Attenuating Renal Interstitial Fibrosis by Shenqi Pill via Reducing Inflammation Response Regulated by NF-κB Pathway *In Vitro* and *In Vivo*

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Introduction. One of the most significant clinical features of chronic kidney disease is renal interstitial fibrosis (RIF). This study aimed to investigate the role and mechanism of Shenqi Pill (SQP) on RIF.
Methods. RIF model was established by conducting unilateral ureteral obstruction (UUO) surgery on rat or stimulating human kidney-2 (HK-2) cell with transforming growth factor β1 (TGFβ1). After modeling, the rats in the SQP low dose group (SQP-L), SQP middle dose group (SQP-M) and SQP high dose group (SQP-H) were treated with SQP at 1.5, 3 or 6 g/kg/d, and the cells in the TGFβ1+SQP-L/M/H were treated with 2.5%, 5%, 10% SQP-containing serum. In *in vivo* assays, serum creatinine (SCr) and blood urea nitrogen (BUN) content were measured, kidney histopathology was evaluated., and α-smooth muscle actin (α-SMA) expression was detected by immunohistochemistry. Interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) content, inhibitor of kappa B alpha (IKBα) and P65 phosphorylation were assessed. Meanwhile, cell viability, inflammatory cytokines content, α-SMA expression, IKBα and P65 phosphorylation were detected *in vitro* experiment.
Results. SQP exhibited reno-protective effect by decreasing SCr and BUN content, improving renal interstitial damage, blunting fibronectin (FN) and α-SMA expression in RIF rats. Similarly, after the treatment with SQP-containing serum, viability and α-SMA expression were remarkably decreased in TGFβ1-stimulated HK-2 cell. Furthermore, SQP markedly down-regulated IL-1β, IL-6, and TNF-α content, IKBα and RelA (P65) phosphorylation both *in vivo* and *in vitro*.
Conclusion. SQP has a reno-protective effect against RIF *in vivo* and *in vitro*, and the effect is partly linked to nuclear factor-kappa B (NF-κB) pathway related inflammatory response, which indicates that SQP may be a candidate drug for RIF.

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Original Paper

INTRODUCTION

Chronic kidney disease (CKD) is a serious health problem that affects more than 10% of the adult population globally, and renal interstitial fibrosis (RIF) is the final result of almost all kinds of progressive CKD.[1,2] RIF is characterized by the expansion of myofibroblast, accumulation of extracellular matrix, as well as tubular

atrophy.[3] At present, no specific treatment has proved to be effective for RIF.[4] The etiology of RIF is highly complicated and still poorly understood, but there is a strong belief that RIF is associated with inflammatory response.[5,6] Hence, looking for effective agent that can diminish inflammation may serve as a rational strategy to treat RIF.

Nuclear factor-kappa B (NF-κB), a common transcription regulator, is able to induce the expression of numerous genes, thereby producing diverse cytokines facilitated inflammatory response, and eventually promoting fibrogenesis.[7,8] Extensive studies have suggested that overexpression of NF-κB would induce and promote the progression of RIF, and NF-κB deficiency would attenuate RIF.[6] In addition, it has been reported that an NF-κB inhibitor (BAY-11-7082) can inhibit the expression of α-smooth muscle actin (α-SMA, a fibrotic marker), which is an important mechanism for inhibiting NF-κB to prevent RIF development.[9] Hence, inhibition of NF-κB pathway may help to preserve renal function and prevent RIF.

Recently, the advantages of traditional Chinese medicine (TCM) in the prevention and treatment of kidney diseases have been consistently acknowledged worldwide.[10] Shenqi Pill (SQP) is a famous prescription that was first introduced by Zhang Zhongjin, and has the nephroprotective effects.[11] SQP consists of *Aconiti Lateralis Radix Praeparata* (Fuzi in Chinese), *ramulus cinnamomi* (Guizhi in Chinese), *Rehmannia glutinosa Libosch* (Dihuang in Chinese), *common yam rhizome* (Shanyao in Chinese), *asiatic cornelian cherry fruit* (Shanzhuyu in Chinese), *Alisma orientale* (Zexie in Chinese), *Indian buead* (Fuling in Chinese), and *tree peony bark* (Danpi in Chinese) and has been used to treat human diseases for over 2000 years in China. Some studies report the major components of SQP, such as Shanzhuyu, Shanyao, Zexie and Danpi have ani-inflammatory effects via regulating the NF-κB pathway in aging rats.[12] However, the specific function and detailed mechanism of SQP in RIF is poorly understood.

In this study, we set up *in vivo* and *in vitro* RIF models to explore whether SQP can exhibit reno-protective effect against RIF by modulating inflammatory response through NF-κB pathway, thereby providing a novel approach in the treatment RIF.

MATERIALS AND METHODS

Animals and Treatments

Thirsty-six seven-week-old male SD (Beijing Vital River Laboratory Animal Technology Co., Ltd, China) rats weighing 200 to 220 grams were used in this study. The rats were housed in a temperature-, humidity-, and light-controlled animal room (20 ℃, 60% humidity, 12 hrs light/dark cycle) with free access to food and water. All animal experiments were performed with the approval of the Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center (Certificate No. SYXK (Zhe) 2021-0033), and the experiments were conducted according to the guidelines of the Chinese Council on Animal Care.

Following the accommodation for 7 days, the rats were fasted for 12 hrs but allowed to drink water freely, and were then randomly divided into: sham operation (Sham), unilateral ureteral obstruction surgery (UUO), SQP low dose (SQP-L), SQP middle dose (SQP-M) and SQP high dose (SQP-H) groups (n = 9/group). Rats in the UUO, SQP-L, SQP-M and SQP-H groups underwent unilateral ureteral obstruction (UUO) operation as described previously.[13] Simply speaking, the rats were immobilized by injecting 1.5% pentobarbital (60 mg/kg) intraperitoneally. Following shaving and disinfecting, a longitudinal incision was applied on the left side of the rat abdomen to expose the left kidney and ureter. Next, 3-0 silk threads were used to ligate the proximal and distal portion of the left ureter. The ureter between the ligatures was detached. Subsequently, the ligated kidney was carefully returned to its original position, and the abdominal incision was sutured in layers. The rats in the sham group received similar surgical manipulation, except for the ligation of the left ureter. One day after UUO surgery, the rats in the SQP-L/M/H groups were administered with SQP (dissolved in 0.5% carboxymethyl cellulose sodium (CMC-Na)) at 1.5, 3, and 6 g/kg, respectively.[11] The rats in the Sham and UUO groups were given the equal volume of 0.5% CMC-Na solution. SQP and CMC-Na solution were given daily for consecutive 28 days via intragastric administration. After performing the last administration, all rats were fasted overnight and then euthanized, and their blood samples and left kidneys were collected. After weighing, a portion of the kidney tissue was stored

at -20 ℃ for biochemical measures, while the rest was placed in 10% formaldehyde for 1 week and embedded in paraffin for histopathological analysis.

Preparation of SQP-containing Serum

Ten SD rats were given SQP suspension (0.3 g/mL, suspended in a 0.9% NaCl solution) twice daily at a dose of 3 mL/kg for 5 days. Prior to the last administration, the rats were fasted for 12 hrs, but were allowed to take water freely. Following 1 hr of the last administration, the rats were anesthetized, and their blood samples were collected from abdominal aorta. After resting (4 ℃, 30 min), centrifugation (3000 r/min, 15 min) and inactivation (56 ℃, 30 min), the SQP-containing serum was prepared and stored at −20 ℃. Another 10 SD rats were treated in a similar manner as described above, except for the gavage of the same volume of 0.9% NaCl solution, and their serum were obtained and marked as blank serum.[14]

Cell Culture and Treatment

Human kidney-2 (HK-2) cells, supplied by Saibaikang Biotechnology Co., Ltd. (iCell-h096), were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) containing 10% fetal bovine serum (FBS) under normoxic conditions (at 37 °C, 21% O_2 and 5% CO_2). HK-2 cells were inoculated into 6-well plates at 1×10^4 cells/well and divided into following groups: 1) control group, the cells were treated with 10% blank serum; 2) TGFβ1 group, the cells were stimulated by 10 μng/mL transforming growth factor β1 (TGFβ1) for 24 hrs. and then treated with 10% blank serum; 3-5) TGFβ1+SQP-L/M/H groups, the TGFβ1-stimulated cells were treated with 7.5% blank serum +2.5% SQP-containing serum, 5% blank serum +5% SQP-containing serum, and 10% SQP-containing serum, respectively.

Measurement of SCr and BUN in the Serum of Rats

Serum creatinine (SCr) and blood urea nitrogen (BUN) were measured for the evaluation of renal function of the rats. Blood samples were centrifuged (4000 g, 10 min, 4 °C) for the separation of serum. SCr and BUN levels were detected by corresponding commercial kits (Beijing Suolaibao Technology Co., Ltd., BC4910, BC1530) according to their instructions.

Histological Analysis

Pathological changes of the renal tissues were assessed by hematoxylin and eosin (H&E) and Masson staining. Briefly, the paraffin-embedded renal tissues were sliced into 5-μm-thick serial sections by microtome. Subsequently, the sections were dewaxed by xylene, rehydrated with 100 to 75% ethanol and stained by H&E (Servicebio, G1003) and Masson (Servicebio, G1006) staining for morphological study in strict accordance with the instructions. In order to evaluate renal pathological changes, the sections stained with H&E and Masson were assessed by using a light microscope.

Immunohistochemistry

Immunohistochemical staining was applied to examine the expression of fibronectin (FN) and α-SMA protein in kidney tissues. The specimens were sliced into 4-μm sections, deparaffinized with xylene, rehydrated in ethanol, heated with citrate buffer for antigen retrieval, treated with 3% H_2O_2 to stop endogenous peroxidase activity, and incubated with 3% bovine serum albumin (BSA) to reduce unspecific binding. The samples were then incubated at 4 °C overnight with primary antibodies against FN (AF5335) and α-SMA (AF1032). After rinsing, the samples were incubated with HRP-conjugated secondary antibody at 37 °C for an additional 50 min. Upon counterstaining with hematoxylin, the sections were examined by microscopy. The software of ImageJ was employed to quantify the FN- and α-SMA-positive cells. All primary antibodies were purchased from affinity and used at a dilution of 1:100.

Methyl Thiazolyl Tetrazolium (MTT) Assay

In conventional approaches, cell viability was determined by using the methyl thiazolyl tetrazolium (MTT) assay. In brief, the treated cells were washed and incubated at 37 °C with MTT solution (BBI Life Sciences, E606334-0500) for 4 hrs. Then, the formed purple formazan was dissolved in DMSO (100 μL/well), and the optical density value of each sample was measured at 540 to 595 nm by microplate reader. Cell survival was presented as a percentage (%) of the control group.

Immunofluorescence Staining

Immunofluorescence staining was carried out to assess the expression level of α-SMA in HK-2

cells. Specifically, the processed cells were rinsed by phosphate buffered solution (PBS), fixed with paraformaldehyde (4%), and subsequently immersed in TritonX-100 (0.5%). After blocking with 3% BSA for 30 min, the cells were incubated at 4 °C with a primary antibody of α-SMA (1:500, CST, 19245S) overnight. Then, the second antibody (abcam, ab150077) was added to re-incubate the cells for 30 min, followed by counterstaining with 4,6-diamino-2-phenyl indole in darkness to visualize nuclei. Finally, the cells were observed and photographed by a fluorescence microscope.

ELISA

Enzyme linked immunosorbent assay (ELISA) was applied to assess the levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in cell culture supernatant or rat serum. In simple terms, cells were treated as described above, the supernatant was transferred to ELISA plates, the release of IL-1β (MM-0181H2, MM-0047R2), IL-6 (MM-0049H2, MM-0190R2), TNF-α (MM-0122H2, MM-0180R2) in the culture medium or serum were then determined by respective commercial kits based on manufacturer's protocol. All ELISA kits were purchased from MEIMIAN.

