Acteoside Alleviates Renal Fibrosis by Inhibiting β-Catenin/CTGF Signaling Pathway in UUO Rats

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Abstract

Objective: Acteoside (ACT) has been reported to regulate the inflammation and immune response. The study aims to explore the effect of ACT on renal fibrosis in unilateral ureteral obstruction (UUO) rats. Methods: Eighteen Sprague-Dawley rats were randomly divided into 3 groups: sham group, opened the abdominal cavity and sutured abdominal; UUO group, performed UUO surgery; and ACT + UUO group, ACT (40 mg/kg) was given by gavage every day after UUO surgery. After 2 weeks of rat model construction, urine and blood samples were collected for biochemical analysis, while kidney tissues were harvested for hematoxylin and eosin (H&E), Masson’s trichrome, and immunohistochemistry staining. The expression of connective tissue growth factor (CTGF), alpha smooth muscle actin (α-SMA), collagen III, heat shock protein 47 (HSP47), and β-catenin in the renal tissue was detected and the correlation between these proteins was analyzed. Results: ACT improved the parameters of renal function in UUO rats, including decreased creatinine and urea nitrogen, and declined urinary protein. Pathological analysis suggested that ACT improved the conditions of renal tubule lesion (including structure destruction, atrophy and lumen obstruction), renal interstitial fibrosis and inflammatory cell infiltration in UUO rats. It also down-regulated the expressions of fibrin-related proteins β-catenin, CTGF, α-SMA, collagen III, and HSP47. Correlation analysis found that β-catenin and CTGF were correlated with the expressions of α-SMA, collagen III, and HSP47. Conclusions: ACT could alleviate renal fibrosis in UUO rats probably via inhibiting β-catenin/CTGF signaling pathway.

Keywords

acteoside, renal fibrosis, β-catenin, connective tissue growth factor

Renal fibrosis caused by urinary tract obstruction is one of the causes of long-term obstructive nephropathy. The number of people with obstructive nephropathy caused by kidney stones accounts for 1%~15% in the world and 6.1%~6.4% in China.1,2 In addition to timely surgical treatment, the treatment of chronic and progressive fibrosis is limited. Long-term chronic inflammation creates a unique fibrosis environment for the occurrence of renal fibrosis.3 Renal fibrosis is the common pathological basis of all chronic kidney disease (CKD), and many factors are involved in the course of renal interstitial fibrosis.3 Acteoside (ACT) is a kind of phenylethanoid glycoside that was first isolated from mullein. It is later found in many medicinal plant families in many countries, such as five Lippia species in South Africa, Nucranica L. in Kazakhstan and Verbenaceae and Plantago species in China.4-6. In particular, it has a high content in Rehmannia glutinosa L. from mullein. It is later found in many medicinal plant families in many countries, such as five Lippia species in South Africa, Nucranica L. in Kazakhstan and Verbenaceae and Plantago species in China.4-6 In addition, it has a high content in Rehmannia glutinosa L. which is widely used in traditional Chinese medicine (TCM). ACT has antioxidant, anti-inflammatory, and other biological activities and is used in the treatment of a variety of diseases,5,7 but its anti-renal fibrosis remains to be further explored. It has been confirmed that β-catenin plays an important role in renal fibrosis.8 Connective tissue growth factor (CTGF) regulates fibrotic collagen III and alpha smooth muscle actin (α-SMA) by regulating β-catenin signaling pathway and its downstream target gene.8 ACT can reduce the β-catenin signaling pathway of glioma to inhibit tumor, but its effect on renal fibrosis and β-catenin signaling is unknown.10 The purpose of this study was to investigate the effects of ACT on UUO renal fibrosis and whether it regulates β-catenin/CTGF signaling pathway.

