Original Paper

Effect of Huaier On the Proliferation of Mesangial Cells in Anti-Thy-1 Nephritis

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Key Words

Huaier • Mesangial proliferative glomerulonephritis • Thy-1 nephritis • Mxi-1

Abstract

Background/Aims: To determine whether an aqueous extract of *Trametes robiniophila* Murr. (Huaier) suppresses anti-Thy-1 mesangial proliferative glomerulonephritis (MsPGN) *in vivo* and platelet-derived growth factor (PDGF)-BB-induced mesangial cell proliferation *in vitro*. **Methods:** Male Wistar rats were randomly categorized into 5 groups: Sham, Thy-1, and 3 Huaier-treated groups (low, medium, and high dose). Two weeks after treatment, urinary proteins were quantified and renal pathological changes were examined. MAX interactor 1 (Mxi-1) and proliferating cell nuclear antigen (PCNA) expression levels in isolated glomeruli, rat mesangial cell viability, cell-cycle distribution, and cell-cycle pathways were assessed. **Results:** Huaier diminished the proliferative damages and urinary protein secretion in Thy-1 rats. PCNA was downregulated, whereas Mxi-1 was upregulated in the isolated glomeruli of Huaier-treated groups compared with the Thy-1 group. Huaier inhibited PDGF-BB-stimulated proliferation of rat mesangial cells in a time- and dose-dependent manner (50% inhibitory concentration = 6.19 mg/mL) and induced G2 cell-cycle arrest. Cell-cycle pathway proteins were downregulated, whereas Mxi-1 was upregulated in Huaier-treated mesangial cells compared with PDGF-BB-stimulated cells. **Conclusion:** Huaier reduces urinary protein excretion and relieves hyperplasia in mesangial cells in anti-Thy-1 MsPGN as well as inhibits PDGF-BB-stimulated proliferation and DNA synthesis of rat mesangial cells *in vitro*, suggesting its novel therapeutic potential in MsPGN.

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Introduction

Mesangial proliferative glomerulonephritis (MsPGN) is characterized by the proliferation of mesangial cells and deposition of extracellular matrix proteins. Mesangial

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cell proliferation plays an important role in progressive glomerular injury that leads to glomerular sclerosis [1]. Although MsPGN is a major cause of end-stage renal failure in many countries, its underlying mechanisms are not fully understood [2, 3]. MsPGN is of two types: IgA nephropathy and non-IgA nephropathy. However, there is currently no effective therapeutic drug to treat MsPGN. Pharmacological interventions that can inhibit inflammation, proliferation of mesangial cells, and matrix deposition may be beneficial to retard the progression of glomerulonephritis [4].

Trametes robiniophila Murr. (Huaier) is a sandy beige mushroom found on the trunks of trees such as *Sophora japonica*, belonging to Class Hymenomycetes, Phylum Basidiomycota. This fungus has antitumor and immunomodulatory activities and has been used in traditional Chinese Medicine for approximately 1600 years [5]. Its anticancer mechanisms include inhibition of cell growth and tumor-induced angiogenesis and inactivation of epithelial-mesenchymal transition [6-8]. However, the effects of Huaier in MsPGN are thus far unknown.

Therefore, the principal aim of our study was to determine whether MsPGN can be effectively treated by Huaier in an experimental model and to investigate its underlying mechanisms of action.

Materials and Methods

Induction of Anti-Thy-1 Glomerulonephritis and Experimental Design

Eight-week-old male Wistar rats weighing 180–220 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and bred at the experimental animal center of the People's Liberation Army General Hospital under the following conditions: temperature, $22 \pm 2^\circ\text{C}$ and humidity, $50 \pm 20\%$. All animal experiments were performed in accordance with the provisions of Experimental Animal Committee of the People's Liberation Army General Hospital.

The rats were randomly categorized into five groups, with 15 rats in each group: (1) Sham group, (2) Thy-1 group, (3) anti-Thy-1 nephritis model + low dose of Huaier given by gavage once a day with Huaier at a gavage dose of 1 mg/kg body weight (HRL group), (4) anti-Thy-1 nephritis model + medium dose of Huaier given by gavage once a day with Huaier at a gavage dose of 5 mg/kg body weight (HRM group), and (5) anti-Thy-1 nephritis model + high dose of Huaier given by gavage once a day with Huaier at a gavage dose of 10 mg/kg body weight (HRH group).

