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Qufeng tongluo decoction decreased proteinuria in diabetic mice by protecting podocytes via promoting autophagy

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ABSTRACT

Background: Diabetic kidney disease (DKD) is one of diabetic complications, which has become the leading cause of end-stage kidney disease. In addition to angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker(ACEI/ARB) and sodium-glucose cotransporter-2 inhibitor (SGLT2i), traditional Chinese medicine (TCM) is an effective alternative treatment for DKD. In this study, the effect of Qufeng Tongluo (QFTL) decoction in decreasing proteinuria has been observed and its mechanism has been explored based on autophagy regulation in podocyte.

Methods: In vivo study, db/db mice were used as diabetes model and db/m mice as blank control. Db/db mice were treated with QFTL decoction, rapamycin, QFTL + 3-Methyladenine (3-MA), trehalose, chloroquine (CQ) and QFTL + CQ. Mice urinary albumin/creatinine (UACR), nephrin and autophagy related proteins (LC3 and p62) in kidney tissue were detected after intervention of 9 weeks. Transcriptomics was operated with the kidney tissue from model group and QFTL group. In vitro study, mouse podocyte clone-5 (MPC-5) cells were stimulated with hyperglycemic media (30 mmol/L glucose) or cultured with normal media. High-glucose-stimulated MPC-5 cells were treated with QFTL freeze-drying powder, rapamycin, CQ, trehalose, QFTL+3-MA and QFTL + CQ. Cytoskeletal actin, nephrin, ATG-5, ATG-7, Beclin-1, cathepsin L and cathepsin B were assessed. mRFP-GFP-LC3 was established by stubRFP-sensGFP-LC3 lentivirus transfection.

Results: QFTL decoction decreased the UACR and increased the nephrin level in kidney tissue and high-glucose-stimulated podocytes. Autophagy inhibitors, including 3-MA and chloroquine blocked the effects of QFTL decoction. Further study showed that QFTL decoction increased the LC3 expression and relieved p62 accumulation in podocytes of db/db mice. In high-glucose-stimulated MPC-5 cells, QFTL decoction rescued the inhibited LC3 and promoted the expression of ATG-5, ATG-7, and Beclin-1, while had no effect on the activity of cathepsin L and cathepsin B. Results of transcriptomics also showed that 51 autophagy related genes were regulated by QFTL decoction, including the genes of ATG10, SCOC, ATG4C, AMPK catalytic subunit, PI3K catalytic subunit, ATG3 and DRAM2.

Conclusion: QFTL decoction decreased proteinuria and protected podocytes in db/db mice by regulating autophagy.

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1. Introduction

List of abbreviations

DKD	Diabetic kidney disease
ACEI	Angiotensin-converting enzyme inhibitors
ARBs	Angiotension II receptor blockers
SGLT2is	Sodium-glucose cotransporter-2 inhibitors
TCM	Traditional Chinese medicine
ATG	Autophagy-related gene
UACR	urinary albumin creatinine ratio
SCOC	Short coiled-coil protein
AMPK	Adenosine monophosphate-activated protein kinase
PI3K	Phosphatidylinositol-3-kinase
DRAM	Damage-regulated autophagy modulator
CQ	Chloroquine
3 MA	3-Methyladenine
VPS-41	Vacuolar protein sorting-associated protein 41
AGE	Advanced glycation end-products

Diabetic kidney disease (DKD) is one of chronic complications of diabetes, manifested as edema, proteinuria, and injured renal function, and can develop into nephrotic syndrome or uremia. Latest data shows that DKD has become the leading cause of end-stage kidney disease,[1] which results in a heavy burden on patients and social health system. Unfortunately, there are not many effective drugs for DKD. Angiotensin-converting enzyme inhibitor/angiotensin II receptor blocker (ACEI/ARB) is one of the effective medicines for DKD. Losartan, a typical drug of ACEI/ARB, has the ability of decreasing urinal protein and protecting renal function. Data showed that it reduced the incidence of end-stage kidney disease by 28%, but had no effect on the rate of death. Moreover, ACEI/ARB has side effects of increasing serum potassium and decreasing glomerular filtration rate, and these side effects limit its application in DKD patients when their serum creatinine are higher than 265.2umol/L. Apart from ACEI/ARB, sodium glucose cotransporter-2 inhibitor (SGLT2i) can not only decrease blood glucose, but also protect kidney. Results of CREDENCE trial showed that the relative risk of end-stage kidney disease was lower by 32% in the canagliflozin group compared with the placebo group.[2] However, a real world study showed that 38.6% patients who took SGLT2i suffered side effects,[3] including urinary tract infection, non-hyperglycemic ketoacidosis, hyponatremia, and higher risk of amputation.[4–7] Therefore, although ACEI/ARB and SGLT2i can delay the progression of DKD, the side effects affect their application. Safer and more efficient treatments are needed.