Materials and Methods

Materials and Reagents

Sprague-Dawley (SD) rats were purchased from Animal Experiment Center of Chongqing Medical University. Rabbit

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Experimental Design

Eighteen male SD rats aged 6 to 8 weeks, weighing 180 to 220 g, were fed cleanly. This study was approved by Guizhou Provincial People’s Hospital for animal experiment ethics review. The modeling of the rat model of unilateral ureteral obstruction (UUO) was based on the study of Song et al.11

The specific steps were as follows: rats were anesthetized with 4% pentobarbital sodium intra-abdominal injection. Unilateral ureter was ligated and cut. SD rats were divided into 3 groups with 6 rats in each group: (1) Sham group (SHAM group), abdominal cavity was opened and sutured; (2) UUO group, UUO; (3) ACT + UUO group, after UUO, 40 mg/kg ACT was administered intragastric every day.12

UUO group was given the same amount of normal saline every day. Two weeks after the operation, urine was collected in metabolic cage to detect urinary protein content. Blood was collected from the abdominal aorta and the serum was separated for the determination of creatinine and urea nitrogen. All rats were sacrificed, kidney tissue was taken, fixed with 10% neutral formaldehyde solution, embedded in paraffin, and sectioned for subsequent detection.

Biochemical Index Detection

The collected blood was tested for creatinine and urea nitrogen with a biochemical detector, and the urine protein content was tested with a biochemical detector.

Histopathological Staining

Paraffin sections of 4 μm kidney tissue were taken. H&E and Masson dewaxed, rehydrated, and stained kidney slices. Among them, H&E staining was used to observe renal tissue morphology. Then, the degree of renal fibrosis was observed by Masson trichromatic staining.

Immunohistochemistry

4 μm paraffin section of kidney tissue was taken and dewaxed to water. To remove endogenous peroxidase, drop 3% H₂O₂ and react at room temperature for 10 min. Immersed in 0.01 M citric acid repair solution (pH 6.0), sealed pressure cooker, holding pressure for 2 min, then deflated and depressurized. Appropriate amount of primary antibody (rabbit anti-mouse β-catenin antibody, 1:400; α-SMA antibody, 1:1000; mouse anti-CTGF antibody, 1:200; collagen III antibody, 1:500; HSP47 antibody, 1:200) solution was incubated overnight at 4°C. Appropriate anti-rabbit IgG (1:200) or anti-mouse IgG (1:200) solution was added and incubated at room temperature for 30 min. 3,3'-Diaminobenzidine (DAB) color rendering, cleaning, dehydration, transparency, sealing. The average optical density (OD) value of DAB-positive reactants detected by Image-Pro Plus 6.0 software reflects the expression of detected protein molecules in the kidney tissues.

ELISA

Add 100 μL recombinant human standard and rat serum to 96-well plates, incubate for 2 h at room temperature, and wash for 3 times. Add 200 μL anti-mouse IL-6 (interleukin 6) or IL-18 (interleukin 18) antibody coupled with horseradish peroxidase, and incubate at room temperature for 2 h, then wash 3 times. Add 200 μL of chromogen substrate mixture, and incubate at room temperature away from light for 20 min. Add 50 μL reaction stop solution, change color from blue to yellow. The average OD value was read at 450 nm and corrected at 570 nm. The concentration of IL-6 or IL-18 was calculated according to the standard curve.

Statistical Analysis

SPSS 19 software was used to analyze statistics, t-test was used for homogeneity of variance of variables, t-test was used for measurement data conforming to normal distribution between 2 groups, and one-way analysis of variance test was used for more than 2 g IL-6 or IL-18 groups. Rank sum test is used for non-normal distribution of measurement quality. Counting data were tested by chi-square. Dose data were expressed as (X ± SD), t < .05, the difference was statistically significant. Spearson analysis was used for correlation analysis.

Results

The Biochemical Parameters of Each Group

UUO rat model was developed and evaluated with renal function parameters. Compared with the sham group, serum creatinine, urea nitrogen, and urinary protein were significantly increased in model group (P < 0.05). The biochemical parameters of the ACT + UUO group were greatly reduced in comparison with those in the UUO group (P < .05, Table 1).

ACT Modified UUO-induