Abstract. The aim of the present study was to investigate whether modified Huangqi Chifeng decoction (MHCD) could be an effective treatment against Doxorubicin-induced nephrosis in rats and whether it regulates autophagy via the phosphoinositide-3 kinase/mammalian target of rapamycin (PI3K/mTOR) signaling pathway. A total of 40 male Sprague-Dawley rats were randomly divided into blank, model, telmisartan and MHCD groups. The rat model of nephrosis was induced by intragastric administration of Doxorubicin for 8 weeks. Rats were housed in metabolic cages and urine was collected once every 2 weeks to measure 24-h protein levels. Blood samples were obtained from the abdominal aorta and levels of albumin (ALB), total cholesterol (TCH), triacylglyceride (TG) and serum creatinine (Scr) were assessed. Renal pathological changes were examined using hematoxylin-eosin, Masson’s trichrome and periodic acid-Schiff staining. Podocytes and autophagosomes were observed using an electron microscope. The expression and distribution of microtubule-associated proteins 1A/1B light chain 3B (LC3), LC3-I, LC3-II, beclin-1, PI3K and mTOR were determined using immunohistochemistry and western blotting. At weeks 6 and 8, 24-h proteinuria significantly decreased in the MHCD group compared with the model group (P<0.05). Compared with the model group, the MHCD group exhibited significantly reduced levels of TG, TCH and Scr, as well as significantly increased ALB levels (P<0.05). MHCD was demonstrated to prevent glomerular and podocyte injury. The number of autophagosomes was significantly decreased and the expression of beclin-1, LC3, LC3-I and LC3-II was inhibited following MHCD treatment compared with the model group (P<0.05). MHCD treatment significantly increased the expression of PI3K and mTOR in Doxorubicin nephrotic rats compared with the model group (P<0.05). In conclusion, MHCD was demonstrated to ameliorate proteinuria and protect against glomerular and podocyte injury by inhibiting excessive autophagy via the PI3K/mTOR signaling pathway.

Introduction

Chronic kidney disease (CKD) is defined as kidney damage or a glomerular filtration rate <60 ml/min/1.73 m² for ≥3 months, irrespective of the cause (1). A total of ~500 million adults are diagnosed with CKD all over the world, which has become a global public health problem due to its high prevalence and the accompanying risk of end-stage renal disease (2). The Global Burden of Disease Study 2013 reported that, over the past 23 years, CKD is the most increased non-communicable cause of mortality (3). Angiotensin converting enzyme inhibitors and angiotensin receptor blockers have been used to treat CKD; however, these agents do not completely prevent the progression of CKD (4). In China, traditional Chinese medicine (TCM) has been widely used to treat CKD (5,6). Modified Huangqi Chifeng decoction (MHCD) has previously been reported to be an effective treatment for CKD (7). However, the underlying mechanisms of MHCD in CKD remain to be elucidated.

The role of autophagy in CKD has previously been studied and it was reported that kidney cells differentiate by acquiring specialized membranous components (8). However, the phenomenon of autophagy in the kidney was not clearly defined at that time. Over the past few decades, autophagy in CKD has attracted increased attention. Several studies have

Modified Huangqi Chifeng decoction inhibits excessive autophagy to protect against Doxorubicin-induced nephrotic syndrome in rats via the PI3K/mTOR signaling pathway

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Abbreviations: MHCD, modified Huangqi Chifeng decoction; ALB, albumin; TCH, total cholesterol; TG, triacylglyceride; Scr, serum creatinine; LC3, microtubule-associated proteins 1A/1B light chain 3B; PI3K, phosphoinositide-3 kinase; mTOR, mammalian target of rapamycin; CKD, chronic kidney disease; TCM, traditional Chinese medicine; PAS, periodic acid Schiff

Key words: Chinese herbal medicine, autophagy, Doxorubicin-induced nephrotic rats, phosphoinositide-3 kinase, mammalian target of rapamycin
demonstrated that autophagy is a major protective mechanism against podocyte aging and glomerular injury in rat kidney cells, suggesting that autophagy serves a role in ameliorating human glomerular disease and aging-associated loss of renal function (9-13). It is therefore important to elucidate the relevant molecular mechanisms associated with autophagy in CKD.