The Sham group received 0.2 mL of $1\times$ phosphate-buffered saline (PBS; pH 7.4) on Day 0. The Thy-1 group received an intravenous injection of monoclonal anti-Thy1 antibody (2.5 mg/kg) produced by OX-7 cells diluted in 0.9% saline (via tail veins) on Day 0 and were treated with vehicle from Day -7 to Day 7. The HRL, HRM, and HRH groups were treated with the same dose of anti-Thy-1 antibody as the Thy-1 group, and the Huaier was dissolved in water daily from Day -7 to Day 7. Electuary ointment of Huaier was provided by Gaitianli Medicine Co. Ltd. (Jiangsu, China). At 24 h, urine samples were collected, and the protein level was measured daily. In addition, the blood urea nitrogen and creatinine levels were quantified and the serum creatinine concentration was measured. In each group, 5 rats were sacrificed on Days 1, 3 and 7.

Pathological Analyses

Tissues from the kidneys of rats in each group were fixed in 4% paraformaldehyde. Deparaffinized sections were stained with Periodic Acid-Schiff to examine the pathological changes under light microscopy. The degree of renal impairment was semi-quantitatively and double-blindly scored by two pathologists following a published scoring system [9]. We randomly selected 10 separate fields for each specimen to evaluate the lesions ($400\times$ magnification) and averaged the values for the histological score.

Immunohistochemical Analysis

Routine histological paraffin sections were cut (3–4- μm thick) and mounted on poly-L-lysine-coated slides. The sections were deparaffinized in xylene and rehydrated in alcohol, following which they were treated with 3% hydrogen peroxide to quench the endogenous peroxide activity. Thereafter, the sections were heated in a microwave oven for 10 min in sodium citrate buffer (pH 6.0) and incubated with 1.5% normal goat serum for 30 min, followed by overnight incubation with proliferating cell nuclear antigen

(PCNA) antibody (Abcam, Cambridge, MA, USA). After removal of the unbound primary antibody and rinsing with PBS, the sections were incubated with a biotinylated secondary antibody for 60 min at room temperature. Subsequently, the sections were rinsed and incubated with avidin-biotinylated horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories, USA) for 60 min. Thereafter, the sections were and incubated with 3, 3'-diaminobenzidine tetrahydrochloride for 10 min as a substrate chromogen solution to produce a brown color and counterstained with hematoxylin. To quantify the proliferating cells in the glomeruli, 30 glomeruli per cross-section were counted to identify cells positive for PCNA from each biopsy at each time point. In addition, the glomerular PCNA-positive cells were identified under high-power ($\times 400$) light microscopy in a blinded fashion by two independent pathologists. The mean values of the number of proliferating (PCNA-positive) cells per biopsy were calculated. The PCNA labeling index was calculated as the number of PCNA-positive cells to the total number of glomerular cells. For immunofluorescence staining, glomerular deposition of the anti-Thy-1 antibody in kidney samples from rats with anti-Thy-1 nephritis on Day 1 was detected using Alexa Fluor 555 donkey anti-mouse IgG (H+L) (Invitrogen, Carlsbad, CA, USA).

Cell Culture and Transfection

A rat mesangial cell line was purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium (Gibco, USA) containing 10% fetal calf serum (Hyclone, Canada) at 37°C in a 5% CO₂ atmosphere. Mxi-1 siRNA [10] (GenePharma, Shanghai, China) (100 nM) was transfected into mesangial cells for 48 h using jetPRIME™ (Polyplus-transfection, France) according to the protocol supplied by the manufacturer.