Traditional Chinese medicine (TCM) is an important alternative treatment for DKD and widely used in China. It can be used alone or with modern medicines like ACEI/ARB and SGLT2i. A retrospective cohort study from Taiwan showed that the 8-year cumulative incidence of end-stage kidney disease and mortality among TCM users were 14.5% and 33.8%, respectively, while among TCM non-users, they were 16.6% and 49.2%.[8] Another retrospective cohort study demonstrated that the cumulative mortality rate in TCM cohort was 28%, significantly lower than that in the modern medicine cohort (48%), with a median follow-up duration of 2.49 years.[9] TCM drugs that are capable of dispelling wind pathogens, always named wind-medicines, are important in treating DKD. Extracts of some wind-medicines have been well studied in laboratories. For example, arctigenin has been found can attenuate proteinuria and podocyte injury via targeting phosphatase 2A and drebrin-1.[10] Triptolide, an extract of Tripterygium, can attenuate the epithelial-mesenchymal transition of renal epithelial cells through inhibiting the catalysis of PTENK27-polyUb.[11] QFTL decoction in this study has been developed from our clinical experience, which is made of mainly wind-medicines, consisting of Fructus Arctii (Niu Bang Zi), Rhizoma Dioscoreae Nipponicae (Chuan Shan Long), Caulis Sinomenii (Qing Feng Teng), Pheretima (Di Long) and Bombyx Batryticatus (Jiang Can). Pathogen wind, caused by pathogen heat, blood stasis and phlegm, lurking in the renal collaterals, is the key factor of massive proteinuria in DKD. In QFTL decoction, all ingredients have the function of dispelling wind. Additionally, Fructus Arctii (Niu Bang Zi) clears heat, Pheretima (Di Long) removes blood stasis and Bombyx Batryticatus (Jiang Can) dissolves phlegm. Importantly, Rhizoma Dioscoreae Nipponicae (Chuan Shan Long) and Caulis Sinomenii (Qing Feng Teng) are good at dispelling wind in collaterals. Our previous study has shown that QFTL decoction reduces renal fibrosis and proteinuria via inhibiting PI3K/Akt signaling.[12] Considering that PI3K/Akt signaling is an important upstream signaling of autophagy and podocyte injury is the main cause of proteinuria, the hypothesis has been proposed that QFTL decoction relieves podocyte injury by regulating autophagy.

Autophagy is a lysosome-dependent degradation mechanism of protein or organelle, which is important for cellular homeostasis.[13] Increasing studies have shown that autophagy disorder plays an important role in the development of DKD. In kidney tissue of STZ-induced diabetes model and DKD patients, accumulation of p62 has been observed,[14,15] indicating that the autophagy flux is suppressed. In this study, we intended to make sure the effect of QFTL decoction in protecting podocyte and explore its mechanism based on autophagy.

2. Materials and methods

2.1. Animals

Specific pathogen-free male db/db mice and db/m mice were purchased from Changzhou Cavens Laboratory Animal Co. Ltd (age 5–6 weeks, db/db mice weight about 40 g and db/m mice weight about 30 g, qualified number SCXK(SHU)2016–0010). Animals were housed in the animal house of Dongzhimen Hospital, with free access to water, under standard conditions (constant ambient temperature of 20–25◦C and humidity of 50–65% in a 12-h light/dark cycle). The study followed the national guidelines for laboratory animal welfare and was approved by the Animal Ethics Committee of Dongzhimen Hospital affiliated to Beijing University of Chinese Medicine (NO. 20-20). Pentobarbital sodium (3%, 1 mL/kg body weight) was used intraperitoneally for anesthesia.

2.2. Drugs and regents

QFTL decoction is a TCM compound formula for DKD, composed of Fructus Arctii (Niu Bang Zi), Rhizoma Dioscoreae Nipponicae (Chuan Shan Long), Caulis Sinomenii (Qing Feng Teng), Pheretima (Di Long) and Bombyx Batryticatus (Jiang Can). The TCM drugs were provided by Dongzhimen hospital affiliated to Beijing University of Chinese Medicine. Rapamycin (Cat. NO. R-5000) was purchased from LC laboratory. Trehalose (CT 11312) was purchased from Coollaber. Chloroquine (CQ) (Cat. NO. C6626) and 3-Methyladenine (3-MA) (Cat. NO. M9281) was purchased from Sigma.

2.3. Preparation of QFTL decoction

One dose QFTL decoction for a human with 70 Kg body weight contains Fructus Arctii (Niu Bang Zi) 15 g, Rhizoma Dioscoreae Nipponicae (Chuan Shan Long) 30 g, Caulis Sinomenii (Qing Feng Teng) 15 g, Pheretima (Di Long) 12 g and Bombyx Batryticatus (Jiang Can) 12 g. The mouse dose was 9.1 times the human dose (per unit of body weight)[16] and the volume of drug solution and vehicle was 0.1 mL/10 g body weight. Therefore, the concentration of QFTL decoction for mice was 1.092 g/mL (Niu Bang Zi 0.195 g/mL, Chuan Shan Long 0.39 g/mL,

Qing Feng Teng 0.195 g/mL, Di Long 0.156 g/mL and Jiang Can 0.156 g/mL).

All crude drugs were soaked for 30 min in 500 mL distilled water and decocted for another 30 min. Then medical solution was separated with the crude drugs after filtration. The crude drugs were decocted again with 300 mL distilled water, which also took 30 min. The medical solution got from the second decoction was mixed with the former, and then was concentrated into 77 mL to make the concentration be 1.092 g/mL.

The QFTL decoction for the in vitro study was made into powder by vacuum freeze-drying. The process was described briefly as follows. Medicines were decocted as the protocol described above, and then the medical solution was concentrated into 10 ml and evenly distributed into 10 petri dishes (25 cm^2). After frozen in −80 °C refrigerator overnight, they were put into the freeze dryer. The freeze-dried powder was collected after vacuum freeze-drying and stored in −20 °C.

2.4. Experimental groups and treatment

Based on the blood glucose, all db/db mice (Blood glucose: 25.05 ± 5.11 mmol/L) were allocated into five groups (model, QFTL, rapamycin, QFTL + 3-MA, trehalose, CQ and QFTL + CQ) (n = 10), and urinary albumin was balanced among groups (db/db mice) before intervention. Db/m mice (Blood glucose: 8.65 ± 1.29 mmol/L) were used as the blank group (n = 10). The mice in the blank group and model group were given distilled water by gavage and normal saline by intraperitoneal injection. Mice in QFTL group were treated with prepared QFTL by gavage and normal saline by intraperitoneal injection. Mice in other groups were treated with corresponding reagents (rapamycin 2 mg/kg/d[17], 3-MA 10 mg/kg/d[18], chloroquine 10 mg/kg/d[19], i. p.). The volume of drug solution and vehicle was 0.1 mL/10 g body weight and the intervention period was 9 weeks.