Western Blot

Proteins of HK-2 cells or kidney tissues were extracted by Radioimmunoprecipitation assay (RIPA) lysis buffer for Western blot analysis. The total protein concentration was determined by using bicinchoninic acid (BCA) kits (Solarbio, pc0020). Subsequently, the same amounts of proteins were separated via 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (PVDF). The membranes were blocked with 5% skimmed milk for two hours before being reacted with primary antibodies against TNF-α (AF7014), IL-6 (DF6087), p-inhibitor of kappa B alpha (p-IKBα) (AF2002), IKBα (AF5002), p-p65 (AF2006), p65 (AF5006), GAPDH (AF7021), and Histone H3 (AF0863) overnight for 12 hrs at 4 °C. Then, the blots were rinsed and re-incubated with Horseradish peroxidase-conjugated secondary antibodies for another 2 hrs at 37 °C. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL) reagents. The

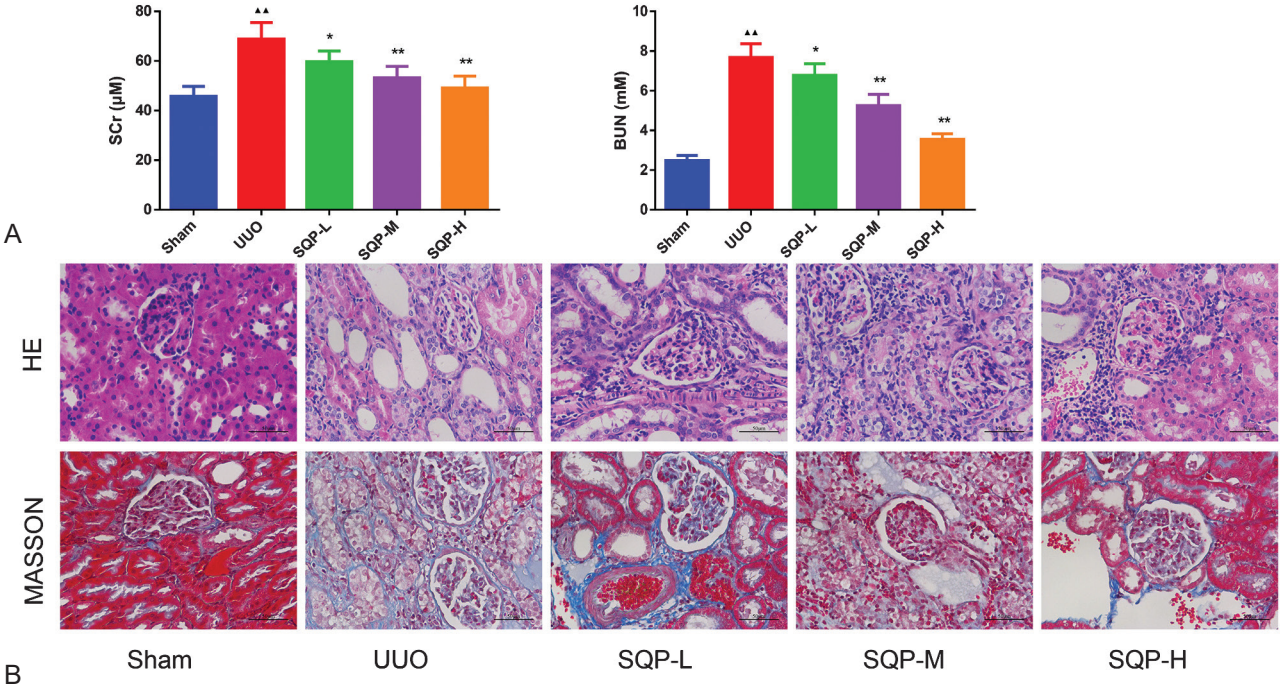

Figure 1. SQP improve the declined renal function and renal interstitial damage in UUO rat. A, The content of SCr and BUN. B. HE and MASSON staining of kidney tissue (×400).
▲$P < .05$, and ▲▲$P < .01$ *vs.* Sham
*$P < .05$, and **$P < .01$ *vs.* UUO
Results were presented as mean ± SD (n = 6).
Abbreviations: SCr, serum creatinine; BUN, blood urea nitrogen, UUO, unilateral urethral obstruction; SQP, Shenqi Pill

densitometry analysis of the immunoreactive bands was performed by Image J program software. All primary antibodies were purchased from Affinity and used at a dilution of 1:5000, expect for TNF-α (1:500) and GAPDH (1:5000).

Statistical Analysis

The histopathological examinations of the study were performed blindly by two pathology experts. The data were presented as mean ± SD and analyzed by SPSS 16.0. One-way ANOVA and SNK tests were applied for multi-group comparison. Kruskal-Wallis H test was applied, if variances were not equal; $P < .05$ was considered a statistically significant difference.

RESULTS

SQP Ameliorated Renal Function and Renal Interstitial Damage in UUO Rat

As shown in Figure 1A, the SCr and BUN levels significantly increased after the rats experienced UUO surgery and then significantly decreased upon SQP administration ($P < .05$). In addition, the results of H&E showed that the tubules and interstitium of the rats' kidney in the sham group were normal, while rats in the UUO group exhibited several features of RIF, including disrupted glomerular structure, atrophy or compensatory glomerular expansion, markedly fibrotic tubulo-interstitium and inflammatory cell infiltration. These changes were slightly or prominently improved in SQP-M or SQP-H groups (Figure 1B). Furthermore, in line with H&E results, Masson staining revealed that increased collagen deposition and area of interstitial fibrosis were seen in UUO rats, whereas the degree of interstitial fibrosis was slightly or significantly reduced in SQP-M or SQP-H groups.