Cell-viability Assay

Cells in the logarithmic phase were selected, and the cell concentration was adjusted to 1×10^4 /mL. Cells were seeded in a 96-well plate and cultured for 24 h until they were completely adherent. With serum-free 1640 culture medium, the cells were synchronized for 12 h. After Platelet-Derived Growth Factor-BB (PDGF-BB, Sigma, USA) (30 ng/mL) [11] or Huaier treatment, a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was used to detect the proliferation of mesangial cells at 12, 24, and 48 h, and the number of viable cells was measured by recording the optical density at 450 nm and generating growth curves. All experiments were performed in triplicate.

Cell-cycle Analysis

A total of 10^5 cells/well were seeded in 6-well plates and starved in serum-free 1640 medium at 37°C. After 12 h of starvation, the cells were treated with PDGF-BB or Huaier and complete medium for 48 h. The cells were trypsinized, washed with cold PBS, and stained using the Cycletest Plus DNA Reagent Kit (BD Biosciences, USA) according to the manufacturer's instructions. The cell cycle was analyzed by flow cytometry (Beckman Coulter, USA). All experiments were independently performed in triplicate.

5-Ethynyl-2'-deoxyuridine (EdU) Assay

Cells were seeded into 12-well culture plates at a density of 1.0×10^4 cells/well and incubated for 24 h in RPMI 1640 medium. After 12 h of starvation, the cells were treated with PDGF-BB or Huaier and complete medium for 48 h. After 48 h of stimulation, cell proliferation was determined using an EdU immunofluorescence assay according to the manufacturer's instructions (Life tech, USA). EdU-positive cells were quantified from 10 images, and the images collected were analyzed by Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Isolation of Glomeruli

The kidney capsules were removed, and cortices were dissected from each kidney. The cortical sections were minced with a razor blade and subsequently pressed through a 180- μ m pore sieve. Thereafter, the sections were rinsed through successive sieves (pore size, 104–37 μ m) using ice-cold PBS. Tissue containing glomeruli on the 63- μ m mesh was collected, and its purity was assessed using light microscopy. Renal cortical tubules were purified from rat kidneys by using a Percoll gradient centrifugation technique [12].

Western Blot Analysis

Proteins were extracted from isolated glomeruli, tubules, or rat mesangial cells using RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate

[SDS], 1 mM phenyl methyl sulfonyl fluoride, and protease cocktail at 1 µg/mL). Protein concentrations were determined using a BCA kit (Pierce, USA). Protein samples (50 µg per lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated overnight in 5% non-fat milk at 4°C, followed by incubation with primary antibodies against neutral and basic amino acid transporter (rBAT), podoplanin, PCNA, Mxi-1, cyclin B1, CDK 1, CDK 2 (Abcam), and β-actin (Sigma). After incubation of individual primary antibodies, the membrane was washed three times with Tris-buffered saline plus Tween 20, followed by incubation of secondary antibody for 1 h. The detection of specific signals was performed using enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK). All experiments were repeated at least three times.

Statistical Analyses

Data are reported as mean ± standard error of mean of at least three independent experiments. Statistical significance, defined as $P < 0.05$, was evaluated using one-way analysis of variance with SPSS software (version 19.0, Chicago, IL, USA). The 50% inhibitory concentration (IC_{50}) was calculated using Prism software version 5.0 (Graph-Pad Software, San Diego, CA).

Results

Effect of Huaier Treatment on Proteinuria and Pathological Changes in Anti-Thy-1 Nephritis

During the study course, no clinical adverse effects were observed in the study animals. The 24-h urine protein level was determined for each rat on Days 3 and 7 (Fig. 1A). Compared with the Sham group, the anti-Thy-1 group demonstrated frank proteinuria on Days 3 and 7 of nephritis. The HRM and HRH groups, in which Huaier was administered before induction of nephritis, showed a significant reduction in the urinary protein levels at 24 h ($P < 0.05$). Pathological analysis revealed that the renal cortical tissue from anti-Thy-1 nephritic rats showed prominent mesangial hypercellularity and glomerular sclerosis on Day 7 of nephritis. In anti-Thy-1 nephritic rats treated with HRM and HRH, but not those treated with HRL, the histological changes were significantly attenuated (Fig. 1B, 1C). Furthermore, HRM and HRH treatment significantly reduced the total injury score of the glomeruli ($P < 0.05$; Fig. 1D). We examined the change in body weight of animals treated with drugs in 2 weeks to show the safety of Huaier use and found no difference among the anti-Thy-1, HRL, HRM, and HRH groups (Fig. 1E). The serum levels of urea nitrogen and creatinine were prone to increase in all groups (Fig. 1E). However, there were no significant differences among these groups. To confirm that Huaier does not affect glomerular deposition of the anti-Thy-1 antibody, we examined the degree of glomerular deposition of anti-Thy-1 antibody by using kidney samples from rats with anti-Thy-1 nephritis on Day 1. The results showed no difference in glomerular deposition of the anti-Thy-1 antibody among the groups analyzed (Fig. 1F). These findings suggest an anti-proteinuric effect of Huaier treatment against anti-Thy-1 nephritis.