2.5. In vitro study

MPC-5 cell line was provided by BeNa Culture Collection (BNCC342021) and cultured following the protocol described before.[20] Hyperglycemic condition was established by the media containing 30 mmol/L glucose and the media containing 5.5 mmol/L glucose was used as control. The concentration of QFTL decoction was 20 μg/mL. Rapamycin (200 nmol/L) was used as autophagy activator, 3-MA (3 mmol/L) and chloroquine (20 mmol/L)[21] as autophagy inhibitors, and trehalose (20 mmol/L) as autophagy inducer. Fully differentiated cells were treated by the reagents mentioned above for 48 h.

2.6. Test of urinary albumin/creatinine (UACR)

6-h urine was taken and centrifuged at a speed of 1000 rpm for 10 min. The supernatant was used to detect urinary albumin with an ELISA kit (abcam, ab207620) and urinary creatinine was detected by sarcosine oxidase method (Nanjing jiancheng bioengineering institute, C011-2-1).

2.7. Western blot

Western blot was done as described previously.[16] The primary antibodies for nephrin (ab235903, 1:500), ATG5 (ab108327, 1:1000), Beclin-1 (ab217179, 1:1000), ATG7 (ab133528, 1:500), p62(ab109012, 1:10,000), LC3(ab128025, 1:1000)β-actin (ab8227, 1:5000) and HRP-conjugated secondary goat antibodies (Proteintech, SA00001-1 and SA00001-2, 1:5000) were used in this study. Western blots were analyzed by Image J software.

2.8. Histopathology staining

The kidney tissue was fixed in 4% paraformaldehyde for 24 h and was embedded in paraffin after gradient alcohol dehydration, xylene vitrification, and waxdip. Blocks were cut into 3 mm sections and were used in immunohistochemistry staining and immunofluorescence staining. Immunohistochemistry staining and immunofluorescence staining was done as described previously.[16] The primary antibody for nephrin (ab235903, 1:100) was used in immunohistochemistry staining, and LC3B (ab192890, 1:2000) and p62 (ab109012, 1ug/ml) were used in immunofluorescence staining. The mean IOD value (nephrin) of each glomerulus was calculated, and if the image has several glomeruli, the average mean IOD value will be treated as the nephrin positive expression of this image. 5–7 images were taken and quantified in each mouse to detect the expression level of nephrin. Images were quantified by the image-pro plus 6.0 software.

The immunofluorescence staining of cell culture was done after intervention as described previously.[20] The primary antibody for ATG5 (ab108327, 1:100), ATG7 (ab133528, 1:300), Beclin-1 (ab217179, 1:200) and donkey anti-mouse (Invitrogen, A-21203, 1:1000) or anti-rabbit (Invitrogen, A-21206, 1:1000) IgG (H + L) Highly Cross-Adsorbed secondary antibody was used in this study. The procedure of phalloidin staining was the same as immunofluorescence staining before secondary antibody incubation. Fluorescein isothiocyanate labeled phalloidin (sigma, p5282, 1ug/mL) was used instead of primary antibody.

2.9. mRFP-GFP-LC3

mRFP-GFP-LC3 was used to evaluate autophagic flux. The stubRFP-sensGFP-LC3 lentivirus[22] was provided by Genechem (GV374). The lentiviral transfection was operated depending on the protocol from Genechem. The multiplicity of infection (MOI) was five and puromycin (gibco, A11138-03) was used at a concentration of 2 μg/ml based on the results of pretest.

2.10. Transcriptomics

Transcriptomics was conducted by Magigene (Guangdong, China).[23] Total RNA integrity was assessed by the Agilent 4200 biological analyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with A260/280 ≥ 1.5 and RNA integrity number ≥6.5 were used for RNA-Seq. The complementary DNA libraries were sequenced on the Illumina NovaSeq 6000 platform. The differential expression was analyzed using the software DESeq2. P ≤ 0.05, fold-change ≥1, and FDR ≤0.05 were set as the thresholds for significant differential expression.

2.11. Statistical analysis

Statistical tests were performed using SPSS 20.0. All data were showed as mean ± standard error of mean. Normally distributed data were analyzed using independent samples student's t-test or one-way ANOVA (Student-Newman-Keuls test was chosen for the multiple comparison of equal variances assumed data and Dunnett's T3 test for the multiple comparison of equal variances not assumed data). Non-normal distributed data were analyzed by nonparametric tests (two independent sample Wilcoxon tests). Cohen's d was calculated using JASP 0.18.1 software. Statistical differences were considered as significant if P value was less than 0.05.

3. Results

QFTL decoction relieved proteinuria and protected podocytes in db/db mice and high glucose condition, which was weakened by 3-MA and chloroquine.

In this study, urinary albumin among groups was balanced before intervention. After an intervention of 9 weeks, urinary albumin and creatinine were detected. Results of UACR showed that db/db mice in the model group had significant higher UACR than those in the blank group (db/m mice) (p = 0.016, Cohen's d = −1.464), and QFTL

decoction decreased the UACR (p = 0.054, Cohen's d = 0.939) (Fig. 1a). Further, the QFTL decoction increased the level of nephrin in db/db mice (WB:p = 0.001, Cohen's d = −2.506; IHC:p = 0.009, Cohen's d = −2.654) (Fig. 1b and c) and high-glucose-stimulated MPC-5 cells (p = 0.01, Cohen's d = −3.811) (Fig. 1d). Results of phalloidin staining (p5282, sigma) showed that high glucose culture decreased the expression of cytoskeletal actin, which was rescued by QFTL decoction (Fig. 1e). Notably, the effects of QFTL decoction described above were attenuated by autophagy inhibitors (3-MA and chloroquine) and autophagy activator rapamycin got a similar effect as QFTL decoction did, indicating that QFTL decoction might protect podocytes through regulating autophagy.

3.1. QFTL decoction improved autophagy flux of podocyte

LC3 and p62 proteins are the most widely used markers for autophagy flux. To make sure if QFTL decoction regulated autophagy flux, high-glucose-stimulated podocytes has been transfected with stubRFP-sensGFP-LC3 lentivirus. Results showed that yellow puncta decreased after high glucose stimulation and rescued by QFTL decoction as well as rapamycin, but not trehalose. Puncta decreased when co-intervened with 3-MA compared with those treated with QFTL decoction alone (Fig. 2). In vivo study, synaptopodin was used as the marker of podocytes. LC3 and p62 puncta co-localized with synaptopodin were treated as LC3 and p62 expressed in podocytes, respectively. Immunofluorescence staining results showed that LC3 puncta in podocytes of db/db mice were significantly lower than those in db/m mice (p = 0.033, Cohen's d = −1.36), and QFTL decoction improved the expression of LC3 (p = 0.023, Cohen's d = −1.334). Autophagy-improving effect of QFTL decoction was blocked by 3-MA (p = 0.198, Cohen's d = −0.703) (Fig. 3a). P62 is the protein linking ubiquitinated substrates and LC3. Decreased p62 levels are associated with autophagy activation. In this study, p62 protein accumulated in the podocyte in db/db mice compared with that in db/m mice (p = 0.004, Cohen's d = 1.719), and QFTL decoction relived the accumulation of p62 (p = 0.032, Cohen's d = 1.194). When db/db mice were treated with both QFTL decoction and the lysosomal inhibitor, chloroquine, p62 accumulation happened as that in db/db mice treated with vehicle (p = 0.89, Cohen's d = −0.087) (Fig. 3b). Consistently, WB results showed the similar trend, and chloroquine (inhibitor of autophagosome degradation) couldn't regulate the LC3 and p62 as QFTL did (Fig. 3c), indicating that the effect of QFTL decoction on LC3 and p62 via promoting autophagy but not blocking the degradation of autophagosome. All these results indicated that QFTL decoction promoted autophagy flux of podocyte.

3.2. QFTL decoction activated the formation of autophagosomes in high-glucose-stimulated podocytes

In this study, the proteins ATG-5, ATG-7, and Beclin-1 in high-glucose-stimulated podocytes were detected by Western blot. Results showed that the level of ATG-5, ATG-7, and Beclin-1 in podocytes was lower after cultured in high glucose media (p = 0.028, 0.007, 0.013; Cohen's d = −1.807, −6.102, −6.045) and QFTL decoction increased their expression (p = 0.037, 0.014, 0.018; Cohen's d = −0.834, −4.333,

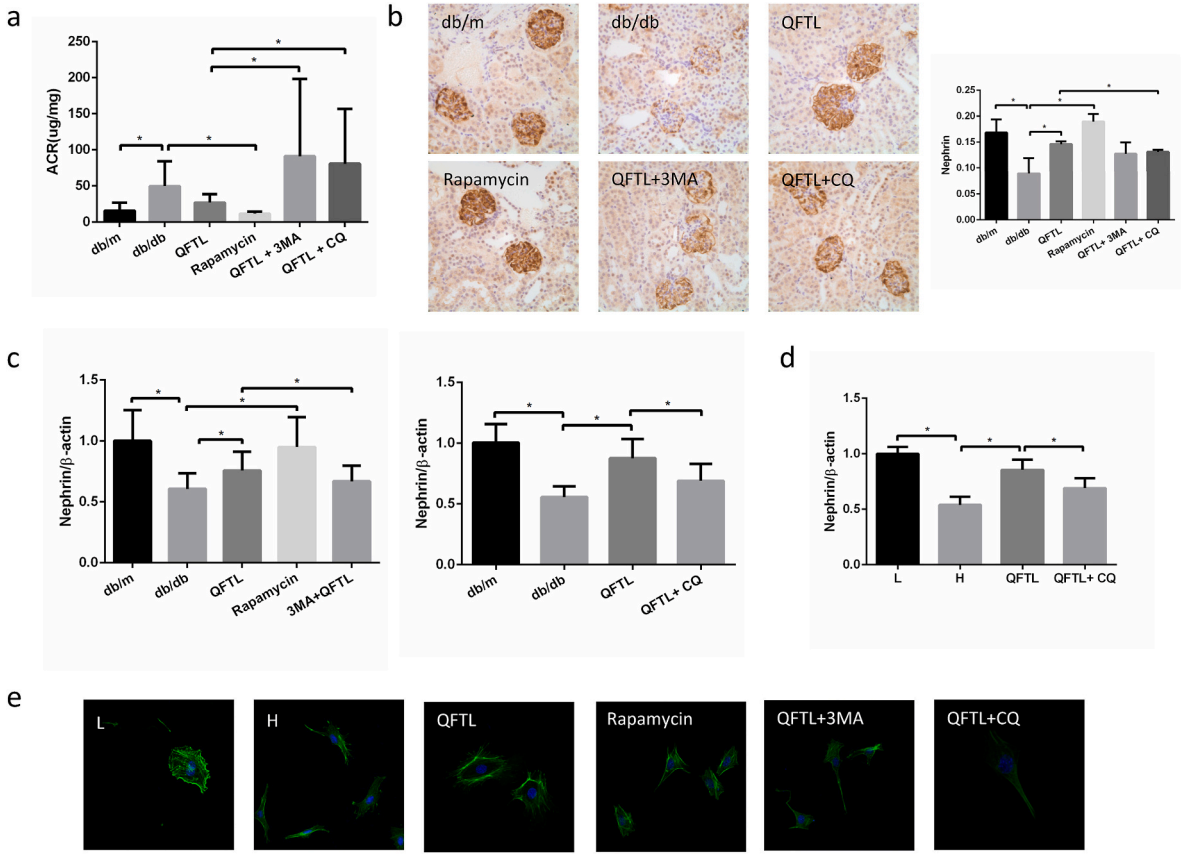

Fig. 1. QFTL decoction relieved proteinuria and protected the podocyte. (a)UACR (n = 6–9) of db/db mice was relieved by QFTL decoction and autophagy activator rapamycin. Autophagy inhibitors including 3-MA and chloroquine blunted the effect of QFTL decoction. (b)IHC results (n = 4) showed that db/db mice had less nephrin compared with db/m mice, and QFTL decoction and rapamycin rescued the inhibited nephrin expression. 3-MA and chloroquine blunted the effect of QFTL decoction. (c-d) Similar WB results of nephrin were observed in kidney tissue (n = 6) and MPC-5 cell lines (n = 3). (e)Phalloidin staining results showed that QFTL decoction could protect the cytoskeleton of high-glucose-simulated MCP-5 cells as well as rapamycin, and autophagy inhibitors blunted the effect of QFTL decoction. UACR, urinary albumin creatinine ratio; 3-MA, 3-Methyladenine; CQ, chloroquine. *, p ≤ 0.05.

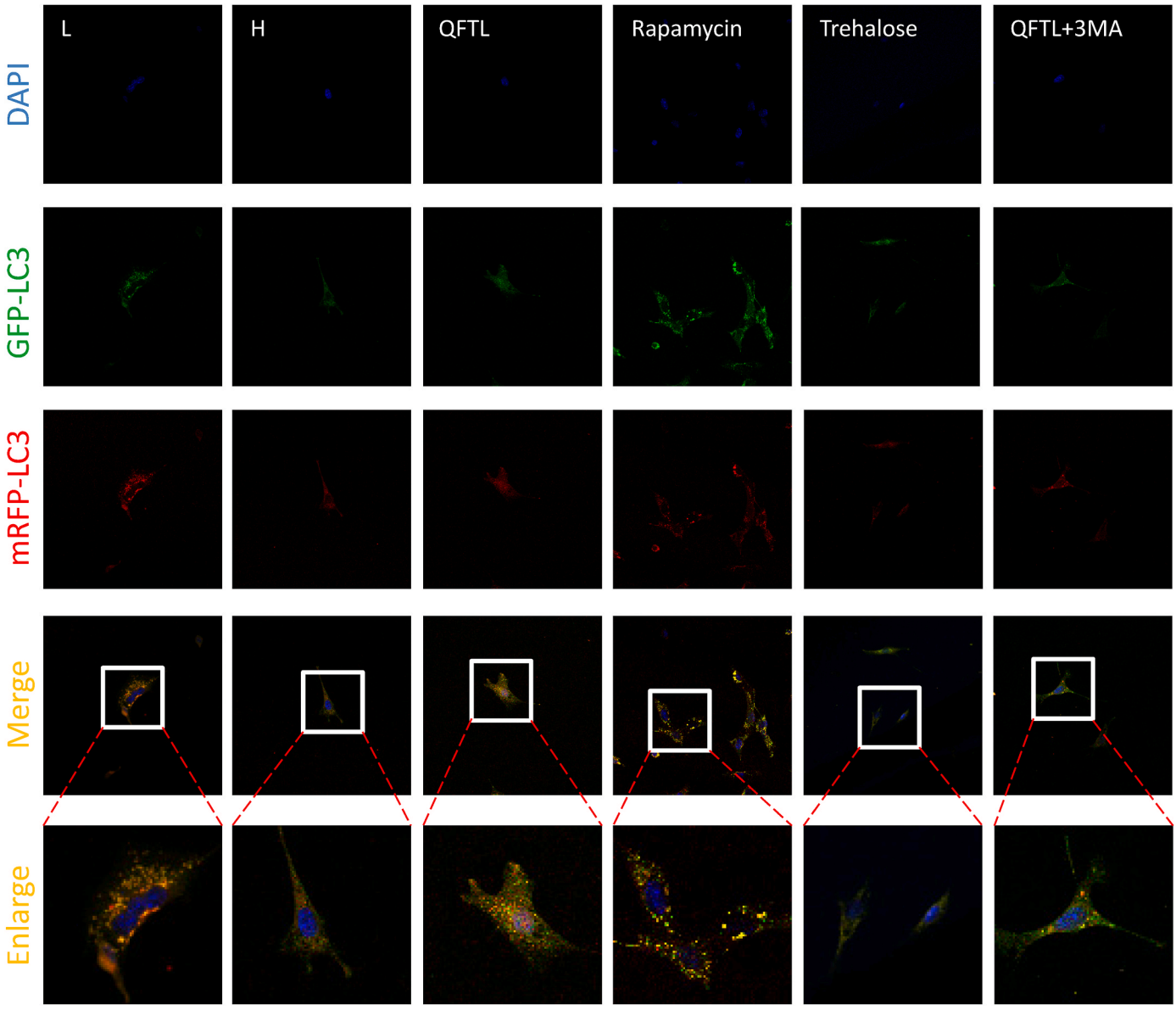

Fig. 2. StubRFP-sensGFP-LC3 lentivirus transfection in high-glucose-stimulated podocytes. Yellow puncta decreased after high glucose stimulation and rescued by QFTL decoction as well as rapamycin, but not trehalose. Puncta decreased when co-intervened with 3-MA compared with those treated with QFTL decoction alone.

−4.794) but chloroquine (inhibitor of autophagosome degradation) did not (Fig. 4a). In order to assess lysosome function, the cathepsins' activity of lysosome was detected by ELISA kits. Results showed that QFTL decoction failed to increase the activity of cathepsin L ($p = 0.507$, Cohen's $d = -0.932$) and cathepsin B ($p = 0.246$, Cohen's $d = 1.155$) (Fig. 4b).

3.3. QFTL decoction regulated autophagy related genes transcription

Results of transcriptomics (Fig. 4c) showed that 51 autophagy related genes were regulated by QFTL decoction significantly, compared with db/db mice treated with vehicle. The genes of SCOC, ATG-10, ATG-4C, ATG-3, and DRAM-2, which play roles in the initiation of autophagy, were upregulated by QFTL decoction. Some key genes belonging to upstream signaling of autophagy, including PI3K/Akt and AMPK signaling, were also regulated. Notably, QFTL decoction activated the transcription of VPS-41 gene, which was required for fusion of autophagosomes with lysosomes.

4. Discussion

In addition to the western medicines, DKD patients in China are also treated with TCM, including QFTL decoction. QFTL decoction is composed of Fructus Arctii (Niu Bang Zi), Rhizoma Dioscoreae Nipponicae (Chuan Shan Long), Caulis Sinomenii (Qing Feng Teng), Pheretima (Di Long) and Bombyx Batryticatus (Jiang Can). In TCM theory, these five medicines have the function of dispelling wind pathogen, which has been considered a key factor causing proteinuria, especially in IgA nephritis. For DKD, blood stasis and kidney qi deficiency are often treated as the reason for proteinuria. However, lab studies have shown that some of wind-medicines, including Fructus Arctii,[10] Caulis Sinomenii,[24] Bombyx Batryticatus,[25] and Pheretima,[26] have anti-inflammation functions, while chronic inflammation has been proved to be an important mechanism for DKD development.[27,28] Based on this cognition, we propose that pathogen wind is another cause of proteinuria in DKD and clinical practice has shown that addition of wind-medicines can improve the effect of TCM formula. In our previous

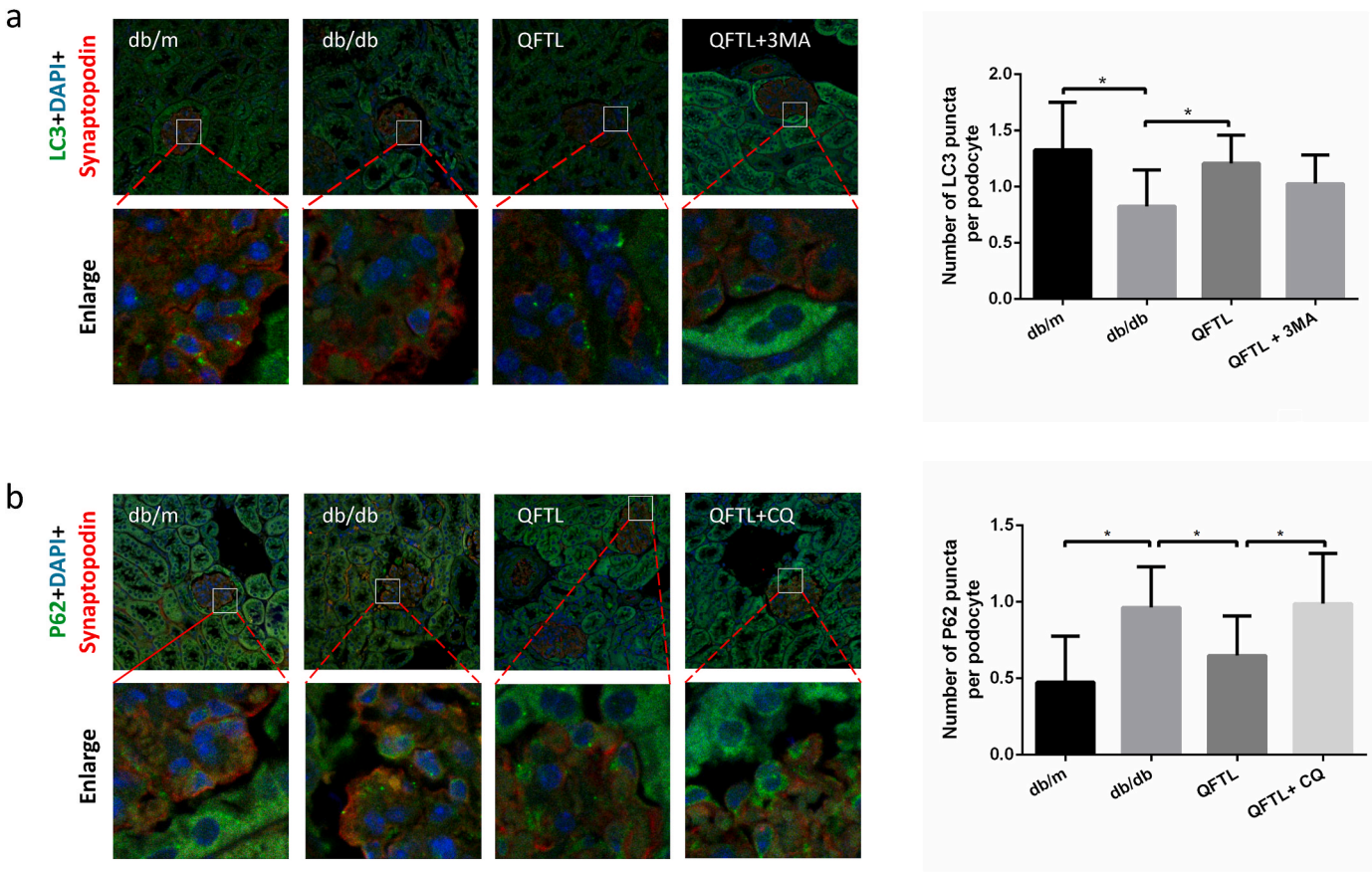

Fig. 3. LC3 and p62 expression in podocytes of db/db mice. (a) Immunofluorescence staining (n = 6–8) showed that fewer LC3 puncta were observed in db/db mice and QFTL decoction could increase LC3 expression. 3-MA blunted the effect of QFTL decoction. (b) p62 accumulation (n = 6–8) in podocytes was observed in db/db mice and was relieved by QFTL decoction. Chloroquine blunted the effect of QFTL decoction in relieving p62 accumulation. (c) Western blot results (n = 6) showed a similar trend of LC3 and p62, and chloroquine couldn't regulate them as QFTL did. *, $p \leq 0.05$.

study, QFTL decoction reduced the 24 h urinary protein and alleviated renal fibrosis in GK rats.[12] In this study, similarly, QFTL decoction decreased the UACR in db/db mice. Moreover, it also showed the effect of rescuing the declined nephrin, important marker of podocytes,[29] in db/db mice, and protect the skeleton structure of high glucose-stimulated MCP-5 cells, while these effects were lost when QFTL decoction was used together with autophagy inhibitors like 3-MA and chloroquine, indicating that the function of protecting podocyte might be associated with autophagy regulation. In the GK rats, PI3K/Akt signaling was inhibited by QFTL decoction, which has been considered as an important upstream signaling of autophagy, also indicating that QFTL decoction may regulate autophagy.

In this study, p62 accumulation happened in db/db mice, which was relieved by QFTL decoction. In addition, LC3, a marker of autophagosome, decreased in db/db mice and was upregulated by QFTL decoction. Consistently, results of mRFP-GFP-LC3 in the high-glucose-stimulated MPC-5 cells also showed that QFTL decoction promoted the autophagy flux. All these results proved that QFTL decoction promoted autophagy in podocytes. The autophagy flux can be divided into two processes, the formation and degradation of autophagosome. Blockage happening in any process can inhibit the autophagy flux and cause the accumulation of damaged organelles and ubiquitinated proteins. ATG proteins orchestrate and mediate the formation of the autophagosome. Podocytes stimulated with serum from DKD model (OLETF rats) had a lower level of LC3 compared with normal cultured podocytes, and selectively ATG-5 knockdown in the podocyte of diabetes rats resulted in podocyte foot process fusion and massive proteinuria.[30] In this study, ATG-5, ATG-7, and Beclin-1 was detected to make sure if QFTL decoction can promote the formation of autophagosome. Results demonstrated that QFTL decoction increased the level of ATG-5, ATG-7, and Beclin-1 in high-glucose-stimulated podocytes, while chloroquine didn't, meaning that the formation of autophagosome was activated and this was consisted with the results of transcriptomics. In the process of autophagosome degradation, cathepsins in lysosome play a key role. One study showed that the activity of cathepsin B and cathepsin L were deficiency in advanced glycation end-products stimulated renal tubular cells.[31] In this study, the activity of cathepsin L decreased in high-glucose-stimulated podocytes, while the activity of cathepsin B did not, and QFTL decoction had no effect on the activity of both cathepsin B and cathepsin L. However, transcriptomics results showed that QFTL decoction upregulated the expression of VPS-41, indicating QFTL decoction might promote autophagy not only by activating the formation of autophagosomes, but also by modulating the fusion of autophagosome and lysosome, but still needs further studies to clarify. Collectively, QFTL decoction promoted autophagy in the podocyte mainly by activating autophagosome formation.

Notably, peer studies have shown that trehalose is an autophagy promoter. However, in this study, trehalose failed to improve autophagy flux and increase the ATG-5, ATG-7 and beclin-1 in high-glucose-induced podocyte. According to the results of peer studies, trehalose induces autophagy by causing low-grade lysosomal stress[32] or limited lysosomal damage,[33] while high glucose inhibits the autophagy in podocyte via AKT/mTOR signaling[34] and AGE-induced blockade of the autophagic flux due to lysosomal dysfunction.[35] This may be the reason why trehalose failed to induce autophagy in the high-glucose-induced podocyte.

There are large number of chemical continents in QFTL decoction. It is worthy to make sure the exact chemical continents that have the