SQP Blunted the Expression of Fibrotic Factors in UUO Rat

Based on the aforementioned MTT results, the effect of SQP on RIF was further checked by measuring the expression of FN and α-SMA in the obstructed kidney. As observed in Figure 2, relative to the sham group, the expression of FN and α-SMA were increased dramatically in the kidney tissue of UUO rats ($P < .01$). Nevertheless, a significant depletion of α-SMA expression were

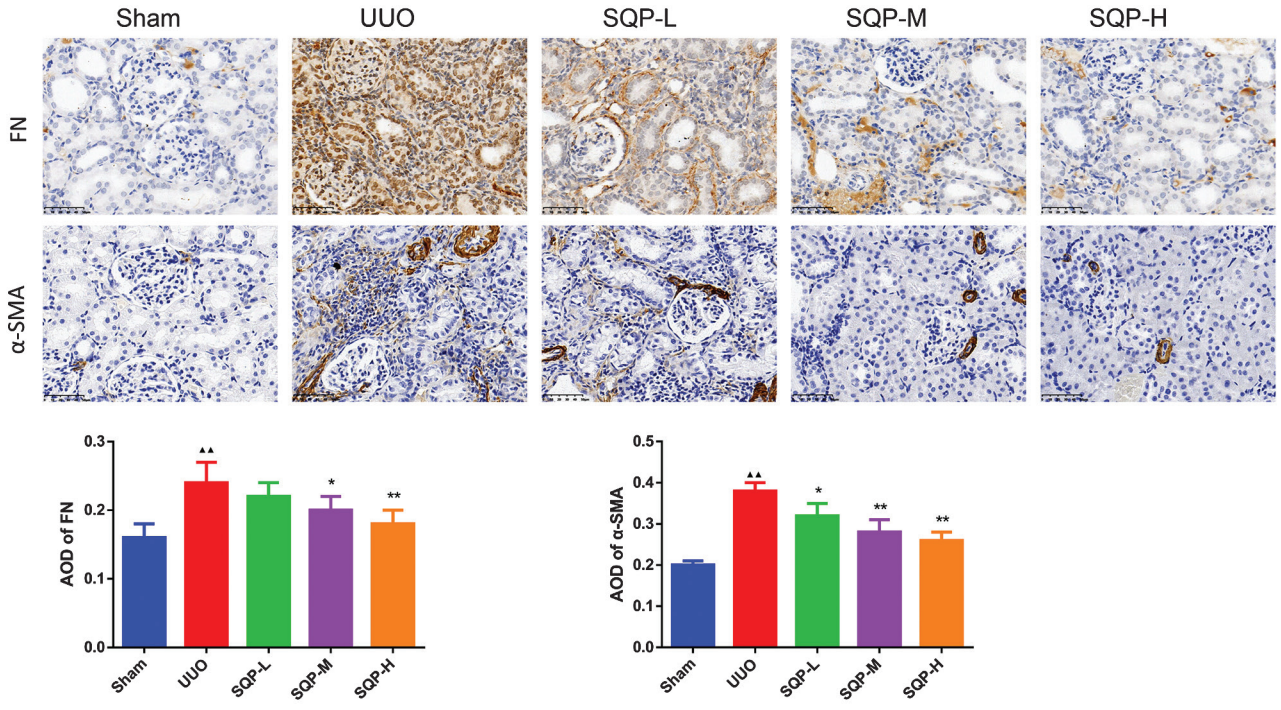

Figure 2. SQP decreased the expression of fibrotic factors in the kidney tissue of UUO rat. The expression of FN and α-SMA were detected with immunohistochemistry and all the images were original magnification ×400.
▲$P < .05$, and ▲▲$P < .01$ *vs.* Sham
*$P < .05$, and **$P < .01$ *vs.* UUO
Results were presented as mean ± SD (n = 3).
Abbreviations: UUO, unilateral urethral obstruction; SQP, Shenqi pill; AOD, average optical density

observed in UUO rats treated with 1.5 g/kg SQP ($P < .05$), the expression of α-SMA and FN were both significantly reduced following the treatment with SQP at the 3 or 6 g/kg ($P < .01$).

SQP Reduced the Release of Inflammatory Cytokines in UUO Rat

In order to investigate the impact of SQP on RIF and its involvement in the inflammatory response, we assessed the levels of IL-1β, IL-6, and TNF-α in the UUO rat. The results of ELISA revealed that the levels of IL-1β, IL-6, TNF-α were notably increased after UUO surgery and decreased by SQP administration ($P < .05$, Figure 3A). Western blot analysis revealed similar changes in the expression of TNF-α and IL-6 in kidney tissues. (Figure 3B).

SQP Suppressed the NF-κB Pathway in UUO Rat

To investigate the potential mechanisms of SQP on RIF, we examined the phosphorylation of IKBα and RelA (P65) in the kidney tissues. The Western blot analysis revealed that the phosphorylation of IKBα and P65, which was increased due to UUO, was significantly reduced by SQP therapy. ($P < .05$, Figure 4).

SQP Decreased the Viability of TGFβ1-stimulated HK-2 Cell

In the in vitro investigation, we conducted cell viability assays for HK-2 cells to ensure that the tested doses did not have a harmful effect on them. The data showed that SQP-containing serum exhibited no cytotoxicity to HK-2 cell when the concentration was under 20% (Figure 5A). Hence, 2.5, 5, and 10% SQP-containing serum were selected for subsequent experiments. To assess the potential protective effect of SQP against RIF in the *in vitro* setting, we evaluated the viability of TGFβ1-stimulated HK-2 cells with and without SQP administration. As exhibited in Figure 5B, following TGFβ1 stimulation, the viability of the HK-2 cell was remarkably increased ($P < .01$). However, the increase viability induced by TGFβ1 was markedly prevented after treating with SQP in low-to-high concentration ($P < .05$).

SQP Prevented Fibrosis in TGFβ1-stimulated HK-2 Cell

To further investigate the anti-fibrosis effect of SQP in the *in vitro* setting, we examined the expression of α-SMA on TGF β 1-stimulated HK-2 cells using immunofluorescence staining. As shown