A previous study by our group demonstrated that MHCD attenuated renal fibrosis by inhibiting excessive autophagy and upregulating basal autophagy in rats with Doxorubicin-induced nephropathy (14), which is an experimental model of progressive kidney disease (15,16). In the present study, Doxorubicin-induced nephrotic rats with proteinuria were used as an experimental model. The aim of the present study was to investigate whether MHCD was able to regulate autophagy in rats to minimize podocyte and glomerular injuries via the phosphoinositide-3 kinase/mammalian target of rapamycin (PI3K/mTOR) signaling pathway.

Materials and methods

Drugs and antibodies. MHCD, comprised of 30 g Sheng Huang-qi (Astragalus membranaceus Bge), 20 g Qian Shi (Euryale ferox Salish), 20 g Jin Ying-zi (Rosae Laevigatae Michx), 10 g Chi Shao (Paonia lactiflora Pall), 10 g Fang Feng (Saposhnikovia divaricata Schischk), 10 g Di Long (Pheretima vulgaris Chen) and 10 g Bai Hua-she-she-cao (Hedyotis diffusa Wild), was provided by the Pharmacy Department of Xiyuan Hospital of China Academy of Chinese Medical Sciences. The herbs were initially soaked in water for 1 h at room temperature, followed by 1 h of decoction at 100°C. The extracts harvested from the decoction were vacuum dried. Water-soluble extracts of MHCD were dissolved in double-distilled water (ddH2O). Telmisartan (Micardis; 80 mg/pill) was purchased from Boehringer Ingelheim International GmbH (Ingelheim am Rhein, Germany). Doxorubicin hydrochloride for injection (instant; also known as Doxorubicin) was purchased from Pfizer Italia Srl (Rome, Italy). Anti-beclin1 (1:1,000; cat. no. ab210498), anti-LC3 (1:1,000; cat. no. ab48394), anti-pI3K (1:1,000; cat. no. ab86714) and anti-mTOR (1:1,000; cat. no. ab2732) primary antibodies were purchased from Abcam (Cambridge, MA, USA). Secondary antibodies used were part of a general-purpose two-step immunohistochemical kit (cat. no. PV-6000; ZSGB Biological Technology; OriGene Technologies, Inc., Rockville, MD, USA). The DAB kit was purchased from ZSGB Biological Technology (OriGene Technologies, Inc.).

Animal grouping and treatment. A total of 40 male Sprague-Dawley rats (weight, 220±18 g; age, 2-3 months) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The rats were housed in humidity-controlled rooms (60±10%) at 24±1°C with a 12-h light/dark cycle and free access to standard food and tap water. All rats were housed in metabolic cages and acclimated to laboratory conditions for 7 days, following which they were randomly divided into either the blank (n=10) or Doxorubicin-induced nephrosis (n=30) group. The Doxorubicin-induced nephrosis group was treated once with Doxorubicin at a dose of 6.2 mg/kg, which was injected into a tail vein, whereas the blank group was treated once with normal saline by intravenous injection into a tail vein. The Doxorubicin-induced nephrosis group was further divided into the model (n=10), telmisartan (n=10) and MHCD (n=10) groups. After 2 weeks, rats in the blank and model groups received normal saline, (0.1 ml/10 g) the telmisartan group received telmisartan (8.33 mg/kg) and the MHCD group received MHCD (11.46 g/kg) by intragastric administration once a day for 6 weeks. All drugs were diluted with distilled water and the dosages were evaluated by body surface coefficient conversion between humans and rats. Urine was collected from rats in the metabolic cages to determine the 24-h protein level. Urine collection was performed once every 2 weeks. Blood was obtained from the abdominal aorta following intragastric administration at week 8 to determine albumin (ALB), total cholesterol (TCH), triacylglyceride (TG) and serum creatinine (Scr) levels using a Roche Cobas-8000 automatic biochemical analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The rats were then sacrificed and the kidneys were harvested. The Animal Care and Use Committee of Xiyuan Hospital of China Academy of Chinese Medical Sciences approved the experimental protocol.

Histopathological analysis. Sections of cortical tissues from the right kidney were fixed in 10% buffered formalin at room temperature for 48 h, embedded in paraffin and sliced to 3 µm thick sections. The sections were stained at room temperature with hematoxylin-eosin (HE) for 3 min, Masson's trichrome for 30 sec-5 min and periodic acid-Schiff (PAS) for 30 sec-5 min, following which they were visualized using light microscopy (magnification, x400). Other sections of the right kidneys were fixed in 2.5% glutaraldehyde at 4°C for 24 h, washed with phosphate-buffered saline and sliced to 70-90 nm. Following staining with osmium tetroxide and lead citrate at room temperature for 5-10 min, the ultrastructure of the kidneys was observed under a Hitachi H-600 transmission electron microscope (magnification, x8,000 and x20,500; Hitachi, Ltd., Tokyo, Japan).

Immunohistochemistry. To evaluate the expression and distribution of microtubule-associated proteins 1A/1B light chain 3B (LC3), beclin-1, mTOR and PI3K, the paraffin-embedded renal cortical sections were dewaxed with xylene and rehydrated with a descending alcohol series, permeabilized with 3% hydrogen peroxide (H2O2) and washed with PBS, following which they were incubated with primary antibodies (1:200) at 4°C overnight and washed with PBS. Next, the sections were incubated with the secondary antibodies (1:2,500) and washed with PBS. Sections were subsequently stained with DAB at room temperature for 10 sec-2 min, washed with PBS, dehydrated with a descending alcohol series, permeabilized with xylene, mounted and viewed using light microscopy (magnification, x400). The expression of proteins was demonstrated by the ratio of integral optical density (IOD). IOD=average optical density x positive area.

Western blotting. To evaluate the expression and distribution of LC3-I, LC3-II, beclin-1, mTOR and PI3K protein, total protein was extracted using radioimmunoprecipitation assay buffer, following which the protein concentration was determined using a BCA Protein assay kit (cat. no. 23225;
Proteins were added to protein sample buffer, boiled in water for 5 min and separated by SDS-PAGE. Proteins were transferred onto polyvinylidene fluoride membranes and blocked at 4°C overnight with Tris-buffered saline/Tween 20 (TBST) containing 5% non-fat dried milk. Membranes were incubated with anti-beclin1 (1:1,000), anti-LC3 (1:1,000), anti-Pi3K (1:1,000), anti-mTOR (1:1,000) and anti-GAPDH antibodies (1:2,500; cat. no. sc-365062; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies, at 4˚C overnight. The protein bands were visualized using an enhanced chemiluminescent kit (EMD Millipore, Billerica, MA, USA). ImageJ software (Version 4.0; National Institutes of Health, Bethesda, MD, USA) was used to analyze the grayscale values of each group and GAPDH was used as the internal reference.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance followed by a post-hoc Bonferroni test with SPSS software 20.0 (IBM Corp., Armonk, NY, USA). Data are expressed as the mean ± standard deviation and P<0.05 was considered to indicate a statistically significant difference.

Results

General conditions. The model group rats were in emotional distress and exhibited diet reduction, messy hair, rough tail, diarrhea and a difference in color and luster of their hair after 3 days (data not shown). Two rats exhibited a slight ulcer in the tails (data not shown). Compared with the blank group, Doxorubicin-induced nephrotic rats exhibited mild edema in the testicles (data not shown). The rats in the blank group exhibited free movement, a normal diet, smooth body hair and no diarrhea or other abnormal reactions (data not shown). No significant differences in the weight were observed among the blank (509±8 g), model (496±30 g), Telmisartan (500±32 g) and MHCD group (498±29 g) at week 8.

MHCD ameliorates proteinuria. As demonstrated in Fig. 1, proteinuria in Doxorubicin-induced nephrotic rats increased to peak levels at week 6. Proteinuria was higher at weeks 2, 4 and 6 (both P<0.05) and 8 (both P<0.01) in the model group compared with the blank group, indicating the successful establishment of the model. Proteinuria in the MHCD and telmisartan groups increased at weeks 2, 4 and 6 compared with the blank group (all P<0.05). However, at weeks 6 and 8, 24-h proteinuria significantly decreased in the telmisartan and MHCD groups compared with the model group (P<0.05). However, no significant differences were observed between the telmisartan and MHCD groups. At week 8, no significant differences were observed between the MHCD and blank groups. These results indicate that MHCD ameliorates proteinuria in Doxorubicin-induced nephrotic rats.

MHCD ameliorates the levels of ALB, Scr, TG and TCH. As demonstrated in Fig. 2A, ALB levels in the model group decreased significantly compared with the blank group (P<0.05). Compared with the model group, the MHCD and telmisartan groups exhibited significantly increased levels of ALB (both P<0.05). Scr levels in the model group increased significantly compared with the blank group (P<0.05; Fig. 2B). Compared with the model group, the MHCD and telmisartan groups exhibited significantly decreased levels of Scr (both P<0.05). Furthermore, levels of TG and TCH in the model group increased significantly compared with the blank group (P<0.05; Fig. 2C). Compared with the model group, TG and TCH were significantly decreased in the MHCD and telmisartan groups (both P<0.05). These results indicate that MHCD ameliorates Doxorubicin-induced changes in ALB, Scr, TG and TCH levels in rats. Long-term proteinuria causes hyperlipidemia due to dysfunctional hepatic lipid protein synthesis (17). MHCD may improve lipid levels by reducing albuminuria in Doxorubicin-induced nephrotic rats. However, it is unclear whether MHCD can directly improve lipid levels.

MHCD prevents glomerular and podocyte injury. As demonstrated in Fig. 3, renal pathological changes were examined using HE, Masson’s trichome and PAS staining. Compared with the blank group, the model group had a greater number of proliferative mesangial cells, increased extracellular matrix deposition, a thickened glomerular basement membrane and disorderly arranged tubular cells (Fig. 3). Compared with the model group, telmisartan and MHCD treatments ameliorated the Doxorubicin-induced renal pathological changes in rats. These results demonstrate the protective effect of MHCD on the kidneys of Doxorubicin-induced nephrotic rats.

As demonstrated in Fig. 4, the blank group exhibited normal morphology. The podocytes in the model group were flattened or fused and an exposed basement membrane and increased number of proliferating mesangial cells were observed. In addition, lipid vacuoles were observed in a number of endothelial cells. Compared with the model group, telmisartan and MHCD treatments alleviated the degree of podocyte foot process fusion. Additionally, only a small number of swollen endothelial cells were observed following MHCD treatment, further supporting the findings.

MHCD inhibits excessive autophagy. Autophagy is a highly conserved cellular process that is important in anaphase cells, including neurocytes and podocytes (18,19). Autophagy in podocytes was observed using transmission electron microscopy. Podocytes in the blank group exhibited a normal
nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus (Fig. 5), whereas podocytes in the model group had a marked increase in the number of autophagosomes, dilated endoplasmic reticula and swollen mitochondria. A lower number of autophagosomes were observed in the MHCD and telmisartan groups compared with the model group. These results demonstrate that MHCD inhibits excessive autophagy in rats with Doxorubicin-induced nephrosis.

To assess whether autophagy is affected by MHCD, the expression of beclin-1 and LC3 were measured using immunohistochemistry (Fig. 6A) and western blotting (Fig. 7A). Beclin-1 (Figs. 6B and 7B), LC3 (Fig. 6C), LC3-I and LC3-II (Fig. 7B; all P<0.05) expression was significantly higher in the model group compared with the blank group. These increases were significantly inhibited following treatment with MHCD or telmisartan (all P<0.05).

MHCD inhibits excessive autophagy by inducing the PI3K/mTOR signaling pathway. Excessive autophagy leads to cell death and it has been demonstrated that the PI3K/mTOR signaling pathway serves a critical role in regulating this process (20,21). To assess whether MHCD inhibits excessive...
autophagy via the PI3K/mTOR signaling pathway, PI3K and mTOR expression were measured by western blotting (Fig. 7A) and immunohistochemistry (Fig. 8A). As presented in Figs. 7B, 8B and C, the expression of PI3K and mTOR was significantly lower in the model group compared with the blank group (all P<0.05). When Doxorubicin-induced nephrotic rats were treated with MHCD or telmisartan, PI3K and mTOR expression increased significantly compared with the model group (all P<0.05). However, as presented in Fig. 6, autophagic activity in the model group was significantly higher compared with the blank group, indicating that the inhibition of the PI3K/mTOR signaling pathway resulted in excessive autophagy. These results indicate that MHCD inhibits excessive autophagy by inducing the PI3K/mTOR signaling pathway.

**Discussion**

The aim of the present study was to investigate whether the regulatory effects of MHCD in autophagy are mediated via the PI3K/mTOR signaling pathway. It was revealed that the protective effects of MHCD in Doxorubicin-induced nephrotic rats are achieved by suppressing excessive autophagy via activation of the PI3K/mTOR pathway.

Proteinuria, hypoalbuminemia and hyperlipidemia are typical manifestations of Doxorubicin-induced nephrosis...
in rats (22) and these characteristics were observed in rats in the present study. Histological changes, including focal segmental glomerulosclerosis, tubulointerstitial inflammation, fibrosis and podocyte fusion, typical of Doxorubicin-induced nephropathy, were also observed in the present study (23). In TCM, MHCD has been demonstrated to reduce urinary protein and cholesterol levels, increase serum albumin levels and prevent glomerular and podocyte injuries in Doxorubicin-induced nephrotic rats (24). These results may be used to develop novel methods for delaying glomerulosclerosis using TCM.

Autophagy is a process by which cellular components are recycled (25) and injured organelles and proteins are removed (26). Autophagy comprises mechanistically distinct steps, including the induction, identification and selection of cargo, the formation of vesicles, autophagosome vacuole fusion, the breakdown of cargo and the release of degradation products into the cytoplasm (27). The formation of autophagosomes serves an important role in autophagy and requires the recruitment of ubiquitin-like-conjugating enzyme ATG (Atg) (28). LC3 has a molecular mass of ~17 kDa and is a mammalian ortholog of yeast Atg8 (29). Previous studies have demonstrated that LC3 is recruited into autophagosomal membranes, indicating that LC3 is a marker for autophagy (30,31). Beclin-1, another key regulator of autophagy, is the mammalian ortholog of yeast Atg6 (32,33). Beclin-1 has been demonstrated to induce autophagy via regulating PI3K VPS34 (Vps-34) to promote the formation of beclin 1-Vps34-Vps15 core complexes (34). As such, LC3 and beclin-1 are effective biomarkers for monitoring autophagy. Cells typically trigger autophagy to reduce cellular damage following infection, ischemia, starvation or growth factor deficiency (35). Autophagy, which occurs under basal conditions, serves an important role in cell growth, development and

Figure 6. MHCD ameliorates Doxorubicin-induced increases in autophagy by inhibiting LC3 and beclin-1 protein levels. (A) The expression of LC3 and beclin-1 in the glomeruli of Doxorubicin-induced nephrotic rats was assessed using immunohistochemistry (magnification, x400). Quantification of (B) beclin-1 and (C) LC3. Data are expressed the mean ± standard deviation (n=10). *P<0.05 vs. the blank group; #P<0.05 vs. the model group. MHCD, modified Huangqi Chifeng decoction; LC3, microtubule-associated proteins 1A/1B light chain 3B.

Figure 7. MHCD decreases the expression of autophagy signaling pathway proteins in rats with Doxorubicin-induced nephrosis. LC3-I, LC3-II, beclin-1, PI3K and mTOR expression was (A) measured using western blotting and (B) quantified. Data are expressed as the mean ± standard deviation. *P<0.05 vs. the blank group; #P<0.05 vs. the model group. MHCD, modified Huangqi Chifeng decoction; LC3, microtubule-associated proteins 1A/1B light chain 3B; PI3K, phosphoinositide-3 kinase; mTOR, mammalian target of rapamycin.
homeostasis by maintaining a balance between the synthesis and subsequent recycling of cellular products (36). However, excessive or sustained autophagy triggers non-apoptotic programmed cell death due to excessive self-digestion and degradation of essential cellular constituents (20,37,38). It has been reported that excessive autophagy serves a role in kidney disease (39); furthermore, excessive autophagy has been demonstrated to result in cell death (40). The results of the present study are in agreement with previous reports, indicating that MHCD attenuates excessive autophagy in the kidney by attenuating LC3 and beclin-1 overexpression and upregulating basal autophagy to protect Doxorubicin-induced nephrotic rats.

The specific mechanism by which MHCD regulates autophagy has not previously been elucidated, however it has been reported that PI3K/mTOR pathway activation is involved in autophagy (41,42). Based on this, the effect of MHCD on PI3K/mTOR pathway activation was investigated. PI3K is a family of lipid kinases (43) and mTOR is a highly conserved serine/threonine kinase (44). In the present study, the expression of PI3K and mTOR in the renal cortical tissues of Doxorubicin-induced nephrotic rats was examined. The results revealed that MHCD activates the PI3K/mTOR signaling pathway. Luo et al (45) reported that excessive autophagy resulted in delayed cell death due to inhibition of the PI3K/mTOR signaling pathway. Furthermore, excessive autophagy is often observed when the PI3K/mTOR signaling pathway is chemically blocked (46). A previous study revealed that autophagy was one of the main mechanisms of cell death when the PI3K/mTOR signaling pathway is inhibited (47). Wang et al (48) revealed that PI3K/Akt signaling was associated with the protection of neurons via autophagy inhibition. Xing et al (49) reported that mTOR upregulation inhibited autophagy. The results of the present study revealed that MHCD attenuates excessive autophagy and activates the PI3K/mTOR signaling pathway in Doxorubicin-induced renal injury. This suggests that MHCD attenuates excessive autophagy by inhibiting the PI3K/mTOR signaling pathway.

Podocyte activity has previously been assessed in vitro using CCK-8 and it was revealed that Doxorubicin promoted autophagy compared with the autophagy inhibitor 3-methyladrenine (50). The aim of the present study was to assess whether MHCD inhibited excessive autophagy caused by Doxorubicin only; no other agents were assessed. In future studies additional autophagy inhibitors and PI3K/mTOR signaling pathway inhibitors should be assessed in vivo to confirm the results of the present study. Furthermore, autophagy is a complicated process; elucidating the specific effects of individual MHCD components in autophagy requires the synthesis of specific small molecule compounds for validation.

In the present study, it rats with untreated Doxorubicin-induced nephrosis exhibited a marked and sustained increase in proteinuria until week 6. In contrast, proteinuria in MHCD- and telmisartan-treated rats increased only slightly between weeks 2 and 4. Future studies should include a group of individuals treated with MHCD for 2 weeks prior to Doxorubicin treatment to further demonstrate that MHCD has protective roles in Doxorubicin-induced nephrotic rats. In conclusion, the results of the present study suggest that MHCD ameliorates proteinuria, increases ALB levels and decreases Scr, TG and TCH levels. Additionally, it protects against glomerular and podocyte injuries by inhibiting excessive autophagy via
the PI3K/mTOR signaling pathway and upregulating basal autophagy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ conceived and designed the animal experiments and helped to draft the paper. ZKY, BY, LSL, JNZ and WH performed the experiments. ZKY and BY analyzed the data and wrote the paper.

Ethics approval and consent to participate

The Animal Care and Use Committee of Xiyuan Hospital of China Academy of Chinese Medical Sciences (Beijing, China) approved the experimental protocol.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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