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ORIGINAL ARTICLE

Protective effects of Huang Qi Huai granules on adriamycin nephrosis in rats

Chunhua Zhu · Songming Huang · Guixia Ding · Yanggang Yuan · Qiuxia Chen · Xiaoqin Pan · Ronghua Chen · Aihua Zhang

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Abstract Huang Qi Huai (HQH) granules, a mixture of Chinese herbs, contains trametes robiniophila murr, wolfberry fruit, and *Polygonatum*. We investigated the mechanism of the protective effects of HQH on adriamycin nephrosis (ADR) in rats. Adriamycin nephrotic rats were induced by a single dose of 5 mg/kg adriamycin. For the HQH-treated adriamycin nephrosis group, 1 day after treatment with 5 mg/kg adriamycin, the rats were administered once-daily oral gavage of 2 mg/kg HQH for 15 days. All the rats were killed at day 15. Histological changes were observed by light microscopy and transmission electron microscope. Nephrin and podocin expression levels were measured by real-time RT-PCR and Western blot. Proteinuria was measured by the Bradford protein assay. Serum TNF-α and IL-1β levels were evaluated by ELISA. Macrophage infiltration was detected by immunohistochemistry and immunoblotting, respectively. ADR rats showed heavy proteinuria, podocyte and tubulointerstitial injury, macrophage infiltration, and increased levels of serum cytokines TNF-α and IL-1β. HQH significantly ameliorated the adriamycin-induced renal injury. These data were validated in the cultured podocytes. The podocytes were treated by adriamycin in the presence or absence of HQH and nephrin and podocin expression and TNF-α and IL-1β synthesis and secretion were determined by real-time RT-PCR, immunoblotting, and ELISA, respectively. Adriamycin significantly reduced nephrin and podocin expression, which was significantly restored by the treatment of HQH. HQH treatment inhibited adriamycin-induced TNF-α and IL-1β expression. Our findings suggest that HQH significantly reduces proteinuria, prevents podocyte injury, and ameliorates tubulointerstitial damage. Inhibition of inflammatory cytokine expression and macrophage infiltration may be the protective mechanism of HQH.

Keywords Nephrotic syndrome · Adriamycin · Huang Qi Huai · Podocytes

C. Zhu · S. Huang (✉) · G. Ding · Y. Yuan · Q. Chen · A. Zhang (✉)
Department of Nephrology, Nanjing Children's Hospital, Nanjing Medical University, Nanjing 210008 Jiangsu Province, China
email: smhuang@njmu.edu.cn
email: zhaihua@njmu.edu.cn

C. Zhu · S. Huang · G. Ding · Y. Yuan · Q. Chen · X. Pan · R. Chen · A. Zhang
Institute of Pediatrics, Nanjing Medical University, Nanjing, China

Introduction

Primary nephrotic syndrome is a common pediatric urinary system disease that manifests as heavy proteinuria, hypoalbuminemia, hyperlipidemia, and varying degrees of edema [1]. Glucocorticoids are the main treatment, but long-term application causes immune dysfunction and complicated infection, thus affecting recovery. Meanwhile, many clinical and experimental studies have shown that the pathogenesis of primary nephrotic syndrome is associated with immune dysfunction [2]. The clinical application of immunomodulatory drugs has thus attracted wide attention.

Huang Qi Huai (HQH) granules, the brand name also known as Huang Er Jin, is primarily composed of trametes robiniophila murr, wolfberry fruit, and *Polygonatum*. Related pharmacological studies have shown that trametes robiniophila murr, being rich in polysaccharides, is a biological response modifier and can stimulate many elements of the immune system to enhance immunity. Clinical studies have shown that it has certain effects on

children with recurrent respiratory tract infections [3, 4], which is one cause of relapse in children with primary nephrotic syndrome [5, 6]. Therefore, in the present study, ADR rat models were selected to observe the effects of HQH on experimental renal disease and to explore its mechanism of action.

Materials and methods

Reagents

Caelyx (liposomal adriamycin) was from Merck (Whitehouse Station, NJ). HQH was provided by Gaitianli Pharmaceutical Co. (Qidong, Jianshu Province, China). HQH is primarily composed of trametes robiniophila murr (30%), wolfberry fruit, and *Polygonatum* (70%).

TRIzol reagent and the IL-1β ELISA kit were from Invitrogen (Carlsbad, CA). Reverse transcription reagents were from Promega (Madison, WI). SYBR Green master mix for real-time PCR was from Applied Biosystems Inc., (Foster City, CA). Anti-nephrin and podocin polyclonal antibodies were from Abcam (Cambridge, MA). ED-1 monoclonal antibody was from Serotec (Oxford, UK). Polink-2 plus Polymer HRP Detection System (PV-9000) was from Beijing Zhongshan Biotechnology Co. (Beijing, China). TNF-α ELISA kit was from Jingmei BioTech Co., Ltd. (Shengzheng, China). All the other chemicals used were of analytical reagent grade.

Animals and groups

Male Sprague-Dawley (SD) rats, weighing 160–180 g, were from Shanghai SLAC Laboratory Animals Co., Ltd., (Shanghai, China). All studies were performed with the approval of the university experimental animal committee. The animals were fed according to the standard methods and fasted 4 h before the experiment. The rats were randomly divided into the following groups (n=6 for each group): rats received a single slow injection of 5 mg/kg adriamycin (adriamycin nephrotic rats: ADR group) or saline (controls). For the HQH-treated group (ADR-HQH), 1 day after treatment with 5 mg/kg adriamycin, the rats were treated with a daily oral gavage of 2 mg/kg HQH for 15 days. The animals were anesthetized with 5 mg/kg urethane at the end of the experiment. The serum was isolated and the kidneys were harvested.

Routine monitoring, and urinary protein and blood sample analysis

Rats were housed at a temperature of 24±1°C and humidity of about 70%. Body weight and food and water intake were monitored daily. Before adriamycin treatment and at days 7 and 17, all the rats were individually placed in metabolic cages and a 24-h urine was collected. Urinary protein concentrations (mg/ml) were measured by the Bradford protein assay (Bio-Rad) and the excretion of 24-h urinary protein was calculated by multiplying the urinary protein concentration by the 24-h urine volume (ml).

Serum creatinine (sCr), blood urea nitrogen (BUN), cholesterol (TC), and albumin (ALB) were measured by Automatic Biochemical Analyzer (Olympus AU400; Olympus Corp., Tokyo, Japan).

Histological assay

Kidney tissues were fixed in 4% paraformaldehyde, embedded in paraffin, cut into 3-μm sections, and then stained with hematoxylin-eosin (H&E), periodic acid-Schiff (PAS), Masson's trichrome, and periodic acid methenamine (PAM) silver stains. All specimens were evaluated blindly by the same observer. The renal tubulointerstitial injury was determined semiquantitatively on ten randomly selected non-overlapping specimens at 200× magnification from each rat on PAS staining sections based on the presence of tubular cellularity, basement membrane thickening, dilation, atrophy, sloughing, or interstitial widening as follows: 0, no changes present; grade 1, <15% tubulointerstitial changes present; grade 2, 15–30% tubulointerstitial involvement; grade 3, 30–60% tubulointerstitial involvement; grade 4, 60–100% tubulointerstitial involvement. The number of macrophages (ED-1-positive cells/mm^2) in the cortex was also quantified.

For ultrastructural examination, ultra-thin sections were cut with glass knives, stained with uranyl acetate and lead citrate, and examined under a JEM-2000EX transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Cell culture

MPC5 conditionally immortalized mouse podocyte clonal cells (kindly provided by Peter Mundel at Mount Sinai School of Medicine through Dr. Jie Ding at Peking University) were cultured and induced to differentiate as described. Briefly, podocytes were grown and propagated at 33°C in RPMI-1640 (Invitrogen, Carlsbad, CA) with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 10 U/ml interferon-γ (Peprotech Inc, Rocky Hill, NJ). To induce differentiation, cells were plated in type I collagen-coated flasks under nonpermissive conditions (37°C without interferon-γ) for 7–10 days. For experiments, fully differentiated podocytes were cultured for 7–10 days at 37°C (to 50–60% confluence) and treated with adriamycin (100 nM) in the presence or absence of HQH (0.2 mg/ml).

Real-time RT-PCR

Total RNA was isolated from the renal cortex and cultured podocytes using Trizol reagents according to the manufacturer's manual. Single-stranded cDNAs were generated by reverse transcription according to the manufacturer's instructions. Real-time RT-PCR was performed with an ABI Prism 7900 sequence-detection system (Applied Biosystems, Foster City, CA), using SYBR Green PCR Master Mix according to the manufacturer's protocol. The primer sequences were as follows: for nephrin, (F, 5′-ACAGCAGCCTCTTGA CCAT-3′, and R, 5′-TGACAACCTTCAGTCCCAGT-3′); for podocin, (F, 5′-CAGCCACGGTAGTGAATGTG-3′, and R, 5′-TCAGGGAGGAGAGGACAAGA-3′); for TNF-α, (F, 5′-GCCTCTTCTCATTCCTGCTT, and R, 5′-CACTTGGTGGTTTGCTACGA); for IL-1β, (F, 5′-CAAAAGATGAAGGGCTGCTT, and R, 5′-ACGG GAAAGACACAGGTAGC); and for GAPDH, (F, 5′-CAAGTTCAACGGCACAGTCAA-3′, and R, 5′-TGGTGAAGACGCCAGTAGACTC -3′). GAPDH served as an internal control. Each sample was normalized to GAPDH. The thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15s and 60°C for 1 min.

Western blot

Renal cortex samples (100 mg) or podocytes were lysed in protein lysis buffer and protease inhibitor cocktail (Sigma). Lysate protein concentrations were determined by Coomassie blue dye-binding assay (Bio-Rad). An equal volume of 2× SDS loading buffer (0.125 mM Tris-HCl, pH 7.4, 4% SDS, and 20% glycerol) was added, and the samples were boiled for 5 min. Protein samples (100 μg) were resolved by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes (Bio-Rad). The filters were blocked with TBST buffer (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20) containing 5% skim milk, incubated with anti-nephrin antibody (1:200) or with anti-podocin antibody (1:500) or with anti-ED-1 antibody (1:200) at 4°C overnight, followed by the addition of horseradish peroxidase-linked anti-rabbit IgG and ECL visualization of the bands. Bands were scanned using a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA), and the results were quantified using Quantity One software (Bio-Rad).

