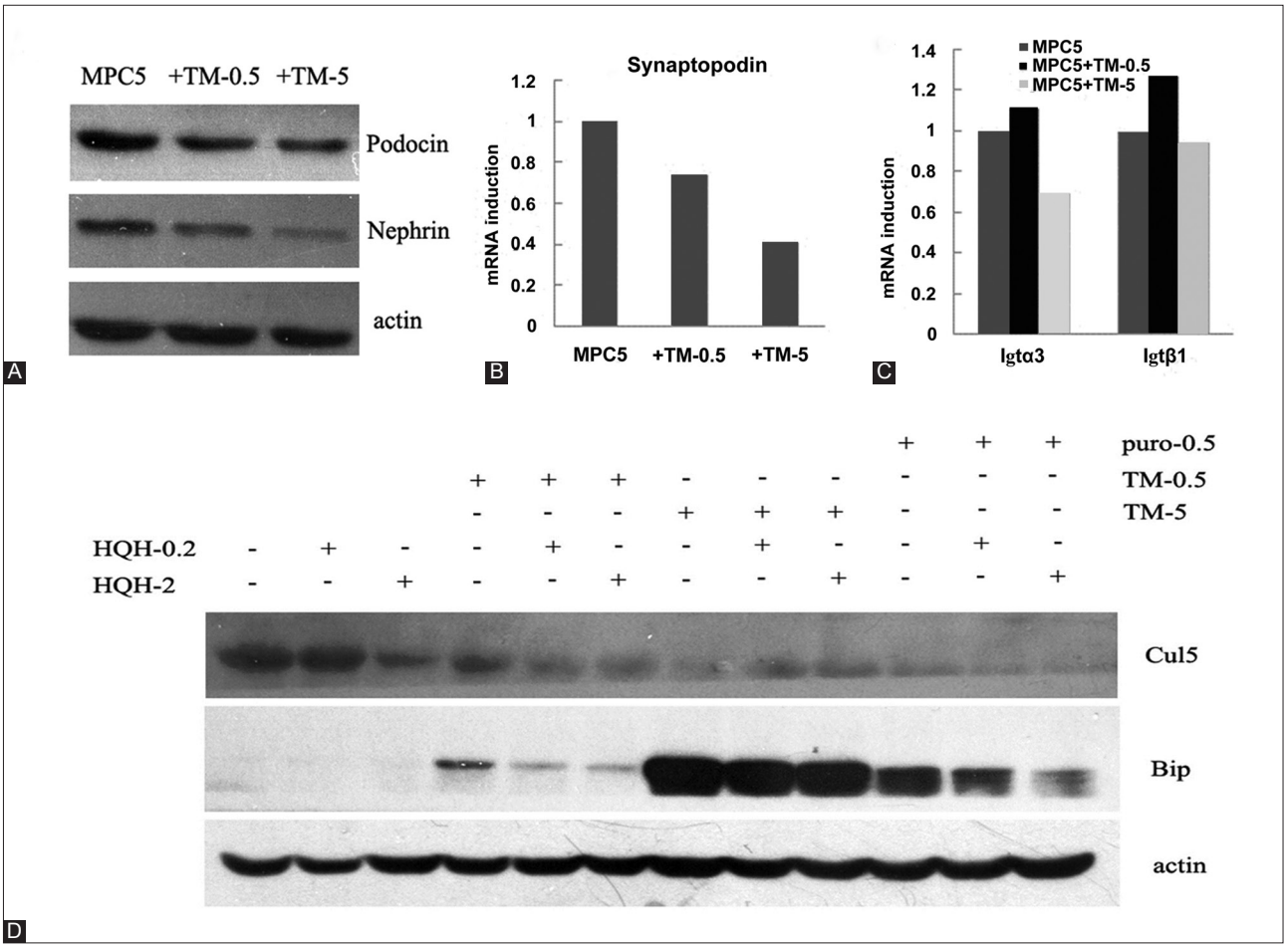

FIGURE 3. The influence of the Huaiqihuang (HQH) treatment on the protein expression related to proteinuria. A: The protein levels of podocin and nephrin were decreased by the increased concentration of tunicamycin (TM) in the MPC5 cells; B: The mRNA levels of synaptopodin gene were decreased by the increased concentration of TM; C: Both the integrin-α3 and integrin-β1 levels were increased by a low dose of TM (0.5 μmol/l), while their levels were decreased by a high dose of TM (5 μmol/l); D: The TM treatment decreased the Cullin-5 and Bip protein expression in the MPC5 cells, but their levels were increased by the HQH application, indicating the protective role of HQH in the TM-induced MPC5 cells.

manner, but this effect was suppressed by HQH ($p < 0.01$; Figure 4B, 4C, and 4D).