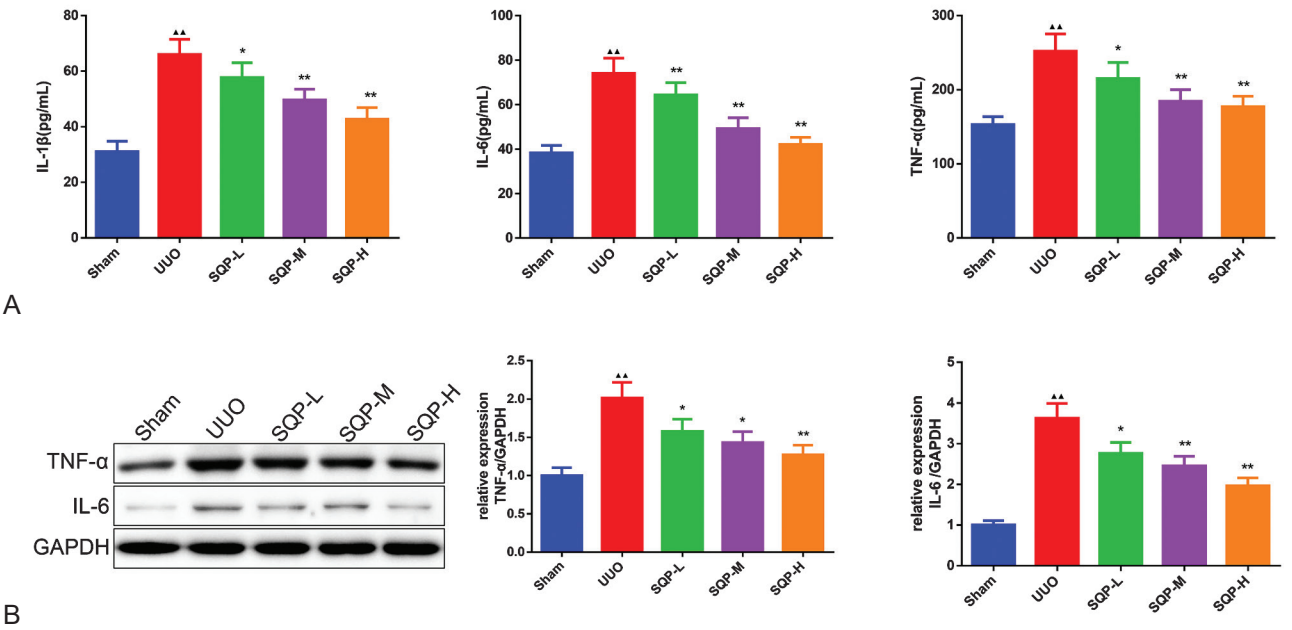

Figure 3. SQP reduced the release of inflammatory cytokines in the serum of UUO rat. A, the serum content of IL-1β, IL-6, and TNF-α were measured by ELISA (n = 6); B, the protein expression of TNF-α and IL-6 in the kidney tissue were detected by Western blot.
▲$P < .05$, and ▲▲$P < .01$ *vs.* Sham
*$P < .05$, and **$P < .01$ *vs.* UUO
Results were presented as mean ± SD (n = 3).
Abbreviations: UUO, unilateral urethral obstruction; SQP, Shenqi pill

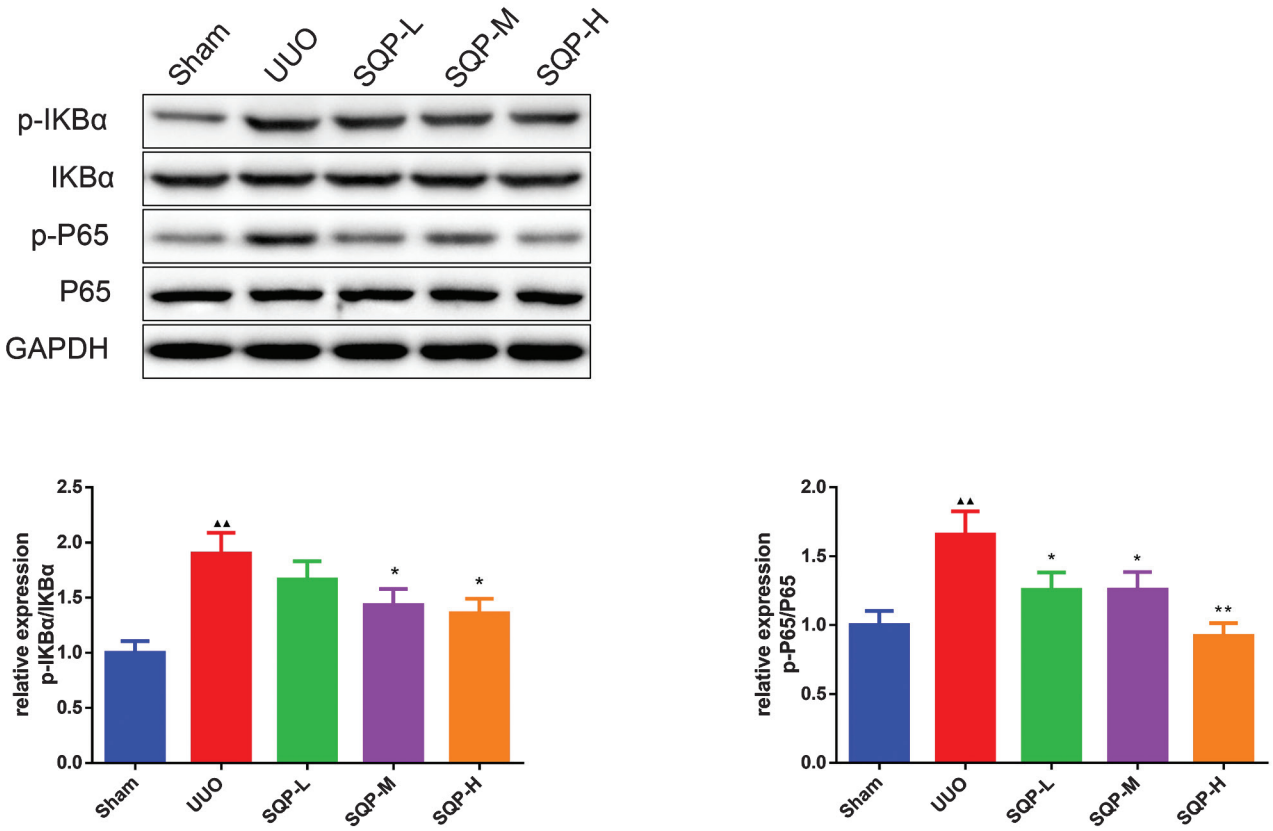

Figure 4. SQP prevented the activation of NF-κB pathway in UUO rat. The phosphorylation of IKBα and P65 were detected by Western blot.
▲$P < .05$, and ▲▲$P < .01$ *vs.* Sham
*$P < .05$, and **$P < .01$ *vs.* UUO.
Results were presented as mean ± SD (n = 3).
Abbreviations: UUO, unilateral urethral obstruction; SQP, Shenqi pill

in Figure 6, after TGFβ1 stimulation, the expression of α-SMA was distinctly elevated in HK-2 cell, while its expression was noticeably declined with SQP treatments ($P < .05$).