Effects of Huaier on Mesangial Cell Proliferation in Anti-Thy-1 Nephritis

The anti-Thy-1 group was characterized by mesangial cell proliferation and matrix expansion on Day 7. Total glomerular cell numbers and the number of cells positive for PCNA markers were compared between anti-Thy-1 rats and Sham rats (Fig. 2A–C). Administration of HRM and HRH reduced the severity of MsPGN. The number of PCNA-positive cells was significantly reduced by HRM and HRH treatment ($P < 0.05$), demonstrating that Huaier inhibited mesangial cell proliferation in an anti-Thy-1 model. Podoplanin is specifically expressed in glomeruli [13, 14], and rBAT is specifically expressed in tubules [15]. We assessed the expression of podoplanin and rBAT to confirm the enrichment of glomerular proteins by western blot analysis (Fig. 2D). In terms of PCNA protein expression in isolated glomeruli, western blot analysis showed that the HRM and HRH groups expressed lower levels of PCNA than the anti-Thy-1 group, consistent with the immunohistochemical results

Fig. 1. Effect of Huaier treatment on proteinuria and pathological changes in anti-Thy-1 nephritis. (A) Twenty-four-hour urinary protein excretion on Days 3 and 7 in anti-Thy1 nephritis rats (* $P < 0.05$). (B) Representative images of glomeruli from vehicle- or Huaier-treated animals with Thy-1 nephritis stained with Periodic Acid-Schiff. Bars = 100 μ m. Magnification: $\times 400$. (C) Representative images of glomeruli from vehicle- or Huaier-treated animals with Thy-1 nephritis stained with Periodic Acid-Schiff. Bars = 100 μ m. Magnification: $\times 200$. (D) The glomerular injury score in all 5 groups (* $P < 0.05$). (E) Time course of body weight and serum concentrations of urea nitrogen and creatinine. (F) Immunofluorescence studies show the degree of glomerular deposition of anti-Thy-1 antibody from rats with anti-Thy-1 nephritis on Day 1. Deposition of anti-Thy-1 antibody shows no change in all 4 groups. Bars = 50 μ m. Magnification: $\times 400$. HRL, low-dose Huaier; HRM, medium-dose Huaier; HRH group, high-dose Huaier.

(Fig. 2E). Our previous study showed that Mxi1 inhibits mesangial proliferation [10]. In this study, we found that Mxi-1 expression in the anti-Thy-1 group decreased on Day 7 compared with the Sham group and increased in the HRM and HRH groups.

Effects of Huaier on the Proliferation of Mesangial Cells in vitro

A previous study showed that PDGF-BB is essential for mesangial cell proliferation preceding the development of glomerulosclerosis in experimental glomerulonephritis [16]. In our study, after confirming that mesangial cells are stimulated with PDGF-BB *in vitro*, we investigated the effects of Huaier on the proliferation of cells. The CCK-8 assay showed that PDGF-BB increased mesangial cell proliferation compared to the control cultures at 12, 24, and 48 h (Fig. 3A). However, Huaier inhibited PDGF-BB-induced proliferation of mesangial cells in a dose-dependent manner, with an IC_{50} of approximately 6.19 mg/mL ($P < 0.05$) (Fig. 3A, 3B). PCNA is a cell cycle-associated nuclear protein and is used as a marker for cell proliferation. Western blot analysis showed that PCNA protein expression increased by PDGF-BB stimulation, which was reduced by Huaier treatment in mesangial cells (Fig. 3C). The EdU assays used to detect the DNA-synthesis capacity of mesangial cells (Fig. 3D)

