Shenqi Pill Mitigates Renal Interstitial Fibrosis Through Weakening Notch1/Jag1 Pathway

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Introduction. Shenqi pill (SQP) can be used to treat various kidney-related diseases, but its exact mechanism of action remains unclear. We intended to analyze the role and mechanism of SQP on renal interstitial fibrosis (RIF).
Methods. After performing unilateral ureteral obstruction (UUO) surgery following the Institutional Animal Care and Use Committee guidelines, all rats were assigned into the sham group, UUO group, UUO + SQP 1.5 g/kg, UUO + SQP 3 g/kg, and UUO + SQP 6 g/kg groups. After treatment with SQP for 4 weeks, the appearance of kidney, serum creatinine (SCr), and blood urea nitrogen (BUN) levels were monitored in each group. The pathological injury, extracellular matrix (ECM), and Notch1 pathway-related protein levels were measured using H&E staining, Masson staining, immunohistochemistry, and Western blot, respectively.
Results. SQP could obviously ameliorate the appearance of the kidney as well as the levels of SCr and BUN in UUO rats (SCr: 67.6 ± 4.64 μM, 59.66 ± 4.96 μM, 48.76 ± 4.44 μM, 40.43 ± 3.02 μM for UUO, low, medium, and high SQP treatment groups; BUN: 9.09 ± 0.97 mM, 7.72 ± 0.61 mM, 5.42 ± 0.42 mM, 4.24 ± 0.34 mM for UUO, low, medium, and high SQP treatment groups; $P < .05$). SQP also effectively mitigated renal tissue injury in UUO rats ($P < .05$). Moreover, we uncovered that SQP significantly inhibited Collagen I, α-SMA, Collagen IV, TGF-β1, Notch1, and Jag1 protein expressions in UUO rats' kidney ($P < .05$).
Conclusion. Our data elucidated that SQP can alleviate RIF, and the mechanism may be related to the Notch1/Jag1 pathway.

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Original Paper

INTRODUCTION

The rising prevalence of chronic kidney disease (CKD) is causing a significant increase in both healthcare and financial burdens, a matter of great concern for the World Health Organization.[1] CKD involves progressive and irreversible loss of nephron and renal tissue injury, followed by renal dysfunction and eventually end-stage kidney disease (ESKD).[2,3] In the United States, the incidence of ESKD is estimated to increase by 11 to 18% by 2030, which is a serious burden to society.[4] Renal fibrosis is the central link and a significant feature in the occurrence and development of CKD, and is also one of the inevitable pathological changes for progressing to ESKD.[5,6] Multiple factors, such as infection, toxins, genetics, autoimmunity, obesity, and high glucose and cholesterol levels can accelerate the progression of renal fibrosis, leading to renal scarring and failure until ESKD.[7,8] At present, there is still a lack of early and accurate

specific markers for the diagnosis of renal fibrosis, and its treatment also lacks pertinence. Therefore, it is of great importance to actively explore drugs and treatments to inhibit renal fibrosis in the treatment of renal diseases.

In recent years, traditional Chinese medicine (TCM) has been employed in the treatment of diverse ailments and has considerable and unique effects.[9,10] Due to its advantages of multi-components, mult-targets, and reduced adverse effects, TCM compound has made continuous progress in the theoretical and clinical research of CKD.[11,12] From the TCM aspect, "kidney-yang deficiency" is a leading contributing factor to renal fibrosis. Therefore, "invigorating kidney yang" is the main principle of TCM in the treatment of renal fibrosis.[13] Shenqi Pill (SQP) is derived from Jingui Yaolue compiled by Zhang Zhongjing, which is a representative prescription for tonifying the kidney in TCM. SQP consists of *Aconiti Lateralis Radix Praeparata*, *Rehmanniae Radix*, *Cinnamomi Ramulus*, *Dioscoreae Rhizome*, *Alismatis Rhizoma*, *Corni Fructus*, *Moutan Cortex* and *Poria*.[14] Our previous studies have discovered that SQP exhibits a renal protective effect in adenine-induced renal interstitial fibrosis (RIF) by suppressing TGF-β1/Smads pathway.[15] Another research has found that about 20 absorbed compounds are highly related to the therapeutic role of SQP on kidney-yang deficiency.[16] However, the specific mechanisms through which SQP mitigates RIF remain unidentified.

Hence, in this research, we established RIF rat models by unilateral ureteral obstruction (UUO) surgery to investigate the specific functions and mechanisms of SQP in RIF treatment, thereby offering a solid experimental foundation for the application and research of SQP in RIF.

MATERIALS AND METHODS

Animals

Thirty male Sprague-Dawley (SD) rats weighing 200 ± 20 g were provided by Shanghai SLAC Laboratory Animal Co., Ltd. Rats were allowed to adapt to the environment for 7 days under a pathogen-free environment (20 to 24 ℃, 40 to 50% humidity, and 12-h light/dark cycle), with conventional chow and water. The Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center (Certificate No. SYXK (Zhe) 2021-0033) approved all the animal experiments of this study, and the experiments were conducted following the Institutional Animal Care and Use Committee guidelines.

Animal Experiments

The specific operation for the UUO model was as follows:[17] Thirty rats were anesthetized with isoflurane (4% for induction and 2% for maintenance). Subsequently, the skin, muscle, and peritoneal tissues were incised along the median abdominal incision to explore the left ureter. Thereafter, the left ureter was ligated with a 4-0 surgical thread and the wound was sutured in layers. Rats in the sham group (n = 6) underwent the same operation, except that the left ureter was not ligated.

The twenty-four rats that underwent UUO surgery were randomly allocated to 4 groups (n = 6): UUO group and UUO + SQP groups (1.5, 3, and 6 g/kg). The rats in the UUO + SQP groups were daily given SQP via intragastric administration at doses of 1.5, 3, or 6 g/kg from the first day after surgery. Meanwhile, the rats of the sham and UUO groups were administered with an equivalent volume of saline intragastrically.

Sample Collection

Upon treatment with SQP for 4 weeks, all animals were fasted overnight and blood samples were taken from rats' orbits. All blood samples were let to stand for 1 h at 4 ℃ before being centrifuged to acquire serum samples. Then, rats were euthanized with an overdose of isoflurane. A detailed autopsy was carried out 2 h of euthanasia, thereafter, we quickly removed both kidneys and observed the appearance and morphology of the kidneys in each group. A section of the renal tissues was put in 10% formalin (A5472, Sigma-Aldrich, USA) for two days. After dehydration and wax embedding, kidney samples were cut to a thickness of four μm with the help of a microtome. The remaining renal tissues were stored at -80 ℃.

Detection of Biochemical Indicators

Serum creatinine (SCr) and blood urea nitrogen (BUN) contents were monitored using SCr kit (C011-2-1, Jiancheng, China) and BUN kit (C013-2-1, Jiancheng, China) according to the manufacturer's instructions.

Kidney Histopathology

The obtained renal slices were routinely dewaxed and hydrated. The slices were then subjected to hematoxylin and eosin (H&E) and Masson staining. H&E staining kit (G1003) and Masson staining kit (G1006) were bought from Servicebio (China). The pathological variables and fibrosis extents of the renal tissues were evaluated with a BX53M microscope (Olympus, Japan). The assessment of kidney injury and kidney fibrosis extent were performed using the H&E semi-quantitative scoring method and Masson staining, respectively.

Kidney injury grade was as follows:[18,19] Zero, normal; one, a small amount of inflammatory cell infiltration, no glomerulosclerosis; two, significant inflammatory cell infiltration, with a small amount of glomerulosclerosis; three, obvious inflammatory cell infiltration and glomerulosclerosis; four, a large number of inflammatory cell infiltration with obvious glomerulosclerosis.

Immunohistochemistry (IHC)

The dewaxed and hydrated sections were treated with citrate buffer followed by immersion in 3% H_2O_2 to block endogenous catalase. After being sealed with BSA, the sections were acted with the primary antibodies against Collagen I (1:500, ab270993, Abcam, UK), α-SMA (1:2000, ab124964, Abcam, UK), TGF-β1 (1:500, ab215715, Abcam, UK), and Notch1 (1:800, #3608, CST, USA) at 4 °C for 12 h. Then, HRP-conjugated secondary antibodies (S0001, Affinity, USA) were taken to treat the slices. Following the reaction with DAB and mounting with neutral balsam, the stained results were captured under a microscope.

Western Blot

The total protein of the renal tissues was lysed by radioimmunoprecipitation (RIPA) (PC104, epizyme, China) and quantified with the bicinchoninic acid (BCA) kit (ZJ101, epizyme, China) referring to the stand protocols.[20] Thereafter, the quantified proteins were undergone denaturation and electrophoresis. After being electro-transferred onto nitrocellulose membranes, the proteins were sealed with 5% non-fat milk (37 °C, 90 min). The membrane was then put in an incubation box with primary antibodies (4 °C, all night). Later on, the blots were re-incubated for another 1 h with secondary antibodies. Lastly, the immunoreactive bands were visualized by ECL luminescence reagents (1705061, BIO-RAD, USA) and analyzed using Image J software. The information on the primary antibodies is exhibited in Table.

Statistics

The data of the study were analyzed by SPSS software (19.0, IBM, USA) and exhibited as mean ± standard deviation. All pathologists, statisticians, and laboratory personnel were blinded to the group allocation. Multiple group comparisons were performed using one-way ANOVA and Tukey tests. The Kruskal-Wallis H test was exploited if the data did not conform to a normal distribution. $P < .05$ was considered statistically significant.

RESULTS

SQP Improved the Appearance of Kidneys in UUO Model Rats

As illustrated in Figure 1, the dimensions of both kidneys in the sham group were identical, and the boundary between cortex and medulla was clear. However, the left kidney of the rats in the UUO group was larger than the contralateral side, and the border between cortex and medulla became vaguer. The administration of SQP resulted in a considerable improvement in the aforementioned characteristics in rats with a UUO model, with a dose-dependent effect.

SQP Reduced the Levels of SCr and BUN in UUO Rats

Biochemical indicators for the rats are described

The information on the primary antibodies

Antibody	Source	Cat No.	Dilutions
Collagen I	Abcam (UK)	ab270993	1:1000
Collagen IV	Abcam (UK)	ab6586	1:1000
α-SMA	Affinity (USA)	AF1032	1:1000
TGF-β1	Abcam (UK)	ab215715	1:1000
Jag1	Abcam (UK)	ab300561	1:1000
GAPDH	Abcam (UK)	ab181602	1:10000

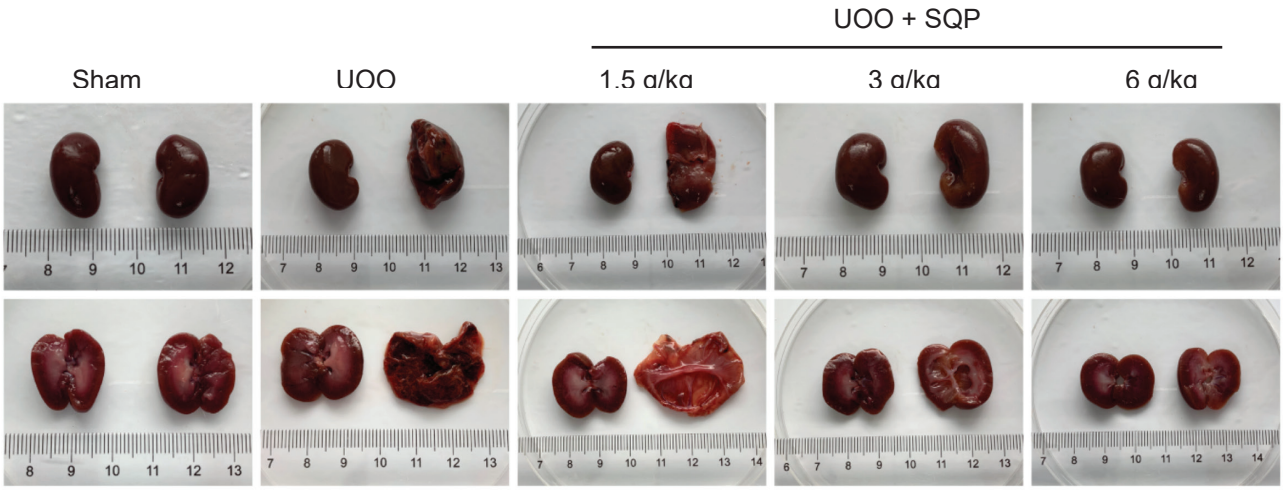

Figure 1. SQP could ameliorate the appearance of kidneys in UUO model rats (The appearance of kidneys in the sham group, unilateral ureteral obstruction (UUO) group, UUO + Shenqi Pill (SQP) 1.5 g/kg group, UUO + 3 g/kg group, and UUO + 6 g/kg group was monitored).

in Figure 2. It could be observed that UUO led to an increase in SCr and BUN ($P < .01$). In comparison to the UUO group, the levels of SCr and BUN in rats in the low, medium, and high SQP treatment groups were notably decreased, and the degree of reduction was positively correlated with the SQP dosage ($P < .05$).

SQP Mitigated Renal Interstitial Injury in UUO Rats

As displayed in the results of H&E staining, a large number of dark brown crystals were deposited in the renal tubules, the structure of the renal tubules was destroyed, accompanied by many inflammatory cell infiltration, and the glomeruli were obviously sclerosed in the UUO rats (Figure 3). Compared to the UUO rats, there were no evident improvements in the renal tissues of the rats in the low-dose SQP group. Rats in the medium-dose SQP group had reduced inflammatory cell infiltration in the kidneys, and milder cellular damage than rats in the UUO group. In the high-dose SQP group, there was reduced damage to the kidney cells, with clear recovery of the cell morphology and structure. Furthermore, the H&E semi-quantitative score in the kidney tissues of the UUO group was evidently enhanced, whereas SQP intervention strongly weakened the H&E semi-quantitative score in the kidney tissues of the UUO rats (Figure 3).

The results of Masson staining revealed that

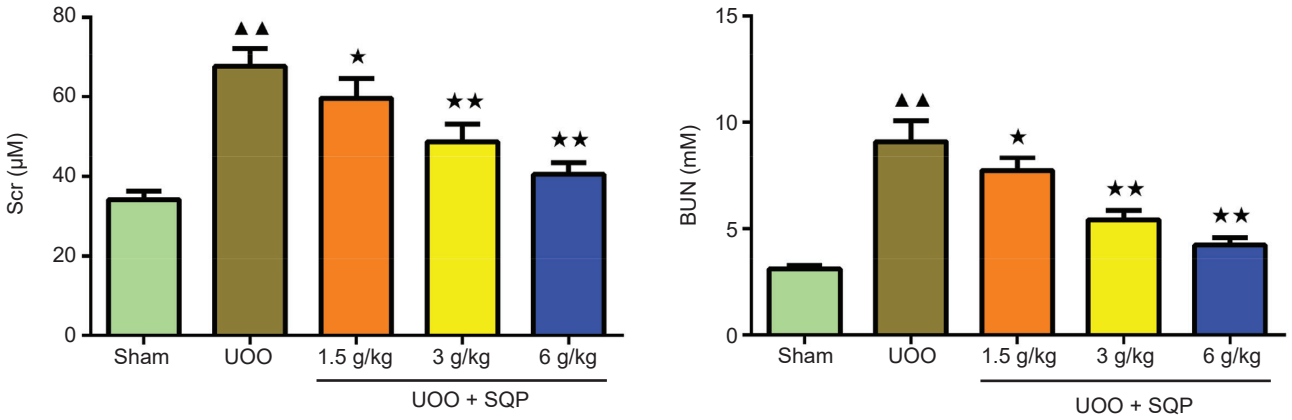

Figure 2. SQP lessened the levels of Scr and BUN in the serum of UUO rats (The effects of SQP on serum creatinine (Scr) and blood urea nitrogen (BUN) contents in UUO rats were examined by ELISA (n = 6)).
Quantitative data were expressed as mean ± standard deviation.
▲▲$P < .01$ vs. sham; *$P < .05$, **$P < .01$ vs. UUO

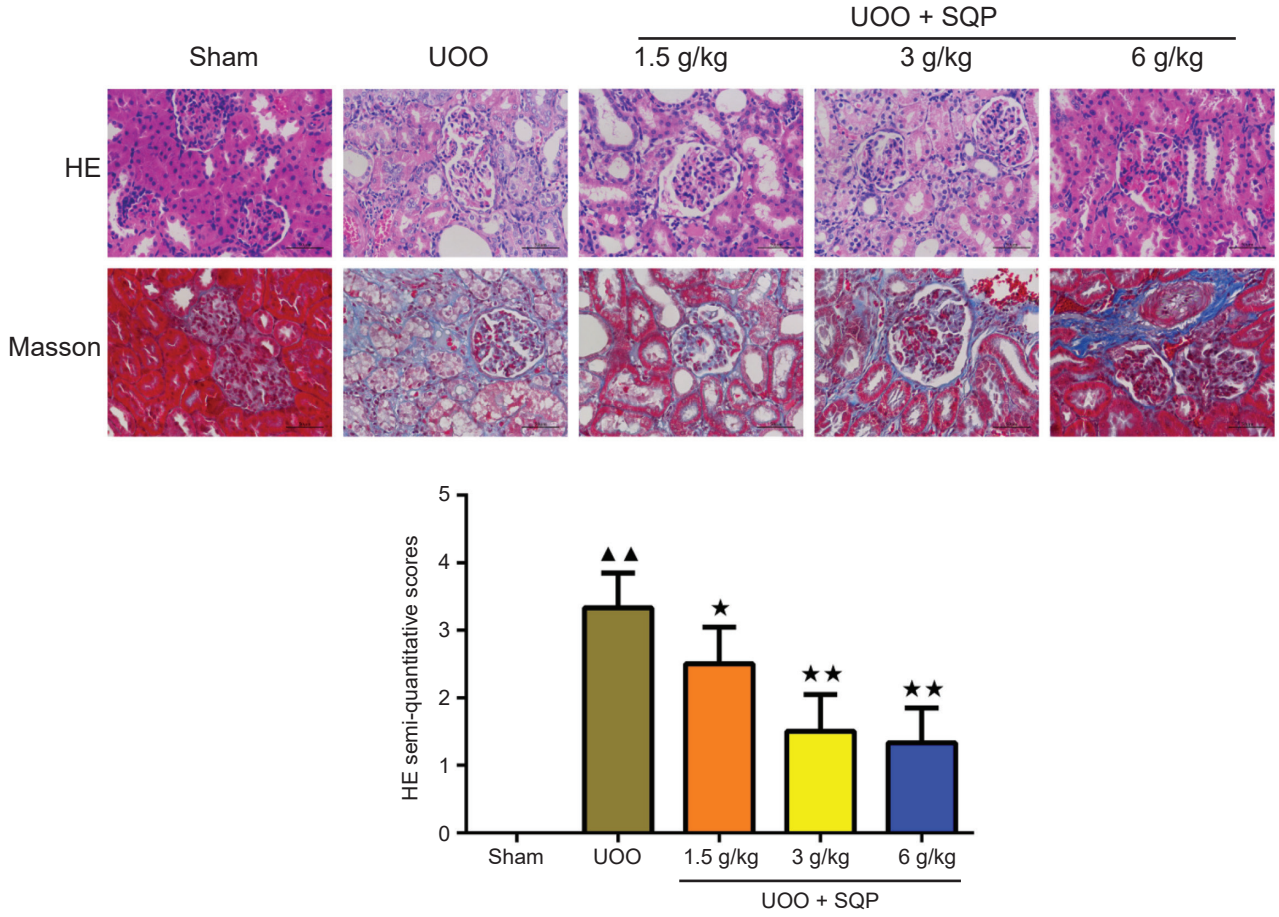

Figure 3. SQP mitigated renal interstitial injury in UUO rats (H&E staining and Masson staining were used to observe the pathological changes and the degree of fibrosis in the kidneys of UUO rats). The assessment of kidney injury was performed using the H&E semi-quantitative scoring method (n = 6).
Quantitative data were described as mean ± standard deviation.
▲▲$P < .01$ vs. sham; *$P < .05$, **$P < .01$ vs. UUO

compared to the sham group, the deposition of collagen fibers in renal interstitium was increased. Additionally, there was an obvious abnormal hyperplasia of fibrous connective tissue in the UUO group. Despite the low- and middle-dose SQP did not exhibit obvious improvements for UUP rats, the high-dose SQP group effectively reduced the collagen fibers in the renal tissues (Figure 3).