DISCUSSION

Increasing evidence has showed that podocyte damage is the major cause of glomerular proteinuria [2,4]. Therefore, timely prevention or alleviation of podocyte damage would help blocking the progression and development of chronic nephrosis. Numerous studies have demonstrated the pivotal protecting role of HQH in proteinuria and podocyte damage, but the mechanism still remains unclear. In this study, we constructed the podocyte damage model *in vitro* using MPC5 cells induced with TM, and analyzed the potential protective effects of HQH in podocyte damage. The results showed that the podocyte damage model was successfully constructed, and HQH could recover the damage caused by TM on the MPC5 cells, including the cell proliferation and apoptosis. Moreover, the HQH treatment could reverse the effects of TM on proteinuria and the expression of proteins related to the podocyte damage.

Since podocytes are terminally differentiated cells [28], the cultivation of podocytes *in vitro* is difficult. In this study, we cultured rat MPC5 podocytes at 33°C or 37°C. Cullin-5 is a core component of Elongin-Cullin 2/5-SOCS-box protein E3 ubiquitin-protein ligase complexes, which mediates the ubiquitination and subsequent proteasomal degradation of target proteins [29]. Braun et al. proved that renal edema, glomerular structure changes, and proteinuria were observed in Zebrafish after the *CUL5* knockdown [30], suggesting the pivotal role of Cullin-5 in maintaining the physiological function of glomerular epithelial cells. In addition, the *CUL5* knockdown is correlated with low cell proliferation ability [31]. Our results showed that Cullin-5 levels were highly detected in the MPC5 cells (Figure 1), indicating that the MPC5 podocytes were successfully cultivated *in vitro*.

The endoplasmic reticulum stress is the major cause of diabetic nephropathy damage, and TM has been proved to