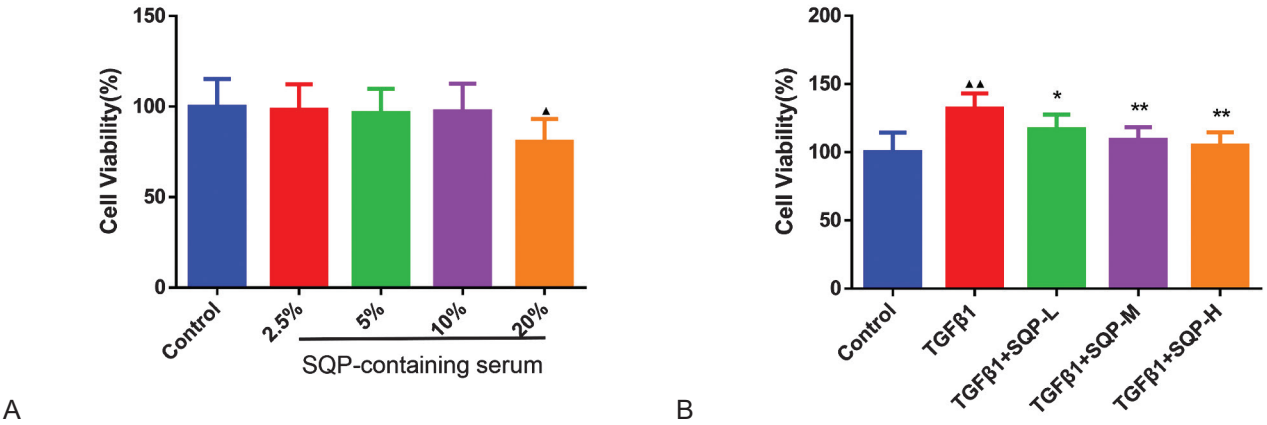

Figure 5. SQP was non-cytotoxic to HK-2 cell and decreased the viability of TGFβ1-stimulated HK-2 cell when the concentration was under 20%. The viability of HK-2 cell (A) and TGFβ1-stimulated HK-2 cell were measured by MTT.
▲$P < .05$, and ▲▲$P < .01$ *vs.* Control
*$P < .05$, and **$P < .01$ *vs.* TGFβ1
Results were presented as mean ± SD (n = 3).
Abbreviations: SQP, Shenqi pill

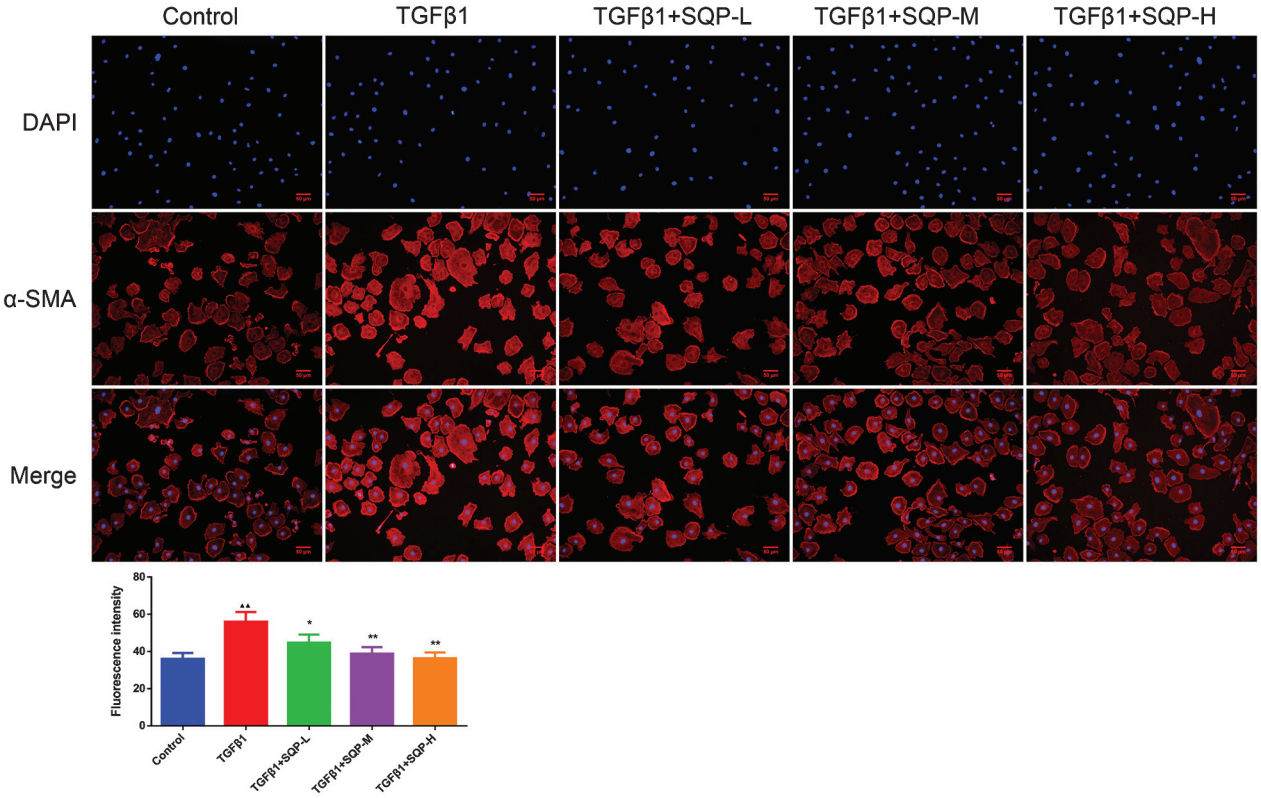

Figure 6. SQP blunted the expression of fibrotic factor in TGFβ1-stimulated HK-2 cell. The expression of α-SMA was measured by immunofluorescence staining, and the images were original magnification ×400.
▲$p < 0.05$, and ▲▲$p < 0.01$ *vs.* Control.
*$p < 0.05$, and **$p < 0.01$ *vs.* TGFβ1.
Results were presented as mean ± SD. n = 3.
Note: SQP, Shenqi Pill.

SQP Inhibited the Release of Inflammatory Cytokines in TGFβ1-stimulated HK-2 Cell

To further explore the *in vitro* anti-inflammatory effect of SQP, we measured the levels of IL-1β, IL-6, and TNF-α in HK-2 cell. We found that relative to the control group, the levels of IL-1β, IL-6, and TNF-α were upregulated considerably in TGFβ1-stimulated HK-2 cell but markedly downregulated after SQP treatment ($P < .05$, Figure 7).

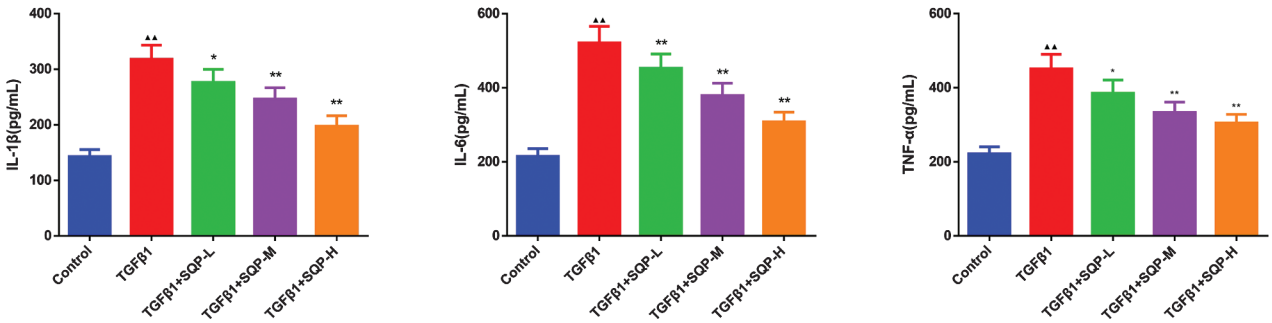

Figure 7. SQP decreased the content of inflammatory cytokines in TGFβ1-stimulated HK-2 cell. The content of IL-1β, IL-6, and TNF-α were measured by ELISA.
▲$P < .05$, and ▲▲$P < .01$ *vs.* Control
*$P < .05$, and **$P < .01$ *vs.* TGFβ1
Results were presented as mean ± SD (n = 3).
Abbreviations: SQP, Shenqi pill.

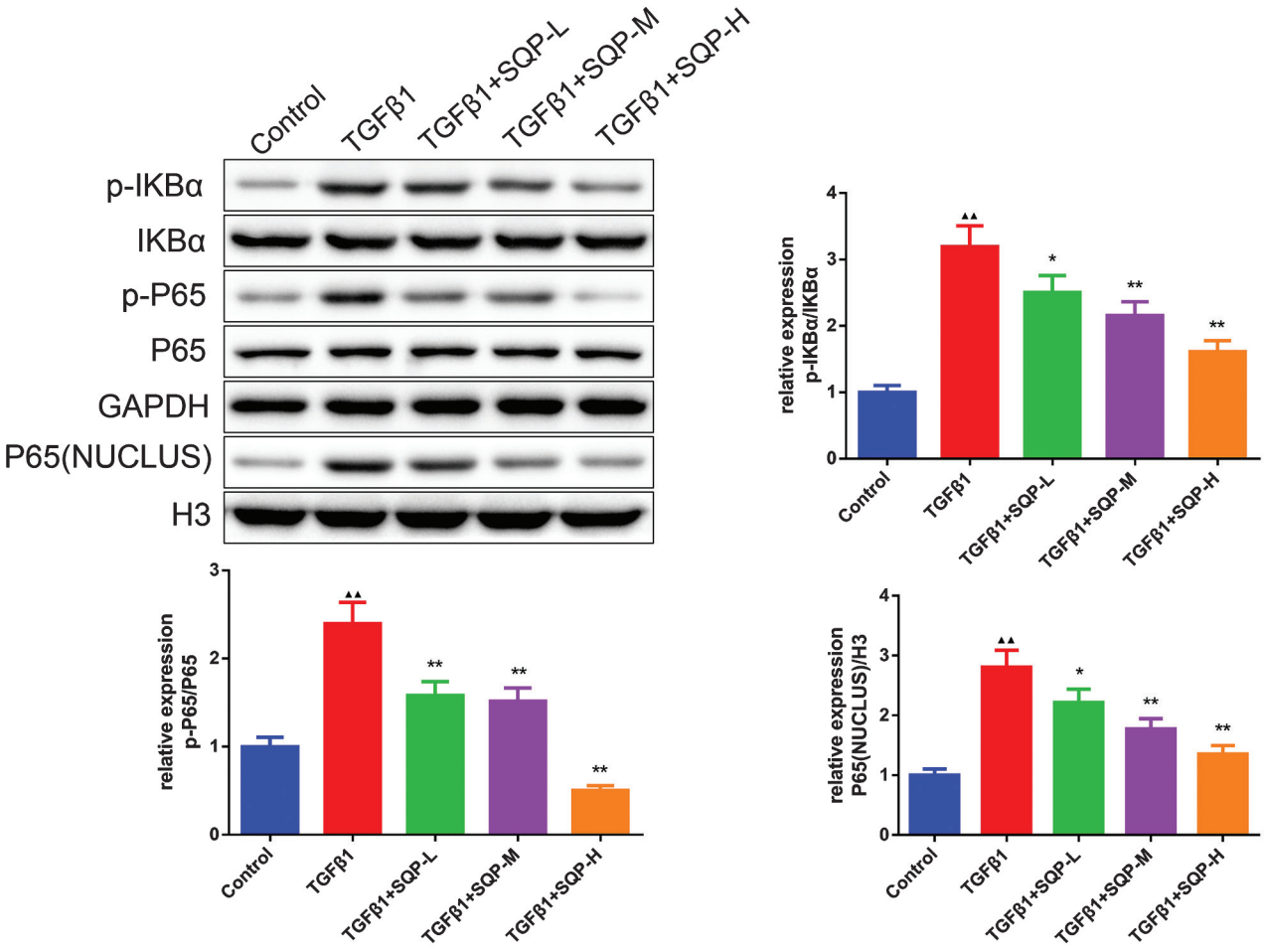

Figure 8. SQP suppressed NF-κB pathway in TGFβ1-stimulated HK-2 cell. The expression of p-IKBα, IKBα, p-P65, P65 and P65(NUCLUS) were measured by Western blot.
▲$P < .05$, and ▲▲$P < .01$ *vs.* Control
*$P < .05$, and **$P < .01$ *vs.* TGFβ1
Results were presented as mean ± SD (n = 3).
Abbreviations: SQP, Shenqi pill

SQP Blocked the NF-κB Pathway in TGFβ1-stimulated HK-2 Cell

Subsequently, we investigated the role of SQP on NF-κB pathway *in vitro*. As expected, the phosphorylation of IKBα and P65, and the expression of P65 (NUCLUS) were increased under TGFβ1 stimulation ($P < .01$, Figure 8). Similar to the study *in vivo*, the activation of NF-κB pathway was suppressed after TGFβ1-stimulated cell was treated with SQP in low-to-high dose ($P < .05$).

DISCUSSION

RIF is recognized as the most crucial pathological characteristic of CKD, which is irreversible and contributes to poor outcome.[15,16] Currently, there is no effective treatment available for RIF. SQP is a traditional prescription for improving kidney dysfunction in TCM.[11] Some studies found that the primary ingredient of SQP had a specific impact on kidney diseases, known as *Rehmannia glutinosa Libosch* (Dihuang in Chinese).[17] Huang *et al.* revealed that SQP was able to repair the injured renal tubular epithelial cells by suppressing Jag2/Notch2/hes1 pathway.[18] The results of the present study revealed that SQP exhibited a strong reno-protective effect through diminishing inflammation response regulated by NF-κB pathway in animal and cellular model of RIF.

The UUO rat model has been used for a long time as an ideal model to study RIF.[19] Ureteral obstruction causes significant changes in both renal hemodynamics and metabolism, which in turn

lead to the transformation of interstitial fibroblasts and excessive deposition of extracellular matrix.[20] The current study showed a substantial reduction in renal function indices (SCr, BUN), increased expression of fibrotic factors (FN, α-SMA) as well as renal interstitial damage following UUO in rats. However, SQP treatment not only ameliorated renal function and renal interstitial injury, but also decreased the expression of fibrotic-related factors. In order to further check the effect of SQP in RIF, we performed cellular experiments. TGFβ1 is a key driving factor of RIF, which can induce the trans-differentiation of fibroblast-myofibroblast that promotes over-deposition of collagen, and finally causes RIF.[21] Therefore, TGFβ1-stimulated HK-2 cell is frequently utilized *in vitro* to mimic and assess the pathological process of RIF.[22] Our study demonstrated that the depletion of viability and α-SMA expression in HK-2 cells after TGFβ1 stimulation were up-regulated by treating with SQP. The results indicated that SQP was able to mitigate RIF progression *in vivo* and *in vitro*.

The exact cause of RIF is still unclear, but growing evidence has documented that the inflammatory response is contributing to the progression of RIF.[23] Increased secretion of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, is correlated with renal, muscle, and intestinal fibrosis.[24-26] A research carried out by Zheng *et al.* revealed that nicotinamide could be regarded as a novel treatment option for RIF due to the inhibition of tubular damage and inflammation in RIF model *in vivo* and *in vitro*.[27] In addition, a published study demonstrated fatty acid binding protein 4 could worsen RIF by facilitating lipid metabolism disturbance and inflammation.[28] Therefore, suppression of inflammation response may have a therapeutic effect against RIF. Amounting evidence revealed that the major components of SQP, such as Poria, could reduce the release of inflammatory mediators.[29] In accordance with the prior studies, this study measured the content of IL-1β, IL-6, and TNF-α and showed that the release of these inflammatory cytokines were prevented upon treating with SQP. Correspondingly, similar results were also obtained in HK-2 cell, which further hinted that SQP might attenuate RIF via inhibiting inflammation response.

To get a more detailed understanding of the molecular mechanism of SQP in RIF, we investigated the effect of SQP on the NF-κB pathway. NF-κB is a pleiotropic signaling pathway, which is primarily known for its role in the regulation of inflammation and fibrosis.[30] Extensive studies have demonstrated that activation of NF-κB pathway in mice usually induces an inflammatory response and finally results in fibrogenesis,[30] while blocking NF-κB pathway could improve fibrosis.[31] It was reported that Babao Dan, a common TCM for chronic liver diseases, could decrease the expression of inflammatory cytokines as well as α-SMA by suppressing the Toll-like receptor 4 (TLR4)/NF-κB pathway in hepatic fibrosis rats.[32] As expected, the results in the present study found that SQP down-regulated the phosphorylation of IKBα and P65 in UUO rats and TGFβ1-stimulated HK-2 cell, which further demonstrated that SQP might exhibit anti-RIF effect by suppressing the NF-κB pathway.

However, there are two limitations to our study. First of all, we did not perform experiments to determine the time-dependent effect of SQP in our cell models. The other was antagonist of NF-κB pathway which was not used to verify the effect of SQP in RIF. In the future, we will conduct time-dependent study and apply the antagonist of NF-κB pathway to better verify the results.

CONCLUSION

In summary, our results revealed the function and detailed mechanism of SQP in RIF. Specifically, SQP exhibited an obvious protective effect on RIF *in vivo* and *in vitro*, and the possible mechanism may be associated with the suppression of inflammation response regulated by NF-κB pathway. All of these findings suggest that SQP may serve as a new agent for treating RIF, and NF-κB pathway may be a critical target for RIF therapy.

ACKNOWLEDMENTS

Not applicable.

ETHICAL CONSIDERATIONS

All animal experiments were performed with the approval of the Animal Experimentation Ethics Committee of Zhejiang Eyong Pharmaceutical Research and Development Center (Certificate No. SYXK (Zhe) 2021-0033), and the experiments were conducted according to the guidelines of the Chinese Council on Animal Care.

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COMPETING INTERESTS

The authors declare no conflicts of interest.

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