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Quercetin suppresses glomerulosclerosis and TGF-β signaling in a rat model

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Abstract. The transforming growth factor-β (TGF-β) signaling pathway is an important regulatory pathway in renal fibrosis and is abnormally activated in glomerulosclerosis. Quercetin is a common Chinese herbal medicine and has been reported to inhibit TGF-β signaling pathway activation. In the present study a glomerulosclerosis rat model was constructed and mice were treated with different concentrations of quercetin. Biochemical parameters, pathological indices and expression levels of TGF-β signaling pathway-associated proteins were detected using immunohistochemistry and western blotting. It was demonstrated that quercetin significantly improved physiological indices and altered the expression levels of TGF-β signaling pathway-associated proteins in rats with glomerulosclerosis. In conclusion, quercetin can regulate the TGF-β signaling pathway and reduce the progression of glomerulosclerosis.

Introduction

Glomerulosclerosis (GS) is the final pathway leading to the loss of renal function caused by a phenotypic transition of mesangial cells and an increase in extracellular matrix formation (1). The lesions are characterized by a loss of podocytes (2,3) and an accumulation of extracellular matrix (4). The effect of these etiological factors manifest as changes to glomerular mechanics (including high glomerular filtration and pressure), metabolism (such as diabetes) and a variety of regulatory molecules (such as cytokines) (5-7). Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates cell proliferation, differentiation and apoptosis. It has been previously demonstrated that TGF-β can promote renal fibrosis by stimulating the synthesis and inhibiting the degradation of the extracellular matrix (8). Furthermore, TGF-β can induce podocyte apoptosis by activating the mothers against decapentaplegic homolog 9 (Smad) signaling pathway (9). Together, these results suggest that TGF-β is an important pathogenic factor involved in the pathogenesis of GS. Thus, the treatment of GS with TGF-β is currently being investigated.

Quercetin (QU) is a natural flavonoid that is present in various flowers, and leaves and fruits of plants (10). Recent pharmacological studies have demonstrated that QU exerts strong anti-tumor, anti-oxidant, anti-fibrosis and anti-viral effects by regulating the activity of multiple signaling pathways (11,12). Another study suggested that QU inhibits the proliferation and invasion of prostate tumors by preventing TGF-β-induced epithelial mesenchymal transition (EMT) (13). EMT is a complex biological process in which epithelial cells are transformed into interstitial phenotypes with the loss of specific endothelial cell markers, including mesenchymal phenotype initiating expression of fibroblast-specific protein-1 (FSP-1) and α-smooth muscle actin (α-SMA). In addition, QU reduces the expression of TGF-β to inhibit renal interstitial fibrosis (14). Based on these results, QU may alleviate GS by inhibiting the TGF-β signaling pathway.

In the present study, the mechanism and feasibility of QU treatment for GS were examined by evaluating the effects of QU on physiological parameters of GS mice and associated proteins in the TGF-β signaling pathway.

Materials and methods

Animal model. A total of 60 male, 6-8 week old Sprague-Dawley rats, weighing 220±20 g, were purchased from the Experimental Animal Center of Gansu University of Chinese Medicine (Lanzhou, China). The protocol was approved prior to commencing this study by the animal ethics committee of Gansu University of Chinese Medicine. All rats were housed under standard conditions (12/12 light/dark cycle,

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Abbreviations: TGF-β, transforming growth factor-β; GS, glomerulosclerosis; QU, quercetin; EMT, epithelial mesenchymal transition; FSP-1, fibroblast-specific protein-1; α-SMA, α-smooth muscle actin; ADR, adriamycin; SHO, sham operation group; UTP, urine total protein; TP, total protein; Abl, albumin; TG, triglyceride; TC, total cholesterol; BUN, urea nitrogen; SCr, serum creatinine; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff base; TBST, Tris-buffered saline containing Tween-20

Key words: quercetin, glomerulosclerosis, transforming growth factor-β signaling pathway

40% humidity and 21-25˚C) and fed a standard rat diet. The rats were randomized into three groups: Control group (NC; n=10), sham operation group (SHO; n=10) and GS model group (GS; n=40). The GS group was prepared by uninephrectomy of the left kidney. Briefly, rats were anesthetized by intraperitoneal injection of 30-60 mg/kg pentobarbital. Following routine disinfection, an incision of 1-1.5 cm was made in the back to expose the kidney. The kidney fat and adrenal glands were stripped away, the left renal portal vessels were ligated, the left kidney was resected and the incision was sutured. Subsequently, these rats were treated with adriamycin (ADR; Shanxi Pude Pharmaceutical Co., Shanxi, China) at a dose of 3 mg/kg by single tail vein injection at days 8 and 29. The SHO group was operated on without removal of the kidney and received tail vein injection with saline solution, while the NC group was maintained under normal conditions without surgery or injection.

Drug treatment and biochemical analysis. A total of 30 randomly selected GS rats were divided equally into three groups (n=10) and treated with different doses of QU (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) once daily by oral gavage for 8 weeks: Low-dose group (QU I, 25 mg/kg/day), intermediate-dose group (QU II, 50 mg/kg/day) and high-dose group (QU III, 100 mg/kg/day). The NC group, SHO group, GS group, QU I group, QU II group and QU III group were treated with equal volumes of saline solutions. Following 8 weeks of drug intervention, the mental status (15), activity, fur glossiness, appetite, defecation and weight alterations were observed and recorded for each group. The 24-h urine total protein excretion (UTP/24 h) was measured by the sulfosalicylic acid method. Briefly, 0.1 ml 4% sulfosalicylic acid reagent was added to 2-3 ml of urine supernatant in a test tube. The tube was gently shaken for 1 min and immediately observed for turbidity. The blood was collected from the caudal vein of the rats and the levels of serum total protein (TP), serum albumin (Alb), triglyceride (TG), serum total cholesterol (TC), urea nitrogen (BUN), and serum creatinine (SCr) were measured with ELISA kits according to the manufacturer's protocol: Rat triglyceride (TG; RA20187); Rat Albumin (Alb; RA20636); Rat Total cholesterol (TC; RA20136); Rat creatinine (Cr; RA20115) from Bio-Swamp (Wuhan, Hubei, China). Rat total protein (TP; RJ16656) and Rat urea nitrogen (BUN; RJ16084) were purchased from Shanghai Renjie Biotechnology Co., Ltd. (Shanghai, China).

Pathological analysis. After rats in each group were anesthetized (pentobarbital, 45 mg/kg, intraperitoneal injection) and rapidly sacrificed by cervical dislocation. These rats were sacrificed and the right kidneys were removed and fixed with 4% formaldehyde (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 21-25˚C for 15 min. The kidney tissues were embedded in paraffin and 4-μm sections were cut. Certain paraffin sections were separately stained with hematoxylin and eosin (H&E; 1% H for 7 min, 1% E for 4 min at 21-25˚C), periodic acid-Schiff base (PAS; 1.1% Schiff's reagent for 30 min at 20˚C) and Masson's trichrome (Masson; 1% H for 5 min, 1% Ponceau S for 7 min, 2% Aniline blue for 5 min at 21-25˚C). The remaining paraffin sections were dewaxed and microwaved for 10 min in citrate buffer for antigen retrieval. Next, the sections were treated with 3% hydrogen peroxide (H_2O_2) in methanol at 21-25˚C for 30 min to block the activity of endogenous peroxidase followed by rinsing in PBS three times. The sections were incubated with antibodies (Abcam, Cambridge, UK) against podocyte marker proteins [zona occludens protein 1 (ZO-1; 1:100; cat. no. ab214228), Nephrin (1:2,000; cat. no. ab216341) and P-cadherin (1:100; cat. no. ab137729)], EMT marker proteins [α-SMA (1:50; cat. no. ab5694) and FSP-1 (1:2,000; cat. no. ab197896)] and TGF-β and its associated signaling pathway proteins [TGF-β receptor 1 (TGFBR1; 1:50; cat. no. ab31013), TGFBR2 (1:100; cat. no. ab186838), Smad2/Smad3 (1:100; cat. no. ab217553), Smad4 (1:100; cat. no. ab40759), Smad7 (1:100; cat. no. ab216428), glycogen synthase kinase (GSK)-3β (1:50; cat. no. ab75745) and β-catenin (1:500; cat. no. ab32572)] overnight at 4˚C. Next, goat anti-rabbit-horseradish peroxidase (HRP)-conjugated IgG antibody (1:1,000; cat. no. ab6721; Abcam) was added for 1 h at 37˚C. Finally, the sections were developed with 1% diaminobenzidine for 5 min, counterstained with 1% hematoxylin for 2 min at 21-25˚C and mounted with neutral gum. Images of the sections were captured at magnification, x400 using a BX53 inverted light microscope (Olympus Corporation, Tokyo, Japan).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the kidney tissue using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and reverse transcription synthesis of cDNA was performed in a single-step method using the Eastep® RT Master Mix kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. qPCR was performed using the GoTaq® 2-Step RT-qPCR System (Promega Corporation) following the manufacturer's protocol. The thermocycling conditions for RT-qPCR were 5 min at 99˚C, then 15 sec at 94˚C, 30 sec at 59˚C (40 cycles), 45 sec at 72˚C. The fold-change in mRNA expression was calculated with the $2^{-\Delta\Delta Cq}$ method (16). Primer sequences are presented in Table I.

