Jianpi Qushi Heluo Formula Alleviates Renal Damages in Passive Hemann Nephritis Rats by Upregulating Parkin-Mediated Mitochondrial Autophagy

Xin-hui Wang
China Department of Nephrology, Xiyuan Hospital of China Academy of Chinese Medical Sciences
Rui Lang
China Department of Nephrology, Xiyuan Hospital of China Academy of Chinese Medical Sciences
Qin Zeng
Graduate School of Beijing University of Chinese Medicine
Nan Chen
Graduate School of Beijing University of Chinese Medicine
Zhi-zhong Ma
Department of Integration of Chinese and Western Medicine, School of Basic Medical Sciences, Peking University
Ren-huan Yu (tezhongeyu@vip.sina.com)
China Department of Nephrology, Xiyuan Hospital of China Academy of Chinese Medical Sciences

Research Article

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Abstract

Jianpi Qushi Heluo Formula (JQHF) is an empirical traditional Chinese medicine prescription for treating membranous nephropathy (MN) clinically in China. The therapeutic effect of JQHF has been reported in our previous studies. However, the exact mechanism is still unknown. In this study, by establishing an experimental rat model of MN induced by Sheep anti-rat Fx1A serum, we evaluated the effects of JQHF and Tetrandrine(TET), and Benazepril was used as a positive control group. As an autophagy agonist, TET is one of the most active components in JQHF. After 4 weeks, significant kidney damage was observed in the rats in the Model group; comparatively, JQHF markedly decreased 24 hour urinary protein, total cholesterol (TC) and increased serum albumin (ALB). Histology showed that JQHF significant improvements of glomerular hyperplasia, renal tubular damage, IgG immune complex deposition and the ultrastructure of mitochondria in MN rats. Flow cytometry analysis showed that treatment with JQHF decreasing the level of Reactive Oxygen Species and apoptosis rate, upregulating the level of mitochondrial membrane potential. Western blot analysis demonstrated that JQHF could protect against mitochondrial dysfunction and apoptosis by upregulating the expression of Parkin and LC3II, and downregulating the expression of Cytochrome c and Cleaved caspase-3 in the kidneys of MN rats. Similarly, TET treatment significantly upregulates the mitochondrial autophagy and decrease the apoptosis of rats after 4 weeks compared with the Model group. Notably, combined with the above results, the ability to alleviates renal damages of JQHF was significantly better than those of Benazepril and TET. It was demonstrated that JQHF could delay pathology damage to the kidney and hold back from the progression of MN by inhibiting apoptosis and upregulating the mitochondrial autophagy by Parkin pathways.

Introduction

As the main pathological form of adult nephrotic syndrome, Membranous nephropathy (MN) accounts for about 24.1% of all primary glomerular diseases, and within 10 years approximately 33-50% of the MN patients will develop the end-stage renal disease[1-2]. Disappointingly, there is currently no safe and effective treatments available that slow the progression of MN. The current treatment to MN, western medical approaches mainly include immunosuppressive and cytotoxic drugs, which are associated with many disadvantages, including a range of side effects, low tolerance, and the fact that withdrawal can readily result in recurrence; collectively, these factors limit the clinical application of these drugs[3-4].

Traditional Chinese medicine(TCM) has accumulated a lot of clinical experience in treating various kinds of kidney diseases, including MN[5]. Among them, the most representative TCM preparations, Jianpi Qushi Heluo Formula(JQHF) was first developed to treat MN by the department of nephropathy of Xiyuan Hospital, Chinese Academy of traditional Chinese medicine. JQHF contains 10 herbs that were selected by professor Renhuan Yu. Professor Yu has treated MN patients with TCM for more than 20 years and accumulated a large number of theoretical and clinical experience. A clinical study reported that for patients with refractory MN, after a treatment period of 6 months by JQHF, the clinical remission rate was

80% and without apparent side effects[6]. However, the exact mechanism of JQHF on the progression of MN is still unknown.

The critical pathogenesis of the occurrence and the development of MN is the podocytes injury caused by the activates of the complement system by the subepithelial immune complex deposits[7-8]. The process of sublytic quantities of C5b-9 insert into podocyte membranes result in podocyte injury and subsequent loss glomerular barrier function, and also with the release of reactive oxygen species(ROS)[9]. Previous studies have demonstrated superfluous accumulation of ROS and associated mitochondrial dysfunction is strongly linked to kidney injury in experimental models of chronic kidney disease[10-11]. Many oxidative stress urge to accumulate a lot of ROS, which leads to decrease in mitochondrial membrane potential(MMP) and causes mitochondrial dysfunction[12]. The increase of permeability of mitochondrial membranes results in promote Cytochrome c(Cyt c) release from mitochondrial to the cytoplasm. Then the Cyt c in cytoplasm combine with apoptotic protease-activating factor 1 leading to the apoptosome form, which activates caspases-3 in downstream result in cells death passively finally[13]. In intracellular, mitophagy and mitochondria-selective autophagy has been proven to play an essential role in maintaining homeostasis[14].

Overproduction of ROS has been found in Passive Hemann nephritis (PHN) rats[15]. PHN rat shows pathogenesis resembles to MN in human, and has been widely used for the experimental study of MN. We used PHN rats to evaluate the association between mitochondrial dysfunction and renal injury, and further verification that JQHF increases resistance to mitochondrial dysfunction and apoptosis by activated mitochondrial autophagy.

Materials And Methods

Preparation of JQHF. Nine herbs ingredient of JQHF were purchased from Hebei Baicaokang Pharmaceutical Co., Ltd. (Hebei, China, Certificate No.JIY20190064). JQHF is composed of 30g Radix astragali, 15g of Rhizoma atractylodis macrocephalae, 30g of Poria, 20g of Stephaniae tetrandrae radix, 15g of Folium perillae, 30g of Rhizoma dioscoreae nipponicae, 10g of Radix gingseng, 15g of Radix angelica sinensis, 15g of Folium nelumbinis. In the clinic, JQHF crude drug content of 180g is used to making water decoction, and oral 2 times a day to treat patients, which is equivalent to 16.2g/kg/d in the rats. All herbal drugs were prepared by the pharmacy department of Xiyuan Hospital, and the decoction was adjusted to 1.62g/ml with water for the rats orally gavage dose.

Animals and treatment administration. Our animal experiment was conducted under the Arrive Guidelines, national and institutional rules regarding animal experimentation, and pre-approved by the Animal Ethics Committee, Xiyuan Hospital, China's Academy of Chinese Medical Sciences. (Beijing, China, approved No. 2019XLC020-2).

SD rats (150±10g, 5 weeks) were provided by Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China, Certifcate No. SCXK (JING) 2016-0006). The SD rats were housed in a special pathogen-

free (SPF) environment, provided by the Experimental Animal Centre of Xiyuan Hospital (Beijing, China, Certifcate No.SYXK (JING) 2015-0011).

The rats were fed within a temperature-controlled facility (25℃, 50% relative humidity) under a 12h light/dark cycle, and rats were allowed free access to pure water and standard chow. To induce PHN, sheep anti-rat Fx1A serum (PTX-002S, Probetex, San Antonio, USA) was injected into a tail vein of each rat with 0.5ml/100g body weight. After two weeks, we randomly selected two rats for analysis. The rats were sacrificed and electron microscopy was used to investigate for the deposition of immune complex and diffuse thickening of the basement membranes. These pathological changes indicated the successful induction of the PHN model. Forty PHN rats were randomly divided into four groups. Additional ten of healthy Male SD rats were chosen as normal controls group. For experimental intervention, normal rats were orally treated with water(1ml/100g body weight) daily. For the Model group, PHN rats were orally treated with water(1ml/100g body weight) daily. For the JQHF group, PHN rats were orally treated with JQHF decoction daily (JQHF orally gavage dosage: 1.62g/100g body weight) daily. The dosage of rats was determined according to the dosage conversion between patients and animals according to international guidelines. In the TET group, PHN rats were given a daily oral gavage dose of 0.5mg/100g body weight of TET(20190717, Zelang, Nanjing, China). Finally, Benazepril was used as a positive control. In the Benazepril group, according to the human clinical dose, PHN rats were orally gavage with Benazepril(X3080, Nuohua, Beijing, China) 1mg/100g body weight daily. Drug administration was carried out for a total of four weeks.

Measurement of biochemical parameters. Rats were housed in separate metabolic cages every two weeks for 24 hours urine collection. At the end of four weeks of drug administration, rats fasted for 12 hours, blood samples were collected from the abdominal aorta. Then, kidney tissue samples were collected immediately after euthanasia by isoflurane inhalation. Serum was separated (3,500r/min, 15min) for the examination of TC and ALB. All biochemical parameters were detected by the automatic biochemistry analyzer (Ccbas 8000, Roche Diagnostics GmbH, Mannheim, Germany).

Pathological testing of renal tissue. The left kidneys were immediately put into 10% phosphate-buffered formalin solution; after fixed kidney specimens, the specimens were embedded in paraffin. Next, we observe glomerular and tubulointerstitial damage with hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) stain. The stained samples were assessed by two pathologists by utilizing a Nikon Eclipse Ti-SR (Nikon Corporation, Tokyo, Japan) inverted fluorescence microscope.

To observe glomerular hypercellularity, we examined at least 10 glomeruli in the histological section from each animal. For each group, every section was examined to acquire typical photographs (at a magnification of ×100). We counted the total number of cells in each glomerulus. When achieving photos, we ensured that the background lighting of each picture was consistent and the tissue was occupied the whole field of vision. Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, USA) was used to select glomeruli in each photograph.

The stained sections of HE were used to evaluate tubulointerstitial injury. Renal tubular atrophy, dilatation, tubular type, interstitial inflammation, and interstitial fibrosis, were evaluated in 10 fields on each renal section at a magnification of ×100. We used a semi-quantitative renal histological grading scale (from 0-3): 0 = normal; 1 = lesions in the < 25% region of the area of the section; 2 = lesions in the 25% to 50% region; 3 = lesions in the > 50% region[37-38].

Immunofluorescence staining. Paraffin-embedded Kidney tissues were cut for 3μm thickness sections for immunofluorescence staining. The sections were baked at 60 °C for 3 hours, followed by deparafnization, hydration and antigen retrieval. Then, the sections were incubated in 0.25% phosphate buffered saline (PBS) tween and soaked in 3% bovine serum albumin(BSA) blocking before incubation with anti-rat IgG labeled with FITC (4416s, CST, Boston, USA). The sections were then soaked in PBS in a dark room. The positive area of immune complex deposition in glomerulus was then quantitated using Image-Pro Plus 6.0 software.

Immunohistochemistry staining. The kidney sections were deparaffinization, hydration, and antigen retrieval with 10 mM citrate buffer (pH 6.0). Then, 3% BSA blocking and allowed to incubate with primary antibody(LC3: 3868S, CST, Boston, USA; Nephrin: ab136894, Abcam, Cambridge, UK). Afterward, the sections were washed with PBS and incubated with appropriate biotinylated secondary antibody at room temperature, and then the sections were stained with 3,3'-diaminobenzidine (DAB) and hematoxylin, respectively. Image-Pro Plus 6.0 software was used to analyze the integral optical density (IOD) of LC3 and Nephrin in each visual field.

Transmission electron microscopy examination. We cut the other 1mm^3 segment of kidney tissue for TEM examination. Kidney tissue was fixed with 2.5% glutaraldehyde and 0.5% osmium tetroxide successively. Afterward, the fixed sections were followed by dehydrated, penetrated with acetone, and embedded with epoxy resin. Then the ultrathin pieces(60–80nm) were stained with uranyl acetate and lead citrate respectively, and observed using an electron microscope(JEM-1230, JEOL Japan Electronics Co., Ltd, Tokyo, Japan).

Flow cytometry analysis. We first sacrificed the rats and then immediately dissected fresh kidney tissue. A single-cell suspension from rat kidney cortex was prepared using gentle mechanical forces, then filtered with a 400-mesh screen which pores is 40 μm and evenly spaced. Finally, the concentration of single-cell suspension was adjusted to 1x10^6/ml. For intracellular ROS, MMP, and cells apoptosis analysis, single-cell suspension was incubated with dichlorofluorescein diacetate(DCFH-DA)(S0033, Beyotime, Jiangsu, China), tetrechloro-tetraethylbenzimidazol carbocyanine iodide (JC-1)(C2006, Beyotime, Jiangsu, China) and Annexin-V-FITC/PI(10010-02, SBA*, Birmingham, USA*) for 30 minutes, respectively. The cells were then harvested for flow cytometric analysis using Flow Cytometer (EPICS ELITE, Beckman Coulter, Inc., Los Angeles, USA).

Western blot analysis. The proteins of renal cortex tissue were lysed with Protein Extraction Kit(P0033, Beyotime, Jiangsu, China) and quantified by BCA protein assay kit(P1511, Applygen, Beijing, China). Total

tissues lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) and then transferred to nitrocellulose membranes. Afterward, the membranes were blocked with 5% BSA for 1 hour at room temperature. The primary antibodies against Parkin(4211S, CST, Boston, USA), LC3B (3868S, CST, Boston, USA), Cleaved caspase-3(9662S, CST, Boston, USA), Cytochrome c(11940S, CST, Boston, USA), Nephrin (ab136894, Abcam, Cambridge, UK) and horseradish peroxidase-conjugated secondary antibodies were used.

Finally, we used an ECL Kit (P1010, Applygen, Beijing, China) and a multi-functional imaging system to detect positive binding. Image-Pro Plus 6.0 software was used to quantify all protein bands.

Statistical Analyses. Statistical analyses were performed using SPSS version 23.0 software (IBM, Inc., Chicago, USA). Data are presented as mean± standard deviation(SD) unless otherwise indicated. Two-group comparisons were performed using an independent-sample t-test. Multiple-group comparisons were analyzed by One-Way Analysis of Variance (one-way-ANOVA) followed by post hoc LSD test when normality (and homogeneity of variance) assumptions are satisfied, otherwise the Kruskal Wallis H test will be used. $P<0.05$ were recognized to be statistically significant.

Data availability: All data included in this study are available upon request by contact with the corresponding author.

Results

JQHF improved biochemical parameters of PHN rats. Compared with the Normal rats, the Model rats produced high levels of total cholesterol (TC) and 24 hours urinary protein, while the level of serum albumin (ALB) had a significantly reduced. As shown in Figure.1a-c, fortunately, after experimental treatment for 4 weeks, these biochemical parameters were ameliorated by the treatment of JQHF. In addition, the treatment effect in the JQHF group was better than that in the Benazepril group. Similarly, Tetrandrine(TET) treatment significantly reduced 24h urinary protein and increased ALB after 4 weeks compared with the Model rats. However, there were no significant reductions in TC by TET treatment.

JQHF reduces the renal damage in PHN rats. The pathological morphology of kidneys in different groups was revealed by light microscopy(Figure.1d). The model group, treatment with sheep anti-rat Fx1A serum, the volume of the glomeruli was increased and the number of mesangial cells was increased, protein cast in some renal tubules, and IgG deposition along the glomerular capillary wall. Compared with the Model group, both the JQHF group and the TET group showed significant improvements in terms of glomerular hyperplasia and renal tubular damage. Compared with TET and Benazepril group, JQHF group had more significant improvement in the renal pathological changes(Figure.1e,f). Notably, the expressions of IgG also significantly decreased in JQHF group than the Model, Benazepril and TET group (Figure.1g).

JQHF protects against ROS-induced mitochondrial dysfunction in PHN rats. According to the Figure. 2a-d, compared with the rats in Normal group, in the rats in Model group, the ROS levels were elevated and the

MMP levels were decreased. After 4 weeks treatment, as compared with the rats in Model group, we have observed a reduction in ROS levels and an elevated MMP levels in the rats in JQHF group. In the TET group, similar results were observed; MMP levels were elevated as compared to the Model group after 4 weeks.

We use the transmission electron microscope(TEM) to observe the mitochondrial ultrastructural injuries in glomeruli of the kidney. In Normal rats, mitochondria were observed with double membrane structure and transparent cristae, and in Model rats the mitochondria were seen swelled and vacuolization and the cristae lessened. However, in the rats in JQHF group, the ultrastructural damage of mitochondria was significantly improved, as compared with those rats in the Model, Benazepril and TET group(Figure. 2e).

JQHF increases resistance to mitochondrial dysfunction and apoptosis by activated mitochondrial autophagy in PHN rats. In Model rats, Western blot analyses showed that the expression of LC3B II, Parkin(E3 ubiquitin ligase) protein were decreased as compared with the Normal rats. The expression of LC3B II and Parkin protein were significantly increased in the group JQHF and TET, compared to the group Model and Benazepril (Figure.3a,b).

More importantly, JQHF, as expected, reversed the apoptosis in PHN rats. Compared with Normal group, the expression of Cyt c and Cleaved caspase-3 protein were increased in Model group, while the expression of Nephrin protein was decreased(Figure.3e,f). As compared with Model group, treatment with JQHF and TET significantly decreased the expression of Cleaved caspase-3 and Cyt c protein, and increased the expression of Nephrin protein (Figure.4a,b). The ability of JQHF and TET on the resistance of apoptosis was further confirmed by the results of annexin-V-FITC/PI staining. The cell apoptosis rate of the group Model produced high levels as compared with the group Normal. However, compared with the Model group, treatment with JQHF and TET significantly downregulation the rate of cell apoptosis in the kidney in PHN rats(Figure.4e,f).

Next, we examined the protein expression of LC3B and Nephrin by Immunohistochemistry (Figure.3c) (Figure.4c). The ability of JQHF and TET on the regulation of autophagy and apoptosis was further confirmed (Figure.3d) (Figure4.d).

Moreover, combined with the above results, this demonstrates that the apoptosis resistance ability of the JQHF was significantly better than those of the Benazepril and TET.

Discussion

JQHF, a traditional Chinese compound formula, has been used to treat MN for several years at Xiyuan hospital. We previously reported that for patients whit refractory MN, after a treatment period of 6 months by JQHF, the clinical remission rate was 80% and without apparent side effects[6]. The safety and efficacy of JQHF have been proved by clinical trial, but the specific mechanism of JQHF in MN is still unclear. In this study, we evaluated the therapeutic effect of JQHF on experimental MN in rats and explored the potential mechanisms. We induced the PHN model in Male Sprague-Dawley (SD) rats by a

single tail vein injection of sheep anti-rat FX1A serum, which pathogenic mechanism and clinical manifestations are similar to MN in humans[16]. After 4 weeks treatment, our data indicated that JQHF treatment obviously reduced the level of 24h urinary protein and TC, increased the level of ALB, and significantly ameliorated renal histological lesion. Our results suggesting the profound renoprotective effects of JQHF on MN.

The key point of the occurrence and the development of MN is the podocyte injury caused by the deposits of the subepithelial immune complex[7-8]. The slit diaphragm is the final barrier to prevent protein loss from vascular to urinary space, which is formed of podocyte foot and the counterparts of neighboring cells[17]. The deposits activate podocytes through complement-dependent processes, which could causes the activation of the signaling pathways of NADPH oxidase[18]. In cell experiments, the expression of the NADPH oxidase was increased in injury podocytes induced by PAN, which then led to the down-regulated of the expression of slit diaphragm proteins[19]. Slit diaphragm is the final barrier to prevent protein loss from vascular to urinary space. Nephrin is the first identified protein molecule, which is specifically located on the slit diaphragm, have been proved to play key role in maintaining normal podocyte structure and function. Slit diaphragm proteins also include Podocin, Transient receptor potential-6 channel(TRPC6), CD2-associated pro[1]tein (CD2AP)[20]. In vitro, C5b-9 complex induced the increase of ROS production in immortalized murine podocytes. Futher, immunohistochemical staining confirmed that subunit $p47^{phox}$ is existence in podocytes, which is one subunit of the NADPH oxidase, and after C5b-9 assembly the subunit $p47^{phox}$ was observed translocation to the plasma membrane[21]. These prompt us that NADPH oxidase-mediated ROS production results in podocyte injury in MN.

In recent years, a close relationship between oxidative damage and mitochondrial dysfunctions has been established in Kidney injury[22]. The production of ROS was considered as an upstream step in oxidative damage to mitochondrial dysfunctions[23]. Oxidative damage can increase the tendency of mitochondria to release proapoptotic molecules(Cyt c) from the intermembrane space into the cytoplasm, then cause cell death through caspase-dependent and caspase-independent mechanisms[24]. According to Guan et al., mitochondrial fragmentation participated in podocyte injury in adriamycin nephropathy rats[25]. NOX4, a mitochondrial-localizing NADPH oxidase isoform. Sureshbabu A et al. study showed that in sepsis-induced AKI in mice, NOX4 result for the propagation of injury [26].

In our study, sheep anti-rat FX1A serum-induced PHN rats displayed obvious mitochondrial dysfunctions and mitochondrial ultrastructural injuries. Immunofluorescence demonstrated scattered granular deposits of IgG along the capillary walls. we also observed that in the Model rats, the ROS levels were elevated, the MMP levels were decreased, and the expression of Cyt c, Cleaved caspase-3 in cytoplasm were upregulated. Cyt c, which is usually found in the mitochondrial intermembrane space, indicating apoptosis caused by mitochondrial damage in MN rats.The apoptosis was further confirmed by the results of annexin-V-FITC/PI staining, that the expression of cell apoptosis in PHN rats produced high levels. As expected, treated with JQHF downregulating the level of intracellular ROS, upregulating the level of MMP and decreased the apoptosis rate from 45% to less than 15%. We also observed increased

expression of Nephrin protein, indicating podocyte damage was improved in the rats with JQHF treatment.

How did JQHF treatment protect against ROS-induced mitochondrial dysfunction in PHN rats. As we know, autophagy is the only mechanism which is via the lysosomal pathway to engulfed and recycled the aging and damaged organelles[27]. Classically, normal mitochondrial autophagy guarantees cells to engulfed and recycled the injuryed mitochondria to keep the balance of the mass and quantity of mitochondrial in intracellular, and maintain cellular homeostasis[28]. In intracellular, PTEN-induced kinase 1(PINK1) and Parkin are regarded as the mediators to selective degradation of the injury mitochondria by mitochondrial autophagy. Parkin was confirmed to mediate a mitophagy quality control pathway, and is only selectively bind to damaged mitochondria[29]. Recent studies have shown that the stability of PINK1 was enhanced by the process of mitochondrial depolarization, and PINK1/Parkin pathway plays an essential role in maintaining mitochondrial homeostasis by operating as an initiator of mitophagy[30]. A study confirmed that Mitophagy, dependent on PINK1 and Parkin, was activated in renal proximal tubular cells in acute kidney injury(AKI). Mice genetically short in PINK1 or Parkin, as well as deficient in both PINK1/Parkin were all susceptible to ischemic AKI[31]. Wen et al. reported that the activat of PINK1/Parkin pathway could individually ameliorate injured podocytes in diabetic nephropathy mice[32]. These results suggest that mitophagy mediated by PINK1/Parkin pathway plays an essential role in mitochondrial quality control, tubular and glomerular cell survival.

To objectively investigate the mitochondrial autophagy PHN rats, we used western blotting to determine the levels of the mitochondrial autophagy protein parkin and LC3B II. Next, we examined the location and expression of LC3B in glomerulus by Immunohistochemistry staining. The results confirmed that JQHF treatment increased the expression of the Parkin and LC3B II protein. Thus, our study suggested that via Parkin pathway reduced apoptosis was most likely the basis for JQHF ameliorating kidney injury in MN.

TET ia a bisbenzylisoquinoline alkaloid isolated from the Stephaniae tetrandrae radix[33]. Moreover, Stephaniae tetrandrae radix is one of the most active components in JQHF. Previous studies have confirmed that TET has definite effects on anti-hypertensive, anti-inflammatory, and anti-cognitive impairment[34-35]. A recent research have shown that, compared with valsartan, a low concentration of TET exhibited better effects in reduced the apoptosis mediated by TRPC6 in podocytes[36]. Our data indicated that TET treatment significantly upregulates the Parkin pathway and decrease the apoptosis in rats after 4 weeks compared with the Model group. Although it's not as effective as the compound of JQHF, we suggest that as a potential autophagy agonist for clinical treatment of MN, TET was deserved further study.

In conclusion, our results showed that both JQHF and TET treatment attenuates 24 hours urinary protein and prevents the progression of MN, while the ability to alleviates renal damages of JQHF was significantly better than those of TET. They may different degrees of regulation of the mitochondrial autophagy by the Parkin pathway, thereby reducing sheep anti-rat FX1A serum-induced apoptosis in rats.

This study provided evidence of the beneficial effects of JQHF and TET as effective therapeutic medication for MN.

Declarations

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Author Contributions

X.H.W., R.H.Y. and Z.Z.M. provided the conception and designed the experiments; X.H.W., R.L., Q.Z. and N.C. performed animals' experiments, biochemical parameters examination, westernblot analysis experiments, flow cytometry analysis, and pathological examination. Z.Z.M. and R.H.Y contributed to the discussion of results and completed the revision of manuscript; X.H.W. and R.L. analysed the data and wrote the manuscript; All authors reviewed the manuscript.

Additional Information

The authors declare no competing interests.

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Figures

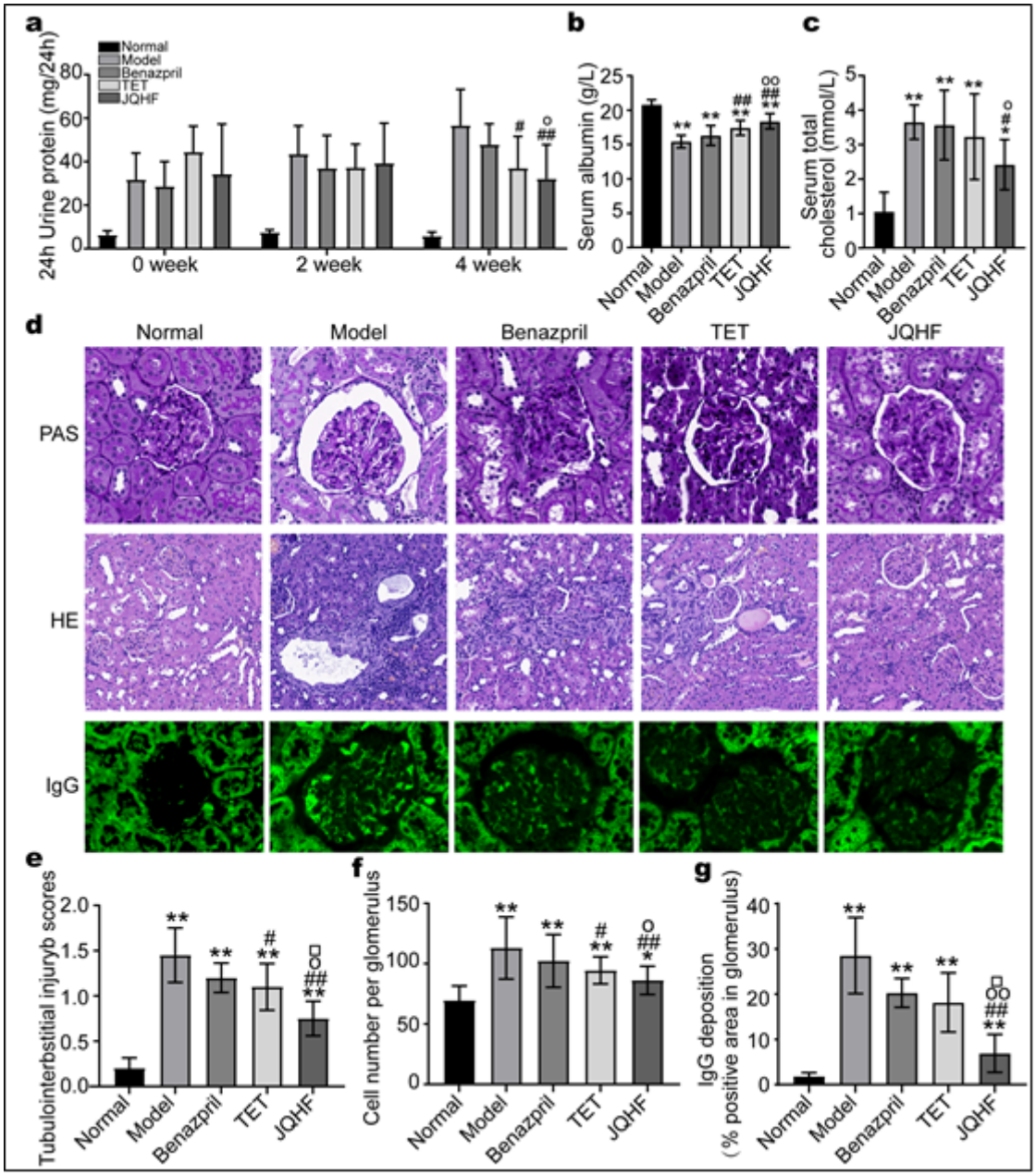

Figure 1

Effects of JQHF on biochemical parameters and renal pathology in PHN rats. (a)24h urinary protein. (b)ALB. (c)TC. (d)Representative microstructural images of diferent groups.(HE staining original magnifcation 100×; PAS and IgG staining, original magnifcation 400×). (e)Tubulointerstitial injury scores, (f) Glomerular hypercellularity. (g)The positive area of IgG deposition in glomerulus . Data were analysed by one-way ANOVA followed by post hoc LSD test(a,b,c,e,f and g) and presented as the mean±SD(n=6). *P < 0.05 vs. Normal group, **P < 0.01 vs. Normal group, #P < 0.05 vs. Model group, ##P < 0.01 vs. Model group, □P< 0.05 vs. Benazepril group and □□P< 0.01 vs. Benazepril group.

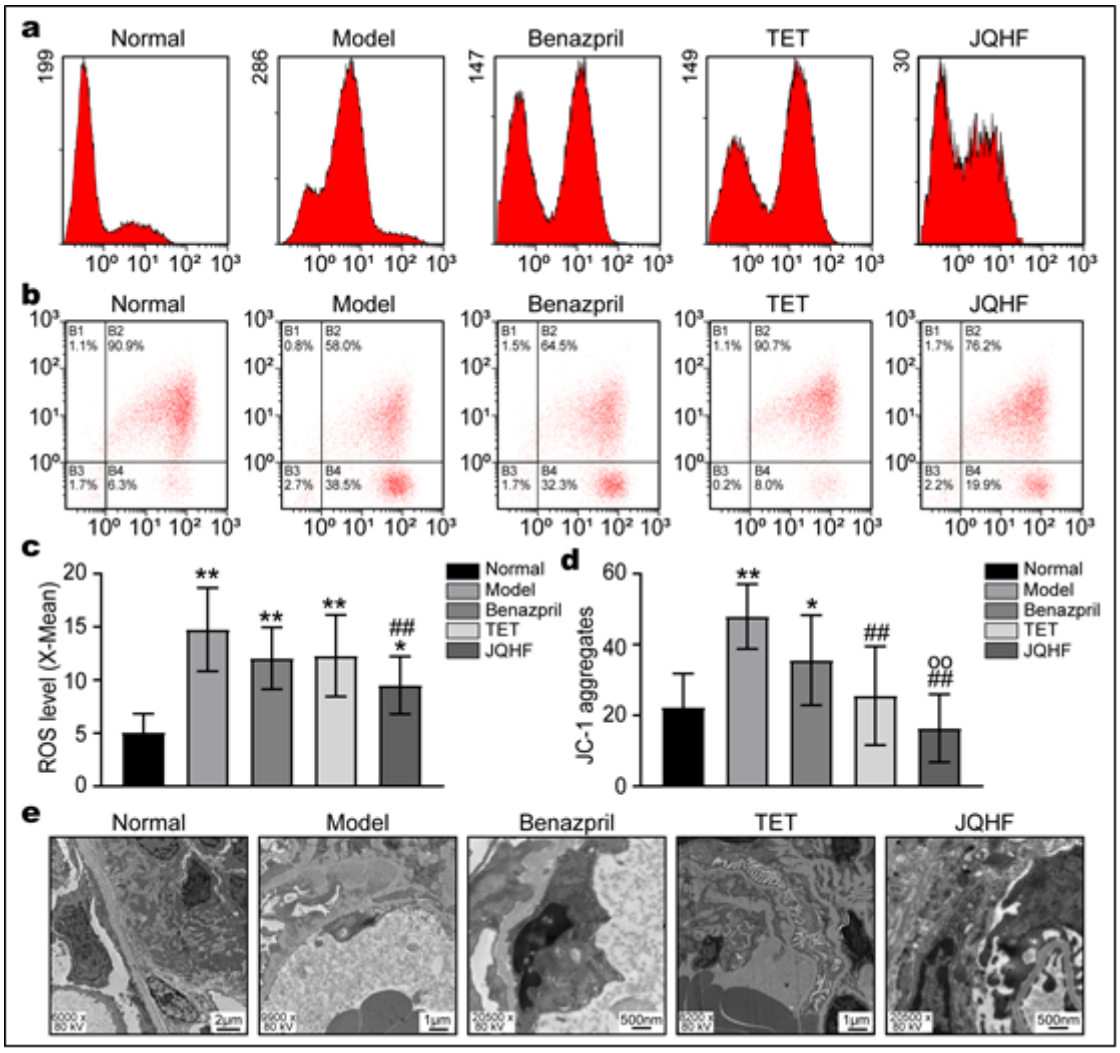

Figure 2

Effects of JQHF on against ROS-induced mitochondrial dysfunction in PHN rats. (a)Determination ROS production by DCFA staining. (b)Determination of MMP by JC-1 staining. (c)Quantitative analysis of intracellular ROS level. (d)Quantitative analysis of MMP. (e)Analyze mitochondrial ultrastructural injuries by TEM. Data were analysed by one-way ANOVA followed by post hoc LSD test(c and d) and presented as the mean±SD(n=6). *P < 0.05 vs. Normal group, **P < 0.01 vs. Normal group, ##P < 0.01 vs. Model group and □□P< 0.01 vs. Benazepril group.

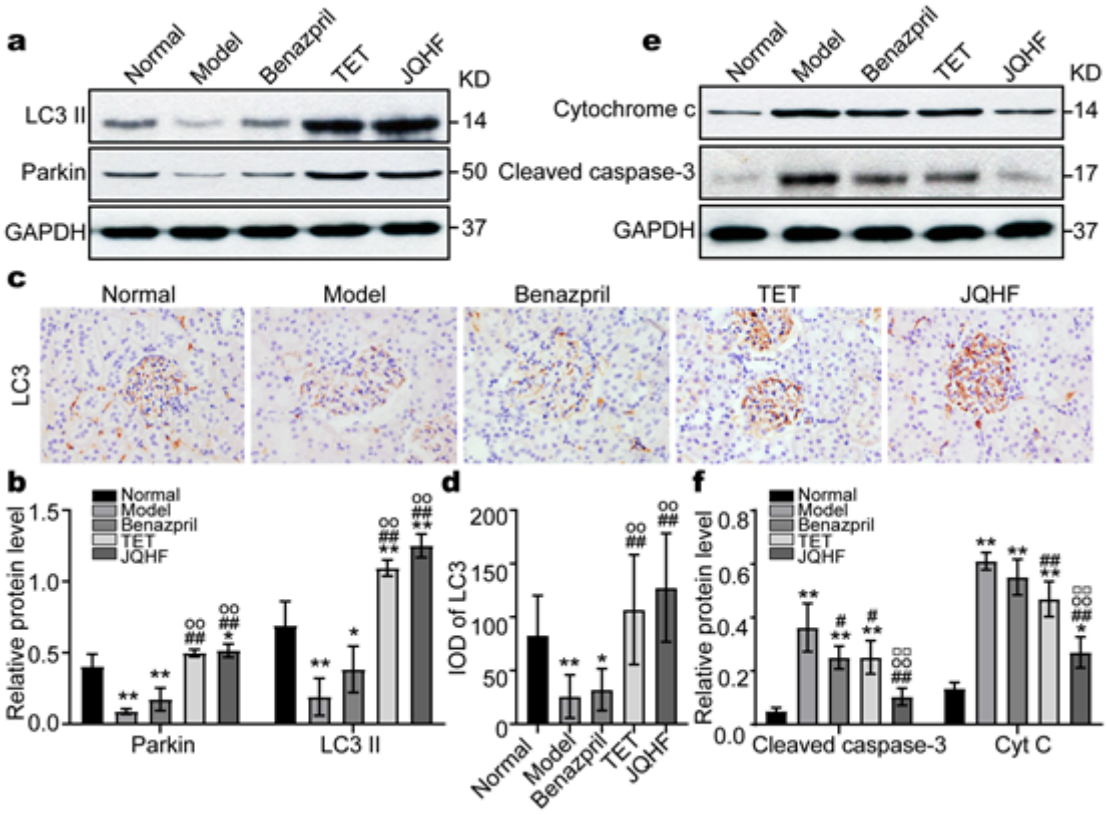

Figure 3

Effects of JQHF on LC3, Parkin, Cyt c and Cleaved caspase-3 protein expression in the kidneys of PHN rats. (a,e)Western blot analysis of LC3II, Parkin, Cyt c and Cleaved caspase-3 protein expression in each group. Full-length blots are presented in Supplementary Figure1,2. (b,f)Quantitative analysis of LC3II, Parkin, Cyt c and Cleaved caspase-3 protein expression. (c)Immunohistochemistry staining of LC3 protein (original magnifcation 400×). (d)Analysis of IOD of LC3. Data were analysed by one-way ANOVA followed by post hoc LSD test(b,d and f) and presented as the mean±SD(n=6). *P < 0.05 vs. Normal group, **P < 0.01 vs. Normal group, #P < 0.05 vs. Model group, ##P < 0.01 vs. Model group, ▯▯P< 0.01 vs. Benazepril group and □□P< 0.01 vs. TET group.

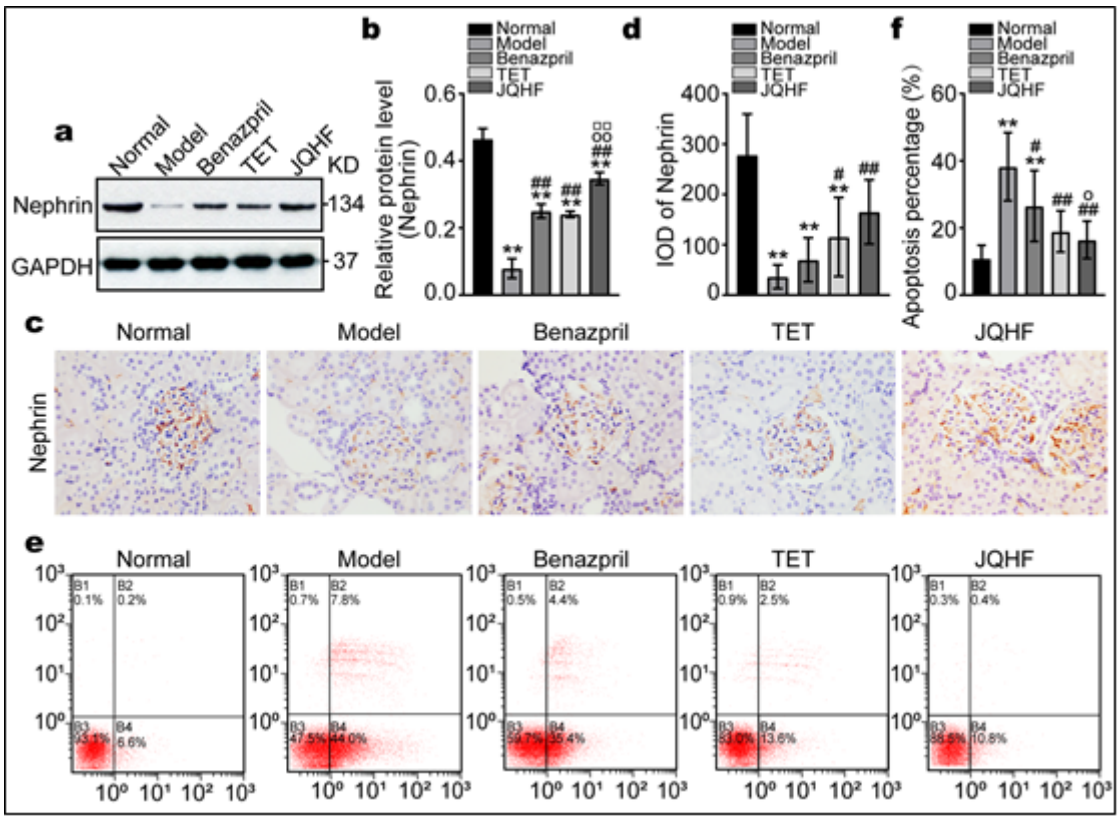

Figure 4

JQHF reduce the apoptosis in the kidneys of PHN rats. (a)Western blot analysis of the expression of Nephrin protein in each group. Full-length blots are presented in Supplementary Figure 3.(b)Quantitative analysis of the expression of Nephrin protein. (c)Immunohistochemistry staining of Nephrin protein (original magnifcation 400×). (d) Analysis of IOD of Nephrin.(e)Determination of apoptosis rate by Annevin-V-FITC staining.(f) Quantifcation of the apoptosis rate in each group. Data were analysed by one-way ANOVA followed by post hoc LSD test(b and d) and presented as the mean±SD(n=6). Data were analysed by Kruskal Wallis H test(f)(n=6). **P < 0.01 vs. Normal group, #P < 0.05 vs. Model group, ##P < 0.01 vs. Model group, ▯P< 0.05 vs. Benazepril group, ▯▯P< 0.01 vs. Benazepril group and □□P< 0.01 vs. TET group.

Supplementary Files

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- SupplementaryDataset1.doc
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