SQP Restrained Collagen I, α-SMA, and Collagen IV Expressions in the Kidneys of UUO Rats

The immunohistochemistry revealed that the positive expressions of Collagen I and α-SMA in the UUO group were higher than in the sham group (Figure 4a, $P < .01$). Relative to the UUO group, the positive expressions of Collagen I and α-SMA in the UUO+SQP groups were lower (Figure 4a, $P < .05$). Western blot assay was conducted to further clarify that relative to rats underwent sham surgery, the Collagen I, Collagen IV, and α-SMA levels of the kidneys in UUO rats were significantly elevated. However, SQP treatment reversed these conditions (Figure 4b, $P < .05$). Notably, the higher the SQP concentration was, the more pronounced the above effect.

SQP Suppressed the Expressions of TGF-β1, Notch1, and Jag1 in the Kidneys of UUO Rats

Finally, we examined Notch1/Jag1 pathway-related markers. From the results of IHC, we discovered that the positive expressions of TGF-β1, Notch1, and Jag1 in the kidneys of the UUO group were evidently enhanced compared to the sham group; the addition of SQP weakened Notch1/Jag1 pathway-related marker expressions in the

kidney of UUO rats in a dose-dependent manner (Figure 5a, $P < .05$). The results obtained from the Western Blot analysis were consistent with those obtained by the IHC experiment (Figure 5b, $P < .05$).

DISCUSSION

UUO animal models are characterized by progressive tubular atrophy and interstitial fibrosis, without hypertension, proteinuria, or abnormal

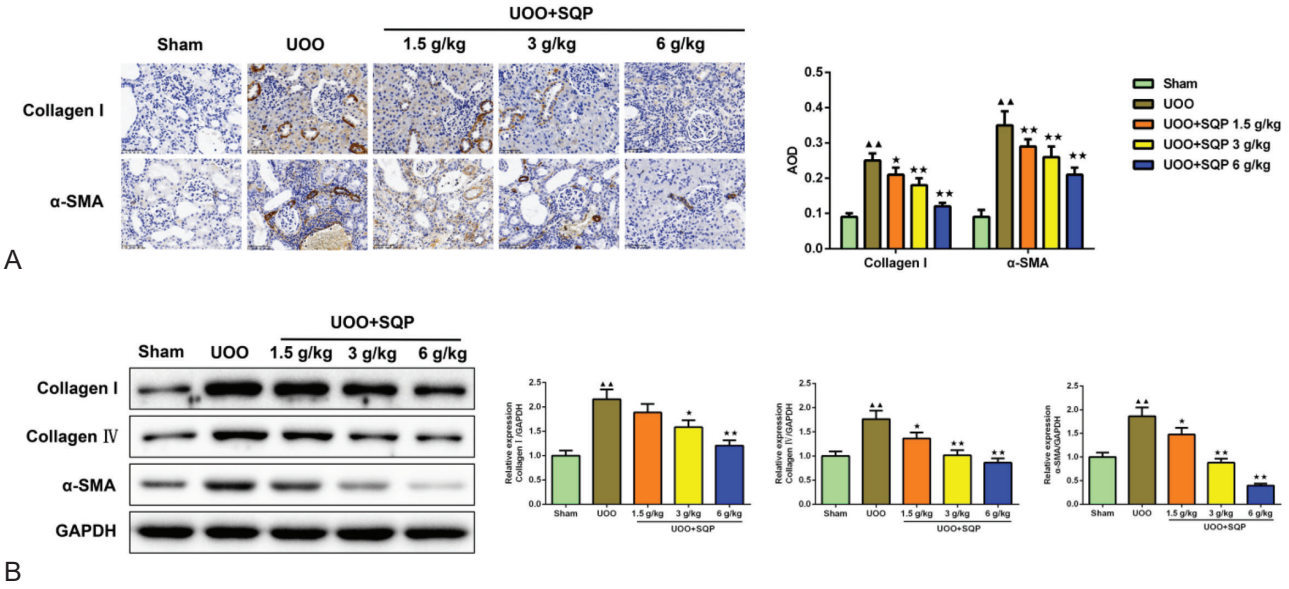

Figure 4. SQP restrained the expressions of Collagen I, α-SMA, and Collagen IV of kidneys in UUO rats [(a) The effect of SQP on the positive expressions of Collagen I and α-SMA of kidney in UUO rats was determined by immunohistochemistry (n = 6). (b) The effect of SQP on the expressions of Collagen I, α-SMA, and Collagen IV of kidneys in UUO rats was determined by western blot (n = 3)].
Quantitative data were manifested as mean ± standard deviation.
▲▲$P < .01$ vs. sham; *$P < .05$, **$P < .01$ vs. UUO

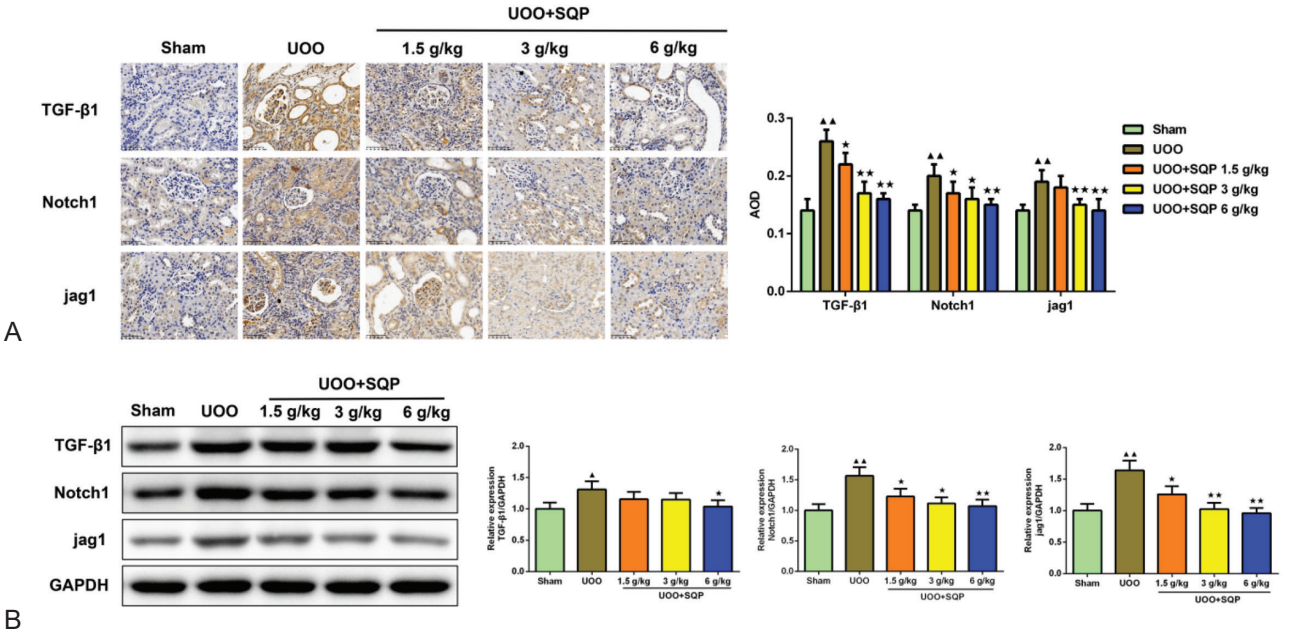

Figure 5. SQP suppressed the expressions of TGF-β1, Notch1, and Jag1 of kidney in UUO rats **[**(a) The effect of SQP on the positive expressions of TGF-β1, Notch1, and Jag1 of kidney in UUO rats was examined by immunohistochemistry (n = 6). (b) The effect of SQP on the protein expressions of TGF-β1, Notch1, and Jag1 of kidney in UUO rats was examined by western blot (n = 3)].
Quantitative data were manifested as mean ± standard deviation.
▲$P < .05$, ▲▲$P < .01$ vs. sham; *$P < .05$, **$P < .01$ vs. UUO

lipid metabolism[21] UUO models can reflect the characteristics of obstructive nephropathy and fibrotic kidney diseases.[22] Therefore, UUO animal models are frequently used to study RIF pathogenesis and evaluate therapeutic agents. In this experiment, we observed the appearance of kidney and discovered that the left kidney of the rats in the UUO group was larger than the contralateral side, and the border between cortex and medulla became vaguer, which indicated that the rat models of UUO were successfully prepared.