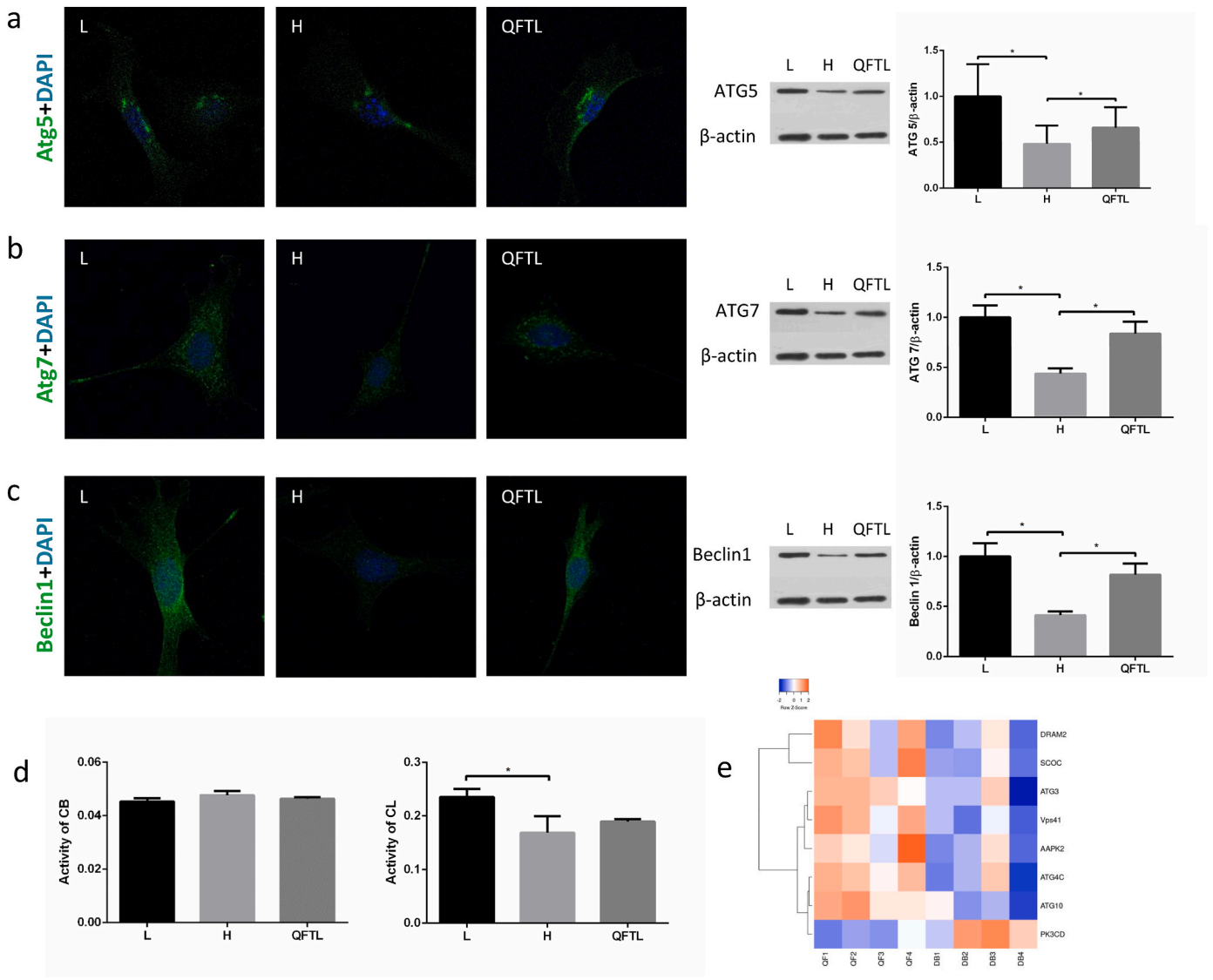

Fig. 4. QFTL decoction promoted the formation of autophagosomes in high-glucose-stimulated podocytes. (a) Immunofluorescence staining images and WB results (n = 3) showed that ATG-5 was inhibited by high-glucose intervention and could be rescued by QFTL decoction. Chloroquine didn't increase ATG-5 as QFTL did. (b-c) Similar results (n = 3) were observed for ATG-7 and beclin-1. Chloroquine didn't increase ATG-7 and Beclin-1 as QFTL did. (d) The activity of cathepsin L and cathepsin B in MCP-5 was detected by ELISA kit. Results (n = 3) showed that QFTL decoction had no significant effect on the activity of cathepsin L and cathepsin B. (e) Results of transcriptomics (n = 4) showed that genes of SCOC, ATG-10, ATG-4C, ATG-3, DRAM-2, VPS-41, PK3CD, and AAPK2 were regulated by QFTL decoction. L, low-glucose; H, high-glucose; CB, cathepsin B; CL, cathepsin L. *, $p \leq 0.05$.

function of activating the autophagy. Arctigenin, which is the continents of Fructus Arctii (Niubangzi), has been proved that can improve the autophagy of neurocytes in streptozotocin-induced diabetic mice.[36] Diosgenin from Rhizoma Dioscoreae Nipponicae (Chuan Shan Long) can also improve the autophagy and mitophagy in DN rat and high-glucose-induced HK-2 cells.[37] Several studies showed that sinomenine was also an activator of autophagy by inhibiting PI3K/AKT/mTOR pathway.[38,39] These continents may be responsible for the autophagy-activating function of QFTL decoction.

This study showed QFTL decoction was effectively decreased the UACR and protected the podocyte in DKD mice model via promoting autophagy. However, the mechanism of promoting autophagy is still not clear and need further study. The active chemical continents of QFTL decoction are also need pharmacological study. Moreover, clinical study is needed to make sure the efficacy and safety of QFTL decoction in treating DKD.

5. Conclusions

In conclusion, QFTL decoction can be an effective alternative medicine for DKD, especially for proteinuria. It protects the podocyte via promoting the autophagy flux in DKD.

Ethics approval and consent to participate

The study followed the national guidelines for laboratory animal welfare and was approved by the Animal Ethics Committee of Dongzhimen Hospital affiliated to Beijing University of Chinese Medicine (NO. 20-20).

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding authors on reasonable request.

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Authors' contributions

Boran Ni and Weijun Huang analyzed the data and wrote the original draft. Weijing Liu, Shidong Wang and Jinxi Zhao reviewed and edited the draft. Boran Ni, Yao Xiao, Ruojun Wei and Weijun Huang completed the animial study. Boran Ni, Liwei Zhu, Yifan Liu, Zhichao Ruan and Jiamu Li carried out the cell study. WeiJun Huang, Shidong Wang and Jinxi Zhao designed and supervised the study. WeiJun Huang and Jinxi Zhao acquired the funds.

Declaration of competing interest

The authors declare that they have no competing interests.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2023.11.007.

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