Immunoperoxidase staining

Immunoperoxidase staining for macrophages (ED-1-positive) was conducted on 3-μm sections of 4% paraformaldehyde-fixed renal tissue using antigen retrieval (microwave oven heating in 0.1 M citrate sodium PH6.0 for 5 min and cooling at room temperature). The sections were incubated with anti-ED-1 monoclonal antibody (1:100) overnight at 4°C and followed by Polink-2 plus Polymer HRP detection. Images (400× magnification) were recorded with an OLYMPUS AX70 microscope (Olympus, Tokyo, Japan).

TNF-α and IL-1β ELISA

The serum or supernatant of culture podocytes levels of TNF-α and IL-1β were determined by commercial kits according to the manufacturer's instructions. All samples were assayed in duplicate.

Statistic analysis

Values shown represent means ± SE. Statistical analysis was performed by one-way ANOVA and Bonferroni tests with a p value of less than 0.05 being considered statistically significant.

Results

Changes in general conditions

From the second day of modeling, rats exhibited varying degrees of diarrhea, reduced food and water intake, and fluffy hair, especially in the model group. The normal control group did not show any significant discomfort. Rats in the adriamycin model group lost a significant amount of weight during the course of the experiment, while HQH significantly mitigated adriamycin-induced weight loss (Table 1), improving the rats' general conditions.

Effects of HQH on urinary proteins and blood biochemical parameters of ADR rats

Urinary proteins in each group were all lower than 15 mg/24 h before modeling, with no significant difference between the groups. On the 7th day after modeling, urinary protein excretion in the nephrosis group was significantly increased, and continued to increase through the 14th day; urinary protein excretion in the HQH-treated group began to decrease after 7 days, and was significantly lower than that of the model group on the 14th day ($p<0.05$) (Table 1). Serum albumin was significantly decreased and cholesterol was dramatically increased in adriamycin nephrosis, which was prevented or ameliorated by HQH treatment. Blood urea nitrogen and serum creatinine did not change significantly (Table 2).

Table 1 Body weight, proteinuria, renal tubulointerstitial injury score, and cortical macrophages infiltration in the control, ADR rats, and the ADR plus HQH-treated rats

	Body weight (g)		Proteinuria (mg/24 h)			Tubulointerstitial injury score	Cortical macrophages (ED-1-positive cells) /mm^2
	Day 0	Day 15	Day 0	Day 7	Day 15		
Control	195.67±6.69	235.33±23.35	11.44±2.87	12.08±4.93	10.98±1.39	0.10±0.32	8.9±4.72
ADR	195.67±5.86	176.33±24.00*	13.86±2.52	34.33±4.03*	56.90±17.30*	1.8±0.63*	42.20±23.03*
ADR+HQH	196.33±9.07	201.67±28.43$^\#$	14.59±1.37	36.66±4.03*	23.64±3.96$^\#$	0.6±0.51$^\#$	15.30±7.57$^\#$

Values are mean ± SEM, n=6. * p<0.01 versus Control; $^\#$ p<0.01 versus ADR rats

Effects of HQH on glomerular visceral epithelial cells

Glomerular volume slightly increased in the model group, as determined by light microscopy, as mesangial cells and matrix presented a focally segmental mild increase; glomerular volume in the HQH-treated group was not significantly different from the control group (Fig. 1a, b, and c). Electron microscopy showed that the glomerular basement membrane was occasionally thickened in the model group, foot processes of glomerular visceral epithelial cells, known as podocytes, were widely fused, and some foot processes disappeared; in the HQH-treated group, podocyte injury was markedly relieved compared to that in the model group, and only focal foot process fusion could be seen (Fig. 2a, b, and c).

Effects of HQH on renal cortical expression of nephrin and podocin

Real-time RT-PCR and immunoblotting analysis showed that the expression of nephrin and podocin in the model group were significantly reduced in comparison to those of the control group, which was significantly restored by HQH treatment (Fig. 3).

Effects of HQH on tubulointerstitial injury and macrophages infiltration

Renal tubular epithelial cells of rats in the model group presented focal granular and vacuolar degeneration, and protein casts were observed in some lumens, showing focal interstitial inflammatory cell infiltration (Fig. 1e), with a small amount of fibrous tissue hyperplasia. The area and range of renal interstitial inflammatory cell infiltration in the HQH-treated group were significantly reduced in comparison to those in the model group (Fig. 1f).

Immunoperoxidase staining and Western-blot results showed that adriamycin significantly induced monocyte/macrophage infiltration both in glomeruli and interstitium, while HQH intervention almost completely blocked this infiltration (Fig. 4).

Semiquantitative analysis of renal tubulointerstitial lesions are shown in Table 1. HQH-treated rats had significantly lower tubulointerstitial scores compared to ADR rats. The urine protein excretion had a significantly positive correlation with tubulointerstitial damage (r^2=0.6, p<0.01).

Serum cytokines TNF-α and IL-1β levels

As shown in Fig. 5, adriamycin significantly increased the levels of serum cytokines TNF-α and IL-1β (vs. the control group, p<0.01). After HQH treatment, the increase in serum TNF-α and IL-1β was significantly inhibited (vs. the model group, p<0.01).

Effects of HQH on the expression of nephrin, podocin, TNF-α, and IL-1β in cultured podocytes

In cultured podocytes, the nephrin and podocin expression was significantly decreased and TNF-α and IL-1β increased when exposed to adriamycin (100 nM). HQH treatment (0.2 mg/ml) significantly restored nephrin and podocin expression, while inhibited TNF-α and IL-1β expression (Figs. 6 and 7).

Table 2 Serum creatinine (sCr), urea nitrogen (BUN), cholesterol (TC), and albumin (ALB) in the control, ADR rats, and the ADR plus HQH-treated rats

	sCr	BUN	TC	ALB
Control	19.67±0.58	6.18±0.54	1.57±0.51	37.85±4.43
ADR	20.13±0.45	7.85±0.49	4.08±0.55*	22.46±2.61*
ADR+HQH	21.70±0.91	7.02±0.56	2.33±0.63$^\#$	32.28±4.94$^\#$

Values are mean ± SEM, n=6. * p<0.01 versus control; $^\#$ p<0.01 versus ADR rats

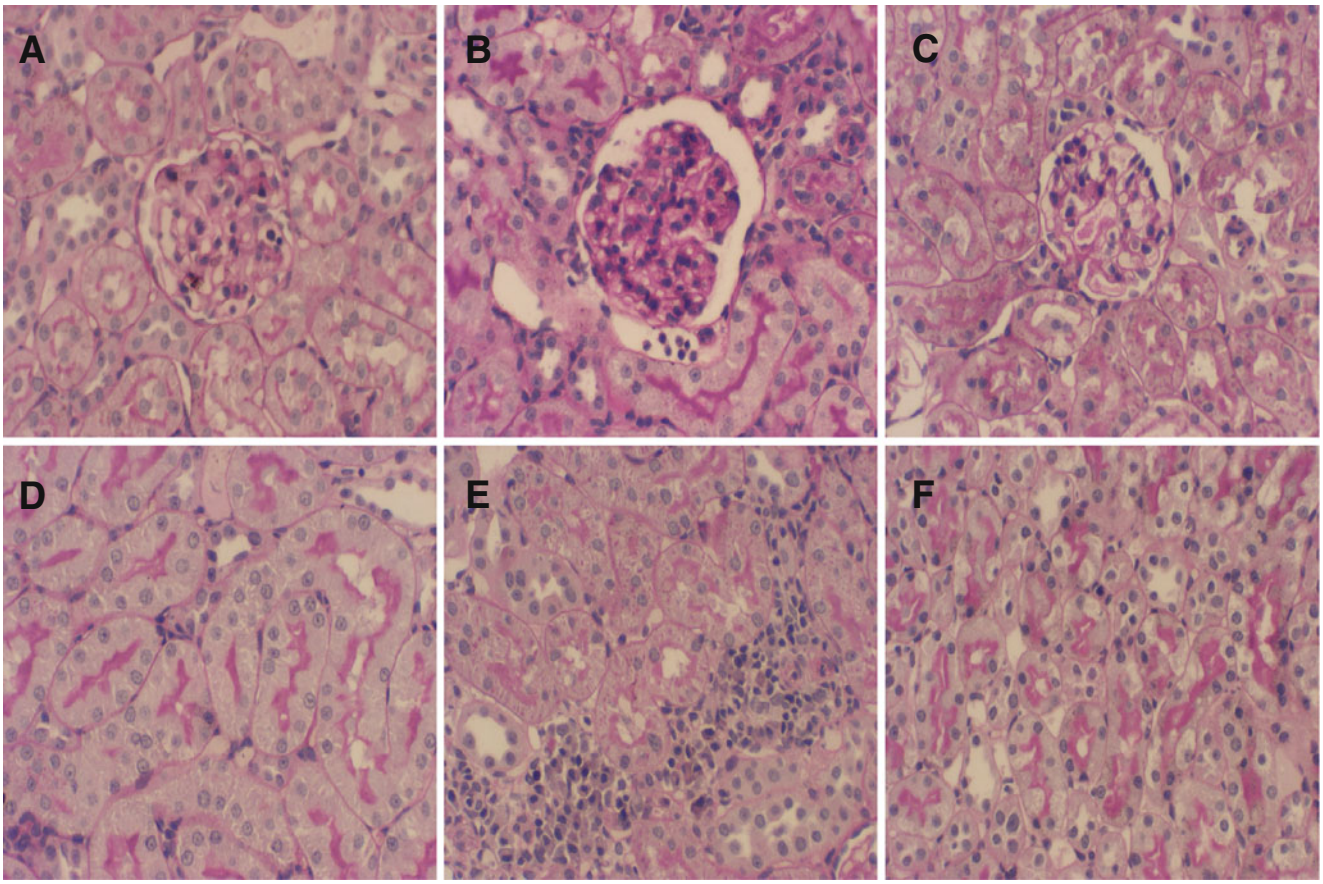


Fig. 1 Histological morphology changes of the glomerulus (**a**, **b**, and **c**) and tubulointerstitium (**d**, **e**) by PAS staining (400×). **a**, **d** Control. **b**, **e** ADR rats. **c**, **f** ADR plus HQH-treated rats

Discussion

In this study, we found HQH significantly inhibited the excretion of serum TNF-α and IL-1β, and macrophage infiltration in the kidneys of ADR rats. It significantly reduced proteinuria, prevented podocyte injury, and ameliorated tubulointerstitial damage.

ADR is the experimental animal model of nephrotic syndrome. Its early pathological changes are mainly podocyte injury and tubulointerstitial lesions [7]. In the present study, the modeling results are consistent with previous reports, that is, a significant increase in urinary protein appears on the 7th day after a single tail vein injection of adriamycin and continue to the 14th day.

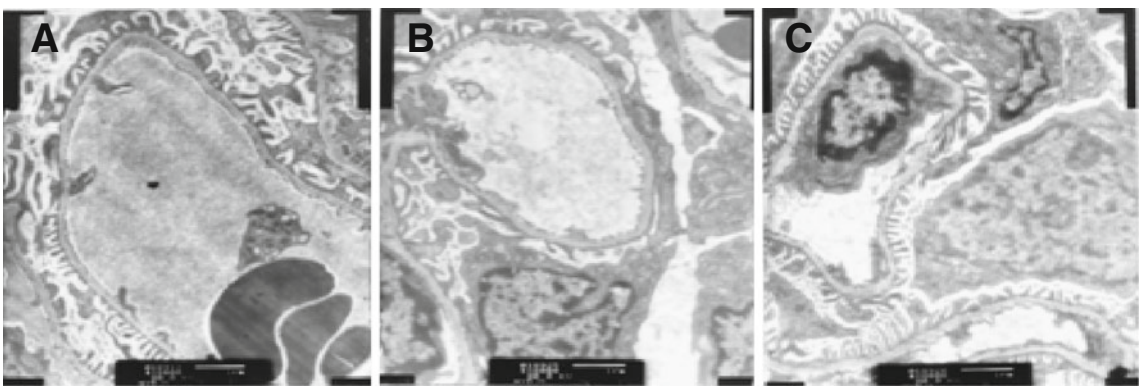


Fig. 2 Effects of HQH on podocytes injury in rats. Morphology changes of podocyte foot processes by electron microscopy (15,000×). **a** Control. **b** ADR rats. **c** ADR plus HQH-treated rats

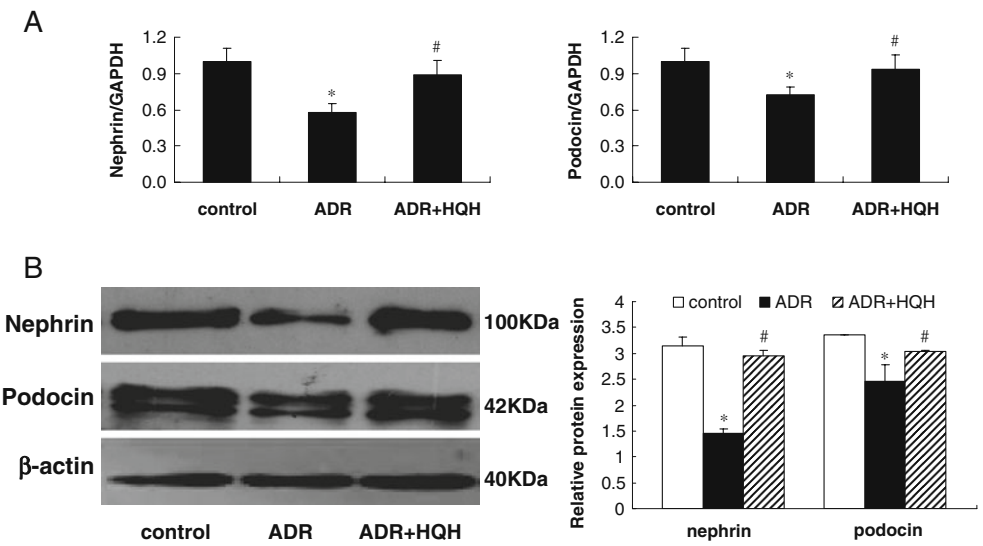
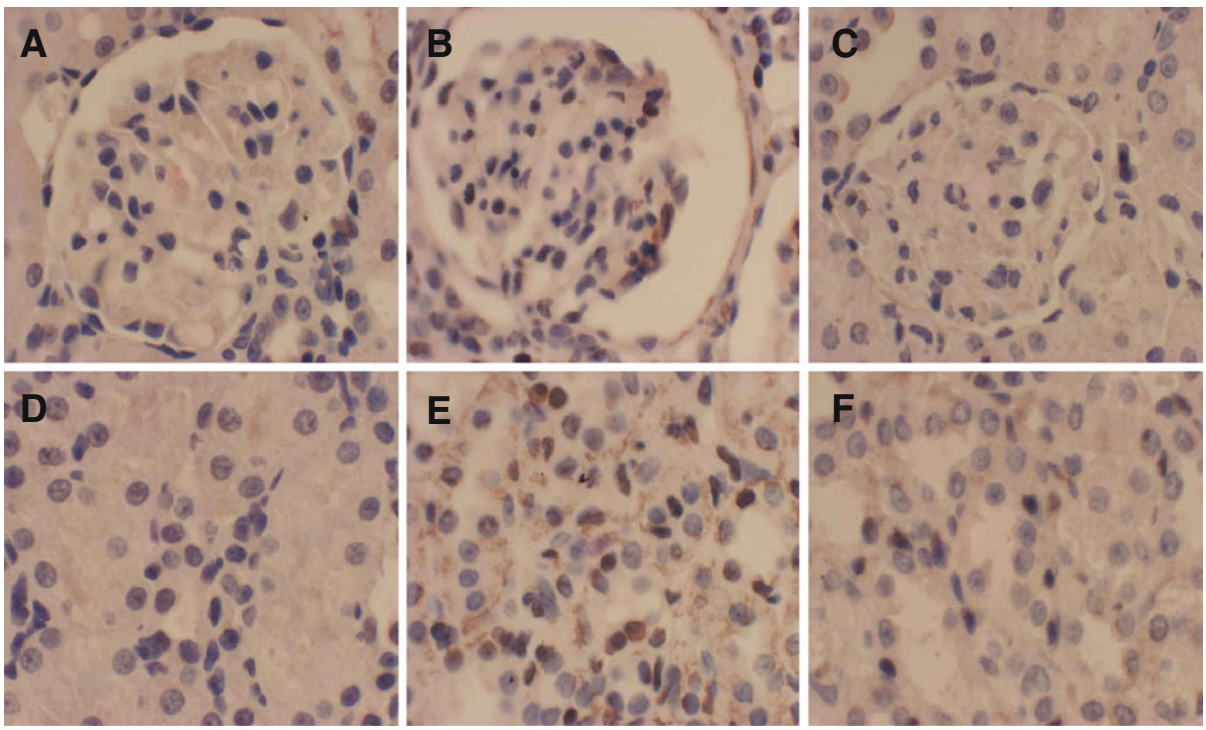


Fig. 3 Effects of HQH on nephrin and podocin expression of the cortex of the kidney. Nephrin and podocin mRNA and protein expression was determined by real-time PCR and Western blot, respectively. **a** mRNA expression. **b** Protein expression. *Left panel* Representative immunoblots. *Right panel* Densitometric analysis. Values represent mean ± SEM, $n=6$. $^{*}p<0.01$ versus control; $^{\#}p<0.01$ versus ADR rats



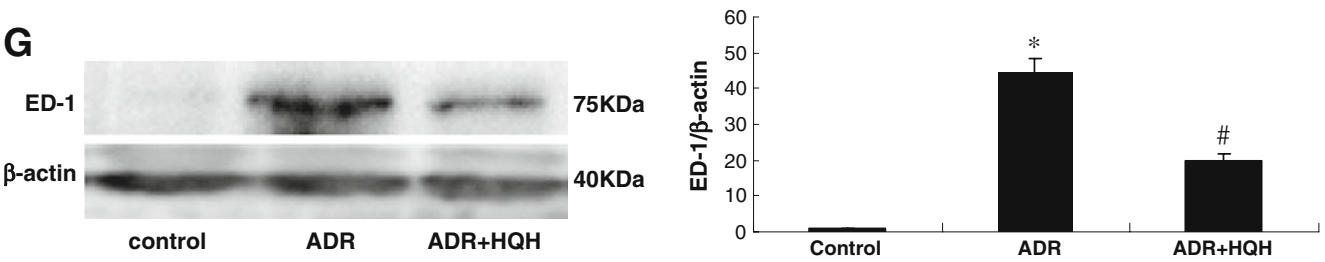


Fig. 4 Effect of HQH on macrophage infiltration in the kidney (**a**–**f**). Macrophage infiltration in the glomeruli (**a**–**c**) and tubulointerstitium (**d**–**f**) was determined by immunoperoxidase staining. **a**, **d**, Control. **b**, **e** ADR rats. **c**, **f** ADR plus HQH-treated rats. **g** Macrophage infiltration was quantified by immunoblotting analysis using anti-ED1 antibodies. *Left panel* Representative immunoblots. *Right panel* Densitometric analysis. Values represent mean ± SEM, $n=6$. $^{*}p<0.01$ versus control; $^{\#}p<0.01$ versus ADR rats

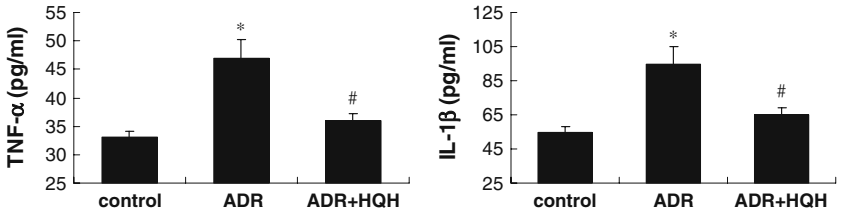


Fig. 5 Effect of HQH on serum TNF-α and IL-1β secretion. Serum TNF-α and IL-1β levels were determined by ELISA. Values represent mean ± SEM, $n=6$. $^{*}p<0.01$ versus control; $^{\#}p<0.01$ versus ADR rats

Histological examination indicates that nephrotic rats presented large-scale fusion and disappearance of foot processes, decreased expression of podocyte-related molecules nephrin and podocin, renal tubular epithelial cell degeneration, and monocyte/macrophage infiltration both in glomeruli and interstitium.

Podocytes, as highly differentiated terminal cells, combine with slit diaphragms among foot processes to form the outermost layer of the glomerular filtration barrier. The fusion and disappearance of foot processes is one of the initial mechanisms of proteinuria formation [8, 9], while urinary protein excretion can aggravate podocyte injury. In this study, HQH reduced proteinuria in ADR rats, simultaneously, significantly alleviated podocyte injury, indicating that proteinuria is closely related to podocyte injury. Meanwhile, we found that tubulointerstitial injury was significantly reduced by treatment with HQH.

Some studies suggest that metabolites of excessive protein deposits in the renal tubules can mediate tubulointerstitial injury by activating the inflammatory cytokine network in a variety of ways, thereby increasing glomerular damage. Monocyte/macrophage infiltration and disorders of various cytokines in particular have attracted wide attention [10]. Monocytes/macrophages are important effectors of the immune response, and infiltration in renal tissue is an important pathological feature of tubulointerstitial lesions, playing an important role in the development of kidney disease. The leakage of excessive proteins from the glomerulus can stimulate tubular epithelial cells to secrete a series of cytokines, such as various chemokines and secretory factors. Monocytes in the blood become macrophages by infiltrating the tubulointerstitial via the vascular endothelial cells and renal tubular epithelial cells under the effect of these factors. Its abnormal infiltration and activation can trigger various cytokines and enlarge the network effect, collectively participating in cellular and humoral immunity, mediating the immune response imbalance. IL-1β and TNF-α are strong pro-inflammatory cytokines, and both can be

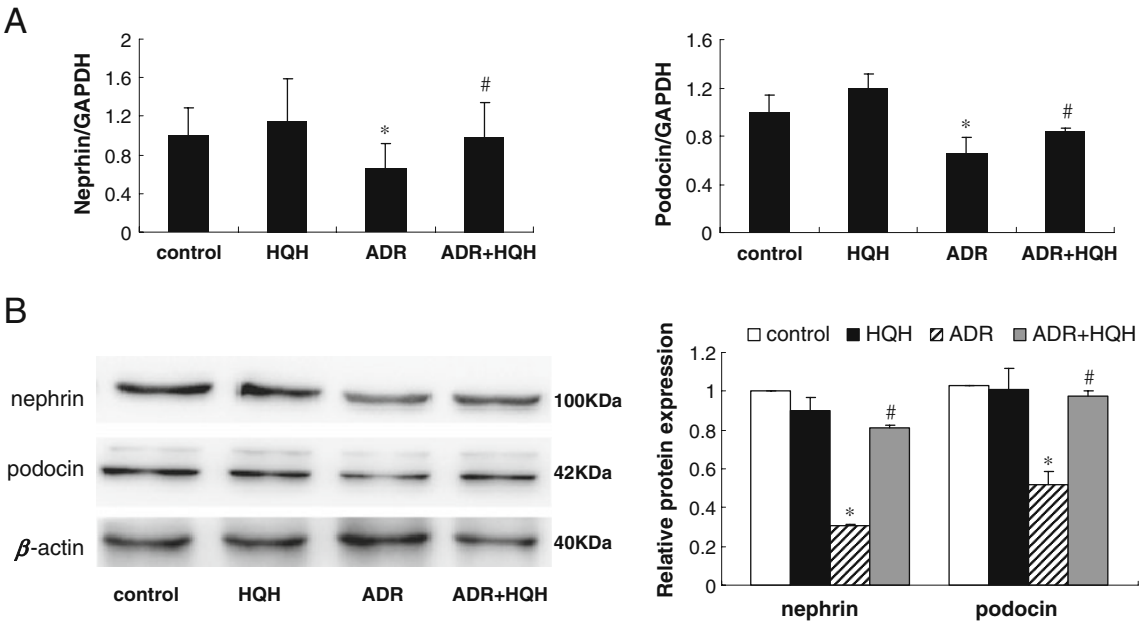


Fig. 6 Effects of HQH on nephrin and podocin expression in cultured podocytes. Nephrin and podocin mRNA and protein expression was determined by real-time RT-PCR and Western blot, respectively. **a** mRNA expression. **b** Protein expression. *Left panel* Representative immunoblots. *Right panel* Densitometric analysis. Values represent mean ± SEM, $n=6$. $^{*}p<0.01$ versus control; $^{\#}p<0.01$ versus ADR groups

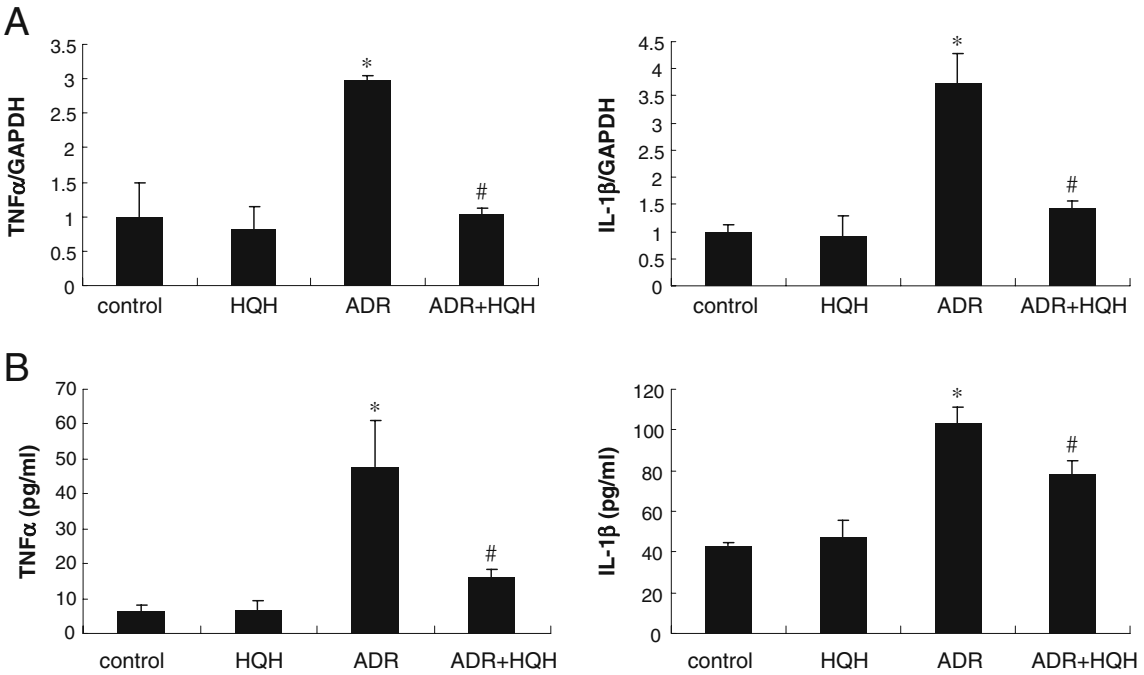


Fig. 7 Effects of HQH on TNF-α and IL-1β mRNA expression and secretion in cultured podocytes. TNF-α and IL-1β mRNA expression and secretion were determined by real-time RT-PCR and ELISA, respectively. **a** mRNA expression. **b** Secretion in the supernatant of cultured podocytes. Values represent mean ± SEM, $n=6$. $^{*}p<0.05$ versus control; $^{\#}p<0.05$ versus ADR groups

generated by activated mononuclear macrophages; they can not only exert direct effects on kidney cells but also induce the production of other inflammatory mediators, thus enlarging the pro-inflammatory effect. Recent studies have shown that anti-TNF-α blocker can alleviate experimental crescentic glomerulonephritis, and delay crescent formation and tubulointerstitial sclerosis, protecting renal function [11]. IL-1β receptor antagonist also has similar effects. We found that ADR rats had heavy renal interstitial mononuclear macrophage infiltration, while HQH significantly reduced the infiltration induced by adriamycin, and simultaneously inhibited the expression of serum IL-1β and TNF-α.

HQH is primarily composed of trametes robiniophila murr (30%), wolfberry fruit, and Polygonatum (70%), and the biological response modifier is trametes robiniophila murr, which contains six kinds of monosaccharide and 18 kinds of amino acid. The wolfberry fruit and *Polygonatum* provide synergetic effects. The exact mechanism involved in the renoprotective effect of HQH remains unclear. It has been reported that macrophages and macrophage-derived cytokines IL-1β and TNF-α significantly inhibited the activity of the nephrin gene promoter [12]. Tang et al. [13, 14] and Le Hir et al. reported that [15] both IL-1β receptor antagonist and gene knockout of TNF-α could inhibit macrophage induced podocyte injury. The anti-inflammatory drugs have also been suggested to lower the proteinuria by restore the nephrin and podocin expression [16]. Our data showed that HQH treatment reduced macrophage infiltration in adriamycin nephrosis in rats, and inhibited IL-1β and TNF-α expression both in vivo and in vitro, indicating that HQH restored nephrin and podocin expression and prevented podocyte injury by inhibition of expression of the pro-inflammatory cytokines, such as IL-1β and TNF-α, etc. More studies are needed to elucidate the precise mechanisms involved in the renoprotective effects of HQH at the cellular and molecular levels.

Glucocorticoid is the basic drug for the treatment of nephrotic syndrome, but it has significant anti-inflammatory and immunosuppressive effects, exerting strong inhibitory effects on monocyte-macrophages, neutrophils, T lymphocytes, and B lymphocytes. We found that the protective effect of HQH was similar to that of glucocorticoid in tubulointerstitial damage; therefore, the mechanism may be associated with the regulation of the immune response, but the specific mechanism remains unknown. IL-1β and TNF-α are not only involved in the development of tubulointerstitial lesions but also show certain correlation with glucocorticoid resistance in children with nephrotic syndrome [17]. IL-1β and TNF-α can activate nuclear transcription factor activator protein-1 (AP-1), which can inhibit glucocorticoid-glucocorticoid receptor complex binding to the glucocorticoid response element, thereby blocking its biological effects [18]. HQH, as an immunomodulatory drug, can effectively reduce the levels of serum IL-1β and TNF-α. Therefore, the combination of HQH and glucocorticoid is expected to provide a new clinical treatment for nephrotic syndrome, especially for

children in whom hormone use is ineffective, the clinical trial is currently conducting at our center and Children's Hospital affiliated to Fudan University to test this hypothesis (registration No: ChiCTR-TRC-10000871).

In summary, HQH can significantly reduce urinary protein excretion in adriamycin nephrosis, protect glomerular podocytes, and ameliorate tubulointerstitial damage. Inhibition of inflammatory cytokine expression and macrophage infiltration may be the renoprotective mechanism of HQH.

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