The assessment of renal pathophysiology serves as a direct reflection of the extent of pathological damage caused by the disease, playing a pivotal role as a key indicator in assessing the effectiveness of drug treatments.[23] In this study, H&E and Masson staining were employed to assess renal damage and fibrosis. The kidneys of rats in the UUO group exhibited massive vacuolar degeneration of renal tubular epithelial cells, severely damaged renal tubular structures, notable inflammatory cell infiltration, and collagen deposition in the renal interstitium. Interestingly, kidney tissues from UUO rats that received SQP interventions showed less tubular injury and collagen deposition. We also assessed renal function indicators, because they can reflect the severity of RIF, especially SCr and BUN.[24] We discovered that SQP presented a potential renal protective effect in UUO rats, as evidenced by the observed reduction in SCr and BUN levels.

Excessive deposition of extracellular matrix (ECM) is a characteristic of renal fibrosis and is recognized as a common pathological feature of CKD.[25] Research has indicated that TGF-β1 is a key mediator associated with progressive renal fibrosis in CKD with multiple biological properties, such as cell proliferation, differentiation, apoptosis, autophagy, and ECM production.[26] In a study of rat UUO models, it has been found that TGF-β1 is increased in obstructed kidneys and can induce renal cells to produce ECM, thereby leading to glomerulosclerosis and interstitial fibrosis.[27] Cell experiments also have discovered that TGF-β can eliminate the E-cadherin of normal rat renal tubular epithelial cells, and enhance the expression of α-SMA, which is transformed into myofibroblasts, namely EMT.[28] Myofibroblasts can secrete and produce large amounts of Collagen I and IV, and it is precisely because the deposition of collagen in ECM leads to significant histological changes of RIF.[29] The enhanced expression of α-SMA and the increased synthesis of Collagen I and Collagen IV are important markers for EMT in the renal interstitium,[30,31] so these markers were also detected in this experiment to verify whether EMT occurred in the kidney of UUO rats. By performing IHC and Western blot experiments, the results clarified that Collagen I, Collagen IV, and α-SMA levels in the kidneys of UUO rats were strongly elevated. However, SQP treatment reversed these conditions, suggesting that SQP exhibited a remarkable therapeutic effect in restraining the progression of renal EMT and improving RIF in UUO model rats.

It has been reported that the Notch1/Jag1 pathway is an important regulatory pathway for both EMT and RIF.[32] The Notch1 pathway is under-expressed or even not expressed in adult kidney tissues but is over-expressed in many renal illnesses.[33] Researchers have found that inhibiting Notch1 pathway contributes to improving TGF-β1-caused EMT and ameliorating RIF *in vivo* and *in vitro*.[34] Morrissey *et al.* have discovered that the gene expressions of Notch1 and Jag1 were up-regulated in the distal renal tubules of UUO rats by gene chip screening study; *in vitro* studies have shown that TGF-β1 can induce Jag1 expression in human renal tubular epithelial cells and suggested the existence of TGF-β1-dependent Notch signaling activation in UUO rats.[35] In this research, our results found that TGF-β1, Notch1, and Jag1 expressions in the kidneys of UUO rats were decreased following SQP treatment, unveiling that Notch1/Jag1 signaling may be involved in the pathological development of UUO and is associated with the activation of TGF-β1. In addition, our results also indicate the regulation effect of SQP on this signaling.

In conclusion, the results of this study indicated that SQP treatment can alleviate the renal interstitial lesions and RIF in UUO rats, and its mechanism might be associated with the repression of TGF-β1/Notch1/Jag1 expressions.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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