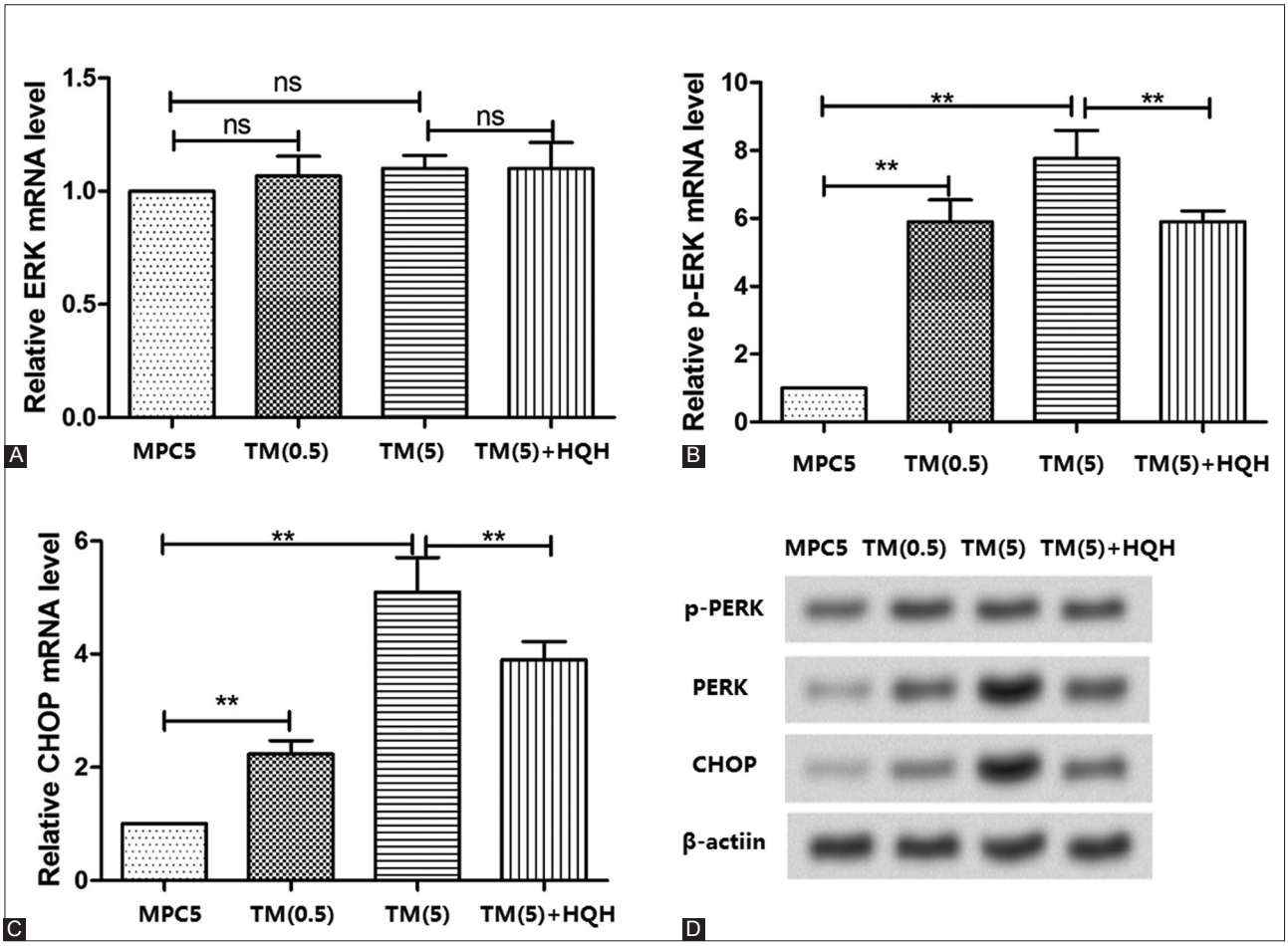

FIGURE 4. The effects of the Huaiqihuang (HQH) treatment on the protein expression related to p-ERK/CHOP signaling pathway in the MPC5 cells treated by tunicamycin (TM). A: There was no significant difference between the extracellular signal-regulated kinases (ERK) mRNA levels among the groups, indicating that the HQH treatment showed no significant effects on the ERK expression.; B: The TM treatment significantly increased the mRNA levels of p-ERK in the MPC5 cells, and this effect was suppressed by the HQH application; C: The TM treatment significantly increased the mRNA levels of C/EBP homologous protein (CHOP) in the MPC5 cells, and this effect was suppressed by the HQH application; D: Western blotting analysis showed that the protein levels of ERK, p-ERK, and CHOP were consistent with their mRNA levels. **$p < 0.01$ compared to the control cells. Ns - no significant difference.

be an inducer of the endoplasmic reticulum stress [32]. In this study, when the MPC5 cells were induced by TM (Figure 2), the cell proliferation was inhibited and the number of apoptotic cells was increased. Previous studies have showed that mutations in nephrin, podocin, and synaptopodin genes are the major cause of congenital nephrotic syndrome [33]. Our results showed that the TM treatment significantly decreased the expression of nephrin, podocin, and synaptopodin with increasing concentrations while the expression of integrin-α3 and integrin-β1 was suppressed by a high dose of TM (Figure 3), suggesting that the MPC5 podocyte damage model was successfully constructed.

We further detected the expression of proteins related to podocyte damage in the MPC5 cells treated by HQH. Sun et al. proved that HQH promotes the nephrin and podocin protein expression in rat renal tissues with Adriamycin-induced nephrosis [9]. In this study, the results showed that the HQH treatment promoted the cell proliferation, suppressed the cell apoptosis, and increased the expression of nephrin, podocin, and synaptopodin proteins (Figure 2 and 3), implying the protective role of HQH in the MPC5 damage.

Previous studies indicated that the activation of ERK or CHOP might result in the damage of podocytes. Anzai et al. proved that the endoplasmic reticulum stress effector, CHOP, could regulate chronic kidney disease-induced vascular calcification [20]. Zhang et al. showed that the ERK activation resulted in the RPC6 up-regulation in angiotensin II-induced podocyte apoptosis [34]. Also, Fujita et al. demonstrated that ERK mediated the high-glucose-induced hypertrophy in renal tubular cells [35]. In this study, the p-ERK and CHOP expression was up-regulated by the TM treatment in the MPC5 cells (Figure 4), implying the p-ERK/CHOP signaling pathway activation. However, their expressions were decreased after the HQH application (Figure 4). Based on our results, we speculate that HQH may protect podocytes from damage by suppressing the p-ERK/CHOP signaling pathway activation.

Although our study revealed the crucial protective roles of HQH in podocyte damage, several limitations remained.

Firstly, further experimental studies should be conducted in the animal model of glomerular proteinuria by using adult rats. Secondly, the protective effects of HQH on proteinuria may be mediated by all three components instead of one. Therefore, we will try to isolate one of the components from HQH to determine which has the main role. Thirdly, further experimental studies are still needed to explore the potential molecular mechanism of HQH in resisting the podocyte damage at transcriptional level, and to provide the theoretical basis for HQH application in adult glomerular proteinuria.

In conclusion, the data presented in this study showed that the Chinese medicine HQH may function as a protector in podocyte damage induced by TM, by suppressing the p-ERK/CHOP signaling pathway. Our study may provide theoretical basis for the possible application of HQH in the clinical treatment of podocytes and proteinuria.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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