Fig. 2. Effects of Huaier on mesangial cell proliferation in anti-Thy-1 nephritis. (A) Increase in glomerular cell number with disease induction (* $P < 0.05$). (B) Representative images of kidneys immunostained for PCNA. Bars = 50 μ m. Magnification: $\times 400$. (C) The number of PCNA-positive nuclei per glomerulus in all 5 groups (* $P < 0.05$). (D) Western blot analysis of podoplanin and rBAT protein expression in each group (glomeruli and tubules were isolated from the renal cortex). β -actin is taken as an internal reference. (E) The relative quantitation of PCNA and Mxi-1 protein expression in each group, as shown by Western blot analysis. β -actin is taken as an internal reference (* $P < 0.05$). HRL, low-dose Huaier; HRM: medium-dose Huaier; HRH group, high-dose Huaier; PCNA, proliferating cell nuclear antigen.

showed that the percentage of EdU-positive cells in the PDGF-BB group was higher than that in the control group, wherever Huaier decreased EdU-positive cells significantly compared with PDGF-BB-stimulated cells ($P < 0.05$; Fig. 3E).

Impact of Huaier on the Cell Cycle of Mesangial Cells

To determine whether the anti-proliferative effect of Huaier attributes to its induction of cell-cycle arrest, we treated mesangial cells with Huaier for 48 h and analyzed the cell-cycle distribution. Huaier treatment increased the percentage of mesangial cells in the G2/M phase ($P < 0.05$) (Fig. 4A, 4B). The effects of Huaier on the expression of cell cycle-related proteins were detected by western blot. We found that the levels of Cyclin B1, CDK 1, and CDK 2 significantly increased in the PDGF-BB group, while those of the cell cycle-related proteins significantly decreased ($P < 0.05$) in the Huaier group (Fig. 4C).

Effect of Mxi-1 on Mesangial Cells Treated with Huaier

We studied the effect of Mxi-1 on mesangial cells treated with Huaier *in vitro* and explored its possible mechanisms. Mxi-1 expression was significantly downregulated in the PDGF-BB group but significantly upregulated in the Huaier group ($P < 0.05$) (Fig. 5A). Mxi-

Fig. 3. Effects of Huaier on the proliferation of mesangial cells. (A) The effect of Huaier on cell viability, as measured by CCK-8 assay. (B) The 50% inhibitory concentration (IC_{50}). (C) Western blot analysis of PCNA in mesangial cell treated with PDGF-BB and Huaier (5 mg/mL) for 48 h; β -actin serves as a loading control. The relative quantitation of PCNA protein, expressed as the ratio of PCNA/ β -actin (* $P < 0.05$). (D) DNA replication activity of mesangial cells in PDGF-BB (30 ng/mL) and Huaier (5 mg/mL)-treated groups for 48 h, as shown by the EdU assay. (E) Percentage of EdU-positive cells in each group (* $P < 0.05$). Con, control; HR, Huaier; PDGF, platelet-derived growth factor; PCNA, proliferating cell nuclear antigen; DAPI, 4',6-diamidino-2-phenylindole.

1 expression could be significantly downregulated by specific siRNA targeting Mxi-1 (Fig. 5B). The EdU assays showed that the percentage of EdU-positive cells in the Huaier group decreased as compared to the control group, wherever si-Mxi-1 increased the number of EdU-positive cells significantly ($P < 0.05$) (Fig. 5C, 5D). Western blot analysis of cell-cycle regulatory proteins showed that the expression of cyclin B1, CDK 1, and CDK 2 decreased in the Huaier group, and this decrease was attenuated by si-Mxi1 transfection ($P < 0.05$) (Fig. 5E). Thus, Mxi-1 may play a role in inhibiting mesangial cells treated with Huaier.