Western blotting. Total protein was extracted from renal tissue using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) and protein concentration was determined using bicinchoninic protein assay reagent (Beyotime Institute of Biotechnology). First, 20 μg protein sample were separated by 10% SDS-PAGE (Beijing Solarbio Science & Technology Co., Ltd.) and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), and then blocked using 5% non-fat milk-Tris-buffered saline containing 0.05% Tween 20 (TBST) at 21-25˚C for 2 h. Following washing with TBST, the membranes were incubated with primary antibodies at 4˚C overnight, the primary antibodies were as follows: Anti-TGF-β1 (1:500; cat. no. ab92486; Abcam); anti-ZO1 (1:1,000; ab96587; Abcam); anti-Nephrin (1:1,000; cat. no. ab216341; Abcam); anti-P-cadherin (1:2,000; cat. no. ab137729; Abcam); anti-α-SMA (1:100; cat. no. ab5694; Abcam), anti-FSP-1 (1:1,000; cat. no. ab197896; Abcam); anti-TGFBR2 (1:1,000; cat. no. ab186838; Abcam), anti-TGFBR1 (1:200; cat. no. ab31013; Abcam), anti-Smad2/Smad3 (1:1,000; cat. no. ab202445; Abcam), anti-Smad4 (1:5,000; cat. no. ab40759;

Table I. Primer sequences of the glomerulosclerosis associated proteins.

Gene	Primer sequence
TGF-β1	F: 5'-ATGAACCGACCCTTCCTGCT-3'
	R: 5'-TGTGTCCAGGCTCCAAATGT-3'
Smad7	F: 5'-CTCTTGCGAACATTACGGCT-3'
	R: 5'-CGAGATCAAGGTCGACCTGC-3'
GADPH	F: 5'-CTACCCACGGCAAGTTCAAT-3'
	R: 5'-GGATGCAGGGATGATGTTCT-3'
Smad2/3	F: 5'-GGAAAGGGTTGCCACATGTT-3'
	R: 5'-AGAATCTCCGTGTGCCGAGG-3'
Smad4	F: 5'-ACGGCCATCTTCAGCACCAC-3'
	R: 5'-AGAATGCACAATCGCCGGAG-3'
ZO-1	F: 5'-GGCATTATTCGCCTTCATAC-3'
	R: 5'-GGAACACAACAATCGGATAC-3'
GSK3β	F: 5'-CTTCAGGACAAGCGATTTA-3'
	R: 5'-CCAGCACCAGGTTAAGGTAG-3'
Nephrin	F: 5'-CCCTCCGGGACCCTACTG-3'
	R: 5'-TCTGGGAGGATGGGATTGG-3'
P-Cadherin	F:5'-AAGTGCTGCAGCCAAAGACAGA-3'
	R: 5'-AGGTAGACCCACCTCAATCATC CTC-3'
SMA	F: 5'-CAGCTATTGCCGTTCCAATTGA-3'
	R: 5'-CCAGGGCTTCATCATTGCA-3'
FSP	F: 5'-ATACTCAGGCAACGAGGGTG-3'
	R: 5'-CTTCCGGGGCTCCTTATC-3'
TGF-βIR	F: 5'-ACCATTTGGAGCCAGAACAC-3'
	R: 5'-GGTCTGAAGAGCTGAGCCTG-3'
TGF-βIIR	F: 5'-CGAGCTCGGTGGAAGGTCTCATTT TATTG-3'
	R: 5'-CCCAAGCTTGGGATGTAAAAGAC AAACAATG-3'
β-catenin	F: 5'-GCTGACCAAAACTGCTAAATGAC GA-3'
	R: 5'-TGTAGGGTCCCAGCGGTACAA-3'

ZO-1, zona occludens protein 1; Smad, mothers against decapentaplegic homolog 9; GSK, glycogen synthase kinase; TGF-βR, tumor growth factor β receptor; SMA, smooth muscle actin; FSP, fibroblast-specific protein.

Abcam), anti-Smad7 (1:500; cat. no. ab216428; Abcam), anti-GSK-3β (1:1,000; cat. no. ab75745; Abcam), anti-β-catenin (1:5,000; cat. no. ab32572; Abcam) and anti-GAPDH (1:2,500; cat. no. ab9485; Abcam), followed by incubation with an HRP-conjugated secondary antibody (1:10,000; cat. no. ab6721; Abcam) for 1 h at room temperature. Finally, proteins were visualized by enhanced chemiluminescence ECL (cat. no. WBKLS0010; EMD Millipore). The images were acquired and analyzed with a ChemiDoc Touch imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. The results of immunohistochemistry and western blotting were analyzed using Image J software version 1.8.0 (National Institute of Health, Bethesda, MD, USA). The results are expressed as the mean ± standard deviation and each experiment repeated three times. Significant differences were established by one-way analysis of variance followed by Bonferroni post-hoc test using the GraphPad Instat program version 3.0 (GraphPad, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

General appearance. Rats in the NC and SHO groups consumed a normal diet and exhibited increased weight, good mental status, sensitive response, shiny fur and solid stools; there was no significant difference between these two groups (data not shown). Serum biochemical indexes also exhibited no significant difference between these two groups (Table II). Therefore, the operation did not affect the experiment. Rats in the GS groups demonstrated decreased food intake and became lethargic, dispirited, and unresponsive. Additionally, they demonstrated severe alopecia, dark hair, loose stools, discolored urine and reduced weight. The levels of serum TP and Alb significantly decreased, but TG, TC, BUN, SCr and UTP significantly increased compared with the NC and SHO groups. Following QU intervention in rats with GS, compared with in the GS group, the general appearance of rats (QU I, QU II and QU III groups) was improved in a dose-dependent manner. The general appearance of the rats in the QU III group was near normal levels. The levels of serum TP and Alb were significantly increased, and TG, TC, BUN, SCr and UTP significantly decreased in a dose-dependent manner in the QU I, QU II and QU III groups compared with the GS group (Table II).

Biochemical parameters. Following 8 weeks of the study, there was no significant difference in biochemical parameters between the NC group and SHO group ($P>0.05$; Table II). The effect of the surgery on the experiment can be considered insignificant. Compared with the SHO group, the weight of mice in the GS group was significantly reduced, while kidney weight increased ($P<0.05$; Table II). UTP in the GS group increased by ~10-fold, accompanied by increased levels of TG, CHO, BUN and SCr and significantly decreased levels of TP and Alb ($P<0.05$; Table II) in comparison to NC and SHO. Biochemical parameters, including TG, Alb, TC, BUN and SCr in rats with GS returned to nearly normal levels following treatment with QU III.

Pathological evaluation. Based on direct observation, compared with the normal group, the kidneys of the GS group were fibrotic (Fig. 1A). Additionally, enlargement, damage, atrophy and hardening of the glomerulus, and interstitial lymphocytic infiltration in the GS group were observed by H&E staining (Fig. 1B). In addition, Masson staining demonstrated that the interstitial cells of the renal interstitium proliferated, the mesangial area increased in mass and the basement membrane of the glomerulus exhibited focal proliferation in the GS group compared with sham and NC rats (Fig. 1C). PAS staining demonstrated that the basement membrane of GS group rats became wider and the

Table II. Biochemical parameters in all groups.

Parameter	NC	SHO	GS	QU I	QU II	QU III
Kidney (g)	1.11±0.02	1.20±0.09[b]	2.45±0.26[a,c]	2.40±0.26[c,f]	2.22±0.15[c,e]	1.97±0.14[c,e]
Weight (g)	442.00±25.68	451.80±21.08[b]	370.71±19.97[a,c]	388.29±14.43[c,f]	383.61±18.23[c,e]	416.42±10.45[c,e]
TP (mM)	62.00±3.19	60.78±2.08[b]	52.90±2.24[a,c]	61.46±4.25[c,e]	57.91±2.91[c,e]	60.61±1.80[d,e]
TG (mM)	1.24±0.17	1.60±0.23[b]	12.30±1.73[a,c]	6.99±0.36[c,e]	4.64±1.87[c,e]	2.31±0.32[c,e]
Alb (g/l)	38.08±1.49	36.06±1.22[b]	14.20±1.24[a,c]	22.37±2.11[c,e]	23.15±4.35[c,e]	30.84±1.72[c,e]
TC (mM)	1.17±0.04	1.48±0.09[b]	10.60±0.98[a,c]	5.42±0.43[c,e]	5.03±1.01[c,e]	2.76±0.23[c,e]
BUN (mM)	5.64±0.43	6.38±0.42[b]	17.39±2.53[a,c]	12.67±0.58[c,e]	11.03±1.03[c,e]	8.99±0.43[c,e]
SCr (mM)	29.40±1.71	33.40±1.98[b]	98.57±10.36[a,c]	71.57±443[c,a]	67.06±8.60[c,e]	45.29±3.04[c,e]
UTP (mg/24 h)	31.34±2.59	34.66±4.97[b]	363.19±18.21[a,c]	299.84±20.13[c,e]	310.53±28.63[c,e]	267.70±12.61[c,e]

[a]P<0.05 vs. the NC; [b]P>0.05 vs. NC; [c]P<0.05 vs. SHO; [d]P>0.05 vs. SHO; [e]P<0.05 vs. GS; [f]P>0.05 vs. GS. TP, serum total protein; TG, triglyceride; Alb, serum albumin; TC, serum total cholesterol BUN, urea nitrogen; SCr, serum creatinine; UTP, urine total protein; QU, quercetin; NC, normal control; SHO, sham operation group; GS, glomerulosclerosis.

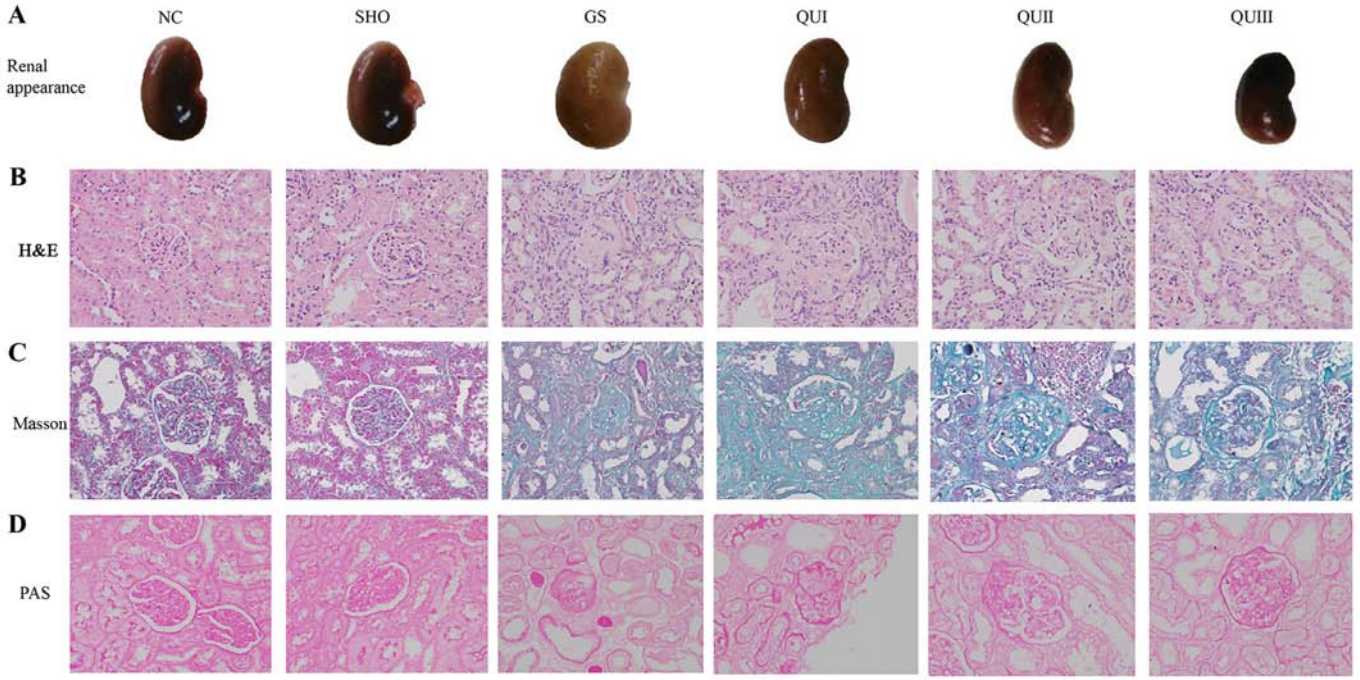

Figure 1. Pathological analysis of renal tissue following QU treatment in rats. (A) Observation of renal tissue in rats. (B) H&E, (C) Masson and (D) PAS staining of rat kidney tissue in all groups, magnification, x400. NC, normal control; SHO, sham operation group; GS, glomerulosclerosis; QU, quercetin; QU I, QU 25 mg/kg/day; QU II, 50 mg/kg/day; QU III, 100 mg/kg/day; H&E, hematoxylin and eosin; PAS, periodic acid-Schiff base.

mesangial matrix increased compared with sham and NC rats (Fig. 1D). Following treatment with increasing doses of QU, it was observed that the pathological alterations of renal tissue were gradually relieved and the fibrosis status of the kidneys gradually subsided and became normal in the QU-treated group.

Effect of QU on transcription and translation levels of GS-associated proteins. mRNA and protein in kidney tissue from each group were extracted and detected by RT-qPCR, western blotting, and immunohistochemistry. Podocyte marker proteins (ZO-1, Nephrin and P-cadherin) and Smad7 mRNAs (Fig. 2A) exhibited significantly decreased expression in the GS group compared with the NC group (P<0.001). Similarly, at the translation level, these proteins exhibited low expression (Fig. 2B) and distribution in the GS group. The proteins were recovered following QU treatment and their levels were altered in a dose-dependent manner. By contrast, EMT marker proteins (α-SMA and FSP-1) and other TGF-β signaling pathway-associated proteins (TGFBR2, TGFBR2, Smad2/Smad3, Smad4, GSK-3β and β-catenin) exhibited increased expression in the GS group compared with the NC group (Fig. 3), and distribution (Fig. 4) of these proteins observed by immunohistochemistry exhibited similar results. Following QU treatment, protein expression decreased in a dose-dependent manner.

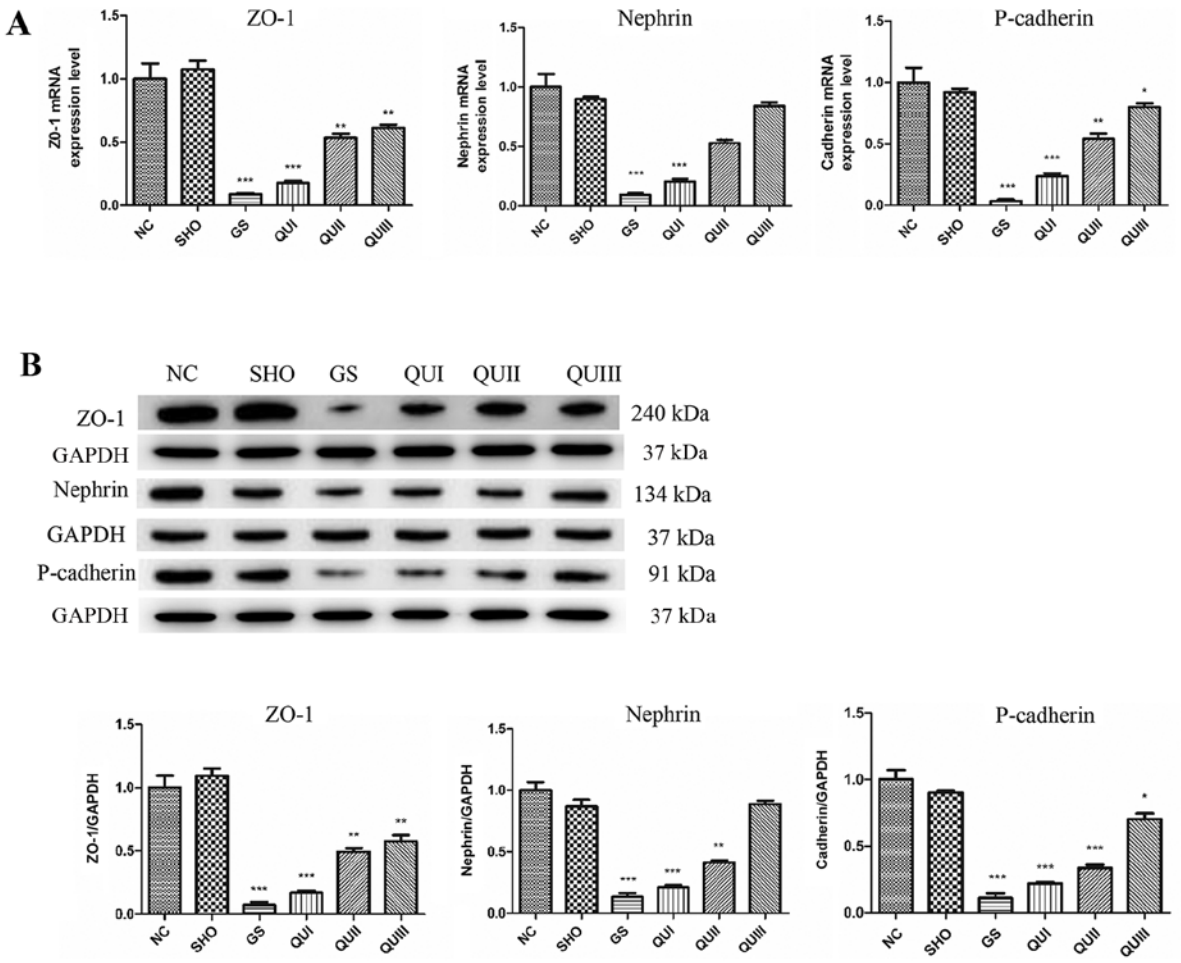

Figure 2. Expression levels of ZO-1, Nephrin and Cadherin following QU intervention. (A) mRNA and (B) protein expression levels were detected by RT-qPCR and western blotting, respectively. All data are presented as the mean ± standard deviation of the mean. *P<0.05, **P<0.01 and ***P<0.001, vs. the NC group. ZO-1, zona occludens protein 1; NC, normal control; SHO, sham operation group; GS, glomerulosclerosis; QU, quercetin; QU I, QU 25 mg/kg/day; QU II, 50 mg/kg/day; QU III, 100 mg/kg/day.

Discussion

To investigate the efficacy and mechanism of QU in GS, an ADR-induced GS mouse model (GS group) was constructed which demonstrated similarities to human renal disease (17). The main experimental manifestations of these mice were proteinuria, hyperlipidemia, hypoproteinemia, hypoxemia and renal hypofunction (18,19). Accordingly, certain biochemical indices of the mice were altered, such as increased SCr, BUN, TG and UTP (20). In the present study, the associated biochemical indices (SCr, BUN, TC, TG, UTP, TP and Alb) were examined following establishment of the rat model, and the results agreed with those of previous reports (20,21). Together with the examination of general appearance and pathological sections of the mice, these results indicated that the renal system of rats in the GS group had swelling and fibrosis, their functions were severely impaired, and absorption and filtration capabilities were weakened. Following treatment with QU, the physiological indices of GS rats began to recover. Therefore, QU may be useful for in the treatment of GS. QU can promote the filtration, protein absorption and lipid metabolism in the kidney, as well as inhibit glomerular fibrosis and improve the renal function of glomerulosclerotic rats.

GS is associated with the activation of various cytokines signal transduction pathways. Numerous studies confirm that TGF-β is a major cytokine causing GS (22-25). Therefore, TGF-β and its signal transduction pathway have become important targets for studies of renal diseases caused by various factors (26). There are two main TGF-β receptors: TGFBR2 and TGFBR1 (27). Activated TGF-β can bind TGF-BR1 and then adsorb TGF-BR1 to form heterodimers on the cell membrane that activate downstream effectors. Activated TGFBR1 induces phosphorylation of Smad2 and Smad3, and the phosphorylation of Smad2 or Smad3 can bind Smad4 to form oligomers, followed by translocation into the nucleus to act as a transcription factor (28-30). However, Smad7 can inhibit the phosphorylation of Smad2 and Smad3 by directly interacting with the TGFBR, and ultimately block the TGF-β signaling pathway (31). In the present study, the expression levels of TGFBR2, TGFBR1, Smad2/Smad3 and Smad4 were elevated in the GS group, and the expression of Smad7 was inhibited. In addition, during EMT there was a significant increase in α-SMA and FSP-1, which are transcriptionally regulated by the TGF-β signal pathway to promote GS (29,32). TGF-β signaling can

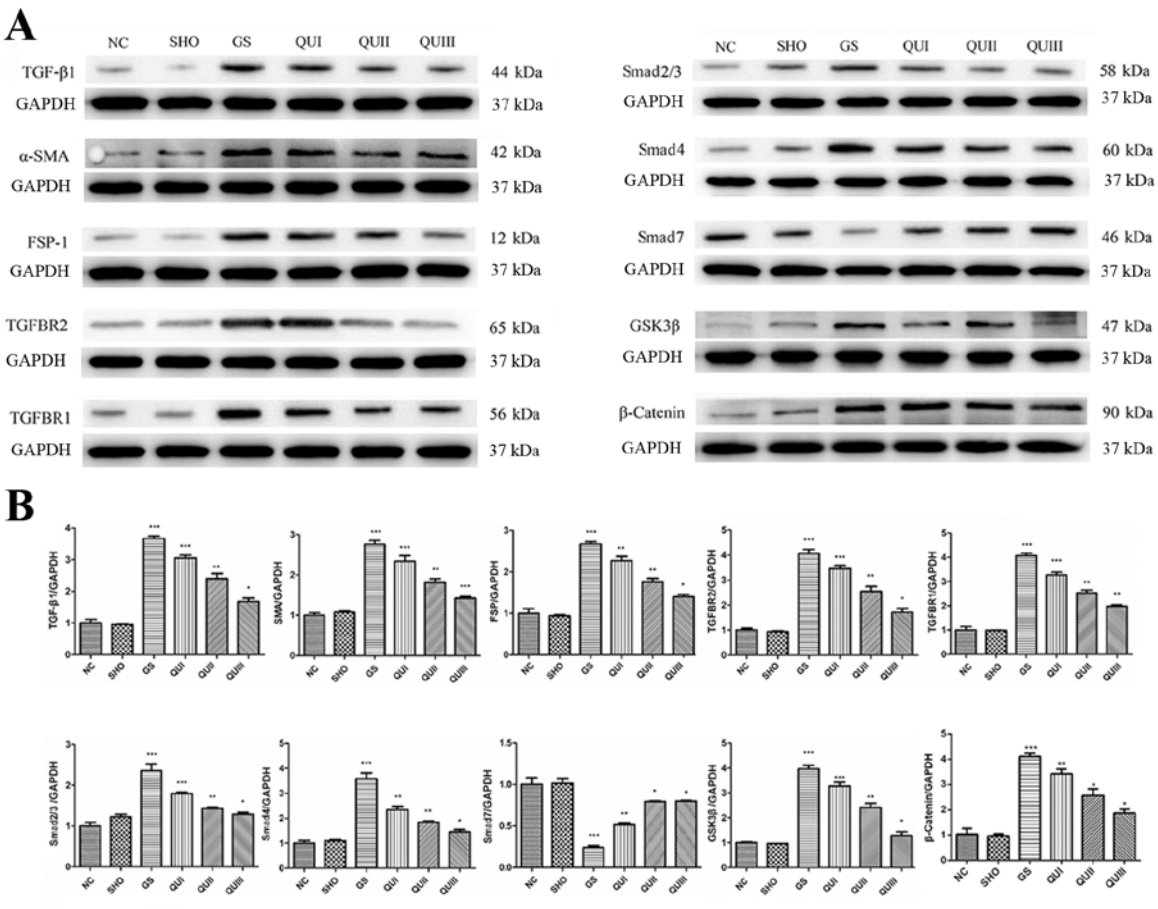

Figure 3. Protein expression levels of GS-associated proteins following QU intervention. (A) Western blotting of GS-associated proteins in rats from all groups. (B) Relative protein expression levels of GS-associated proteins in rats from all groups. Magnification, x400. All data are presented as the mean ± standard deviation of the mean. *P<0.05, **P<0.01 and ***P<0.001, vs. the NC group. NC, normal control; SHO, sham operation group; GS, glomerulosclerosis; QU, quercetin; QU I, QU 25 mg/kg/day; QU II, 50 mg/kg/day; QU III, 100 mg/kg/day; TGF-β, transforming growth factor-β; ZO-1, zona occludens protein 1; SMA, smooth muscle actin; FSP-1, fibroblast-specific protein-1; TGFBR, transforming growth factor β receptor; Smad, mothers against decapentaplegic homolog 9; GSK, glycogen synthase kinase.

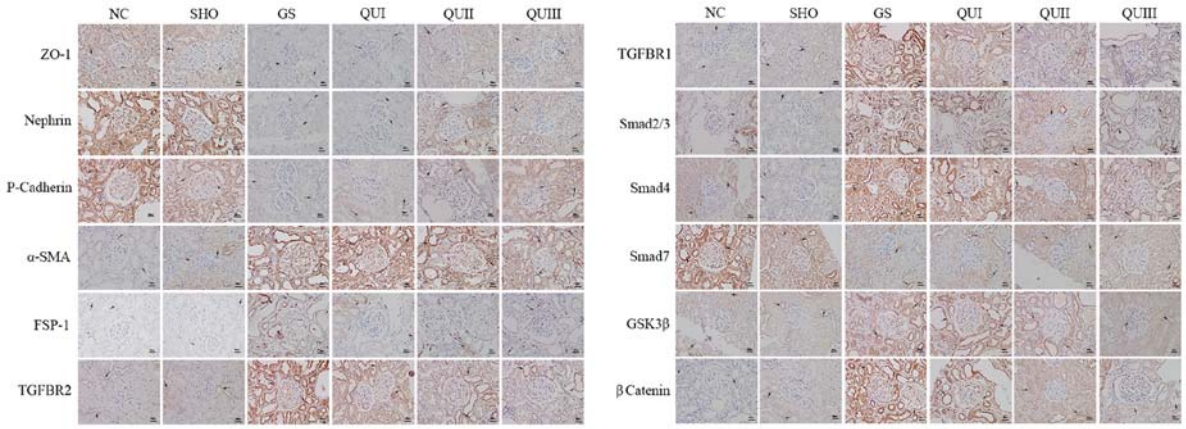

Figure 4. Detection of the distribution of GS-associated proteins by immunohistochemistry. Magnification, x400. Brown represents protein expression and increasingly dark colors represent increased protein expression. The arrows indicated the alterations in morphology. Scale bar, 5 μm. NC, normal control; SHO, sham operation group; GS, glomerulosclerosis; QU, quercetin; QU I, QU 25 mg/kg/day; QU II, 50 mg/kg/day; QU III, 100 mg/kg/day; ZO-1, zona occludens protein 1; SMA, smooth muscle actin; FSP-1, fibroblast-specific protein-1; TGFBR, transforming growth factor β receptor; Smad, mothers against decapentaplegic homolog 9; GSK, glycogen synthase.

lead to tumorigenesis or tumor suppression (33,34). It induces EMT to promote the invasion and metastasis of cancer cells (34). Disruption of the transcriptional network in the EMT process can improve the tumor suppressive functions of TGF-β (35). Myofibroblasts are characterized by their high expression of α-SMA induced by elevated expression of

TGF-β1 receptors (36). The expression of FSP-1 is increased in a TGF-β/Smad-dependent manner in systemic sclerosis skin. Additionally, FSP-1 overexpression or stimulation induced an activated phenotype in resting normal fibroblasts; however, knockdown of S100A4 attenuated the pro-fibrotic effects of TGF-β (37). The expression level of the TGF-β upstream target proteins GSK-3β (38) and β-catenin (39) were also significantly decreased by QU treatment of GS rats in the current study. Additionally, the expression levels of ZO-1 (40), Nephrin (41) and P-cadherin (42), which are podocyte marker proteins are negatively associated with podocyte apoptosis, were decreased in GS rats. Following treatment with QU, the expression of podocyte marker proteins (ZO-1, Nephrin and P-cadherin), TGF-β signaling associated proteins (TGF-β1, TGFBR1/2, Smad2/3, Smad4, Smad7, GSK-3β and β-catenin), EMT associated proteins (α-SMA and FSP-1) returned to normal levels. Following QU treatment, the development of EMT was attenuated in podocytes with GS, potentially via downregulating TGF-β signaling proteins (TGF-β1, TGFBR1/2, Smad2/3, Smad4, GSK-3β and β-catenin) and EMT proteins (α-SMA and FSP-1), and upregulating Smad7. This suggests that QU can protect the kidneys from GS by inhibiting the TGF-β signal pathway.

In the present study, it was demonstrated that QU protects renal function and alleviates the progression of GS in rats. The mechanism may involve inhibition of the TGF-β signaling pathway. QU is a natural flavonoid that is widely distributed in green plants, including fruits and vegetables. Although QU had some negative impact on embryonic development according to an *in vitro* study (43) and prenatal exposure resulted in a small increase in the risk of cancer in mice offspring (44), QU has been well tolerated in human studies. Doses up to 1,000 mg/day did not produce adverse effects on blood parameters, liver and kidney function, hematology or serum electrolytes for several months (45-49). As a result of the low toxicity and side effects of QU, it may be clinically applicable. Therefore, this study suggests that QU can be used to treat GS. The present study used an *in vivo* rat model to demonstrate that QU treatment attenuates glomerulosclerosis via the TGF-β signaling pathway, but lacked *in vitro* experiments. Therefore, the association between QU and TGF-β signal pathway *in vitro* and the interference experiment of TGF-β1 inhibition in QU treatment remains to be investigated.

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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.

Author's contributions

ED designed and supervised these experiments; YL performed most of experiments involved in this study, including the establishment of animal models, pathological section preparation, immunohistochemistry and so on, collated and analyzed the experimental data and wrote the manuscript; JY was responsible for the PCR and western blot analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol was approved prior to commencing this study by the animal ethics committee of Gansu University of Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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