Discussion

In the present study, we found that an aqueous extract of *T. robinophila* Murr. (Huaier) inhibits mesangial cell proliferation in an anti-Thy-1 model of MsPG. Additionally, the anti-proliferative effects of Huaier on mesangial cells were confirmed *in vitro*. To our knowledge, this is the first study to report that Huaier inhibits the proliferation of mesangial cells. These

Fig. 4. Effect of Huaier on the cell cycle. (A) Mesangial cells treated with PDGF-BB (30 ng/mL) and Huaier (5 mg/mL) for 48 h. Flow cytometry analysis shows that cells accumulate in the G2M phase. (B) Distribution of the cell cycle in mesangial cells. (C) The relative quantitation of cell-cycle regulatory proteins in each group, as assessed by Western blot analysis. β-actin is used as an internal reference (*P<0.05). Con, control; HR, Huaier; PDGF, platelet-derived growth factor.

Fig. 5. Effect of Mxi-1 on mesangial cells treated with Huaier. (A) Western blot analysis of Mxi-1 in mesangial cells treated with PDGF-BB (30 ng/mL) and Huaier (5 mg/mL) for 48 h. β-actin serves as a loading control. The relative quantitation of PCNA protein, expressed as the ratio of Mxi-1/β-actin (*P<0.05). (B) Western blot analysis of Mxi-1 protein expression after transient transfection with Mxi-1 siRNA1 and siRNA1 in mesangial cells; β-Actin is used as a loading control. (C) DNA replication activity of mesangial cells treated with Huaier (5 mg/mL) for 48 h with or without Mxi-1 siRNA transient transfection, as examined by EdU assay. (D) Percentage of EdU-positive cells in each group (*P<0.05). (E) The relative quantitation of cell cycle regulatory protein expression in each group, as assessed by Western blot analysis. β-actin is taken as an internal reference (*P<0.05). Con, control; HR, Huaier; PDGF, platelet-derived growth factor; DAPI, 4',6-diamidino-2-phenylindole.

findings support a central role of Huaier in MsPGN and provide a new therapeutic strategy for its treatment.

Chronic kidney disease (CKD) is one of the most-important risk factors for cardiovascular events, induction of renal replacement therapies, and even death [17, 18]. Among the many types of primary kidney disease, MsPGN, including IgA nephropathy, is a representative proteinuric kidney disease that leads to CKD. Anti-Thy-1 glomerulonephritis is a well-established nephritis model that simulates human MsPGN [19]. It is induced by the binding of anti-Thy-1 antibody to the corresponding antigen on the membrane of mesangial cells and is marked by evident transient inflammatory glomerular lesions, mesangial cells proliferation, glomerular capillary aneurysm formation, and extracapillary proliferation, displaying substantial proteinuria during the course. Although anti-Thy-1 glomerulonephritis in rats is not a consequence of collection of abnormal IgA-containing deposits in the glomeruli, and the results obtained cannot be directly extrapolated to human IgA nephropathy, it still represents a relevant model for studying interventions directed at mesangial cell proliferation and mesangial matrix deposition [20, 21].

Thy1.1 nephritis is characterized by the recruitment of inflammatory cells and consequent mesangial proliferation [22]. It was shown that patients with MsPGN have increased peripheral Th17 cells and serum or urinary IL-17 levels [23, 24], CD4 T cells and Th1 cytokines also contribute to the development of the Thy-1 nephritis model, and the suppression of cytokine production could be a valid target for therapy [25-27]. The cell cycle protein kinase inhibitor p27^{kip1} negatively regulates the cell cycle [28]. In the normal glomerulus, p27^{kip1} is highly expressed to maintain a steady-state in the kidney and avoid overproliferation; its level decreases with the initiation of the proliferative response of the mesangial cells and then normalizes with the resolution of the mesangial cell proliferation [29]. A strong induction of CDK 2 kinase activity was noticed during the mesangioproliferative phase in anti-Thy 1.1 GN; mesangial cell proliferation was reduced markedly by MEK inhibition via a decrease in glomerular and renal cortical cyclin E, A and CDK 2 overexpression [30]. The antiproliferative and immunosuppressive rapamycin derivative RAD used in inflammatory renal disease has been studied to inhibit mesangial cell migration in the anti-Thy1 model through the CDK-inhibitor p27^{KIP1} [31]. Matrix metalloproteinase inhibitors, which have anti-inflammatory features, can induce cell-cycle arrest with subsequent apoptosis in mesangial cells in Thy1.1 nephritis [32]. Therefore, it may be concluded that the concept of induction of cell cycle arrest may contribute to the development of new perspectives in the therapy of inflammation in inflammatory renal disease.

The aqueous extract of Huaier mainly comprises proteoglycan, which is the main effective ingredient responsible for the anticancer and immunomodulatory effects of Huaier [33]. The proteoglycan contains 41.53% polysaccharides, 12.93% amino acids, and 8.72% water [8]. Huaier has been used as an anti-carcinogenic medicine for the treatment of cancer, with satisfactory results. The anti-tumor mechanism of Huaier may be associated with the inhibition of endothelial cell proliferation, interference with tumor angiogenesis, and suppression of tumor cell proliferation [7]. The antiproliferative effect of Huaier in cancer cells has been well studied. MsPGN including, IgA nephropathy, is mainly characterized by the proliferation of mesangial cells. Huai Qi Huang (HQH), a compound Chinese herbal medicine that contains Huaier, wolfberry fruit, and Polygonatum, can ameliorate proteinuria and hematuria in cases of mild IgA nephropathy [34]. HQH significantly reduces proteinuria and ameliorates tubulointerstitial damage in adriamycin nephrosis in rats [35]. The main ingredient of Huaiqihuang is Huaier, and no studies have thus far assessed the effect of Huaier in MsPGN. Therefore, we chose mesangial cells and experimental mesangial proliferative glomerulonephritis as a model to determine the potential of Huaier in MsPGN treatment. Our study indicated that Huaier treatment effectively reduces the number of PCNA-positive glomerular cells and alleviates proteinuria. This is the first study to investigate the anti-proliferation effect of Huaier in an experimental MsPGN model.

The regulatory mechanism of mesangial proliferation in MsPGN is complex [36-39]. PDGF has been widely implicated in the pathogenesis of progressive renal injury in both

experimental models and human disease [40, 41]. It is known to participate in either mesangial cell proliferation or mesangial matrix expansion that follows mesangial cell injury with anti-Thy-1 antibody and plays a central role in proliferative glomerular disease [42]. Therefore, specific inhibition of the mitogenic actions of PDGF is a major target for MsPGN therapy. Several compounds have been reported to inhibit PDGF-stimulated proliferation of mesangial cells [43]. Our data showed that administration of Huaier, which potently inhibited PDGF-BB-induced proliferation of mesangial cells *in vitro*, markedly suppressed mesangial cell proliferation.

We used flow cytometry to evaluate the effect of Huaier on the cell cycle and found that it can extend the G2 phase and inhibit cell proliferation by regulating cell cycle proteins. Huaier has been reported to cause G2/M cell-cycle arrest in human cervical cancer cells [44]. Mxi1, a member of the Mad family of transcription factors, has a suppressive effect in human glioblastomas and correlates with cell-cycle arrest [45]. Our previous studies have shown that Mxi1 inhibits the proliferation of mesangial cells, and overexpression of Mxi1 has been shown to inhibit the expression of cyclin B1, thereby inducing a G2/M block [10]. In our study, Mxi1 expression increased with Huaier treatment in anti-Thy-1 rats *in vivo* and in mesangial cells *in vitro*. Furthermore, si-Mxi1 attenuated Huaier-induced inhibition of mesangial cell proliferation. We speculate that Huaier inhibits the proliferation of mesangial cells by upregulating Mxi1 expression. Further studies are needed to clarify the mechanisms of the suppressive effect of Huaier.

Conclusion

Huaier potently inhibits PDGF-BB-induced proliferation of mesangial cells *in vitro* and mesangial cell proliferation and extracellular matrix expansion in an anti-Thy 1 glomerulonephritis model *in vivo*. Our results suggest a novel therapeutic potential for MsPGN.

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Disclosure Statement

The authors declare no conflicts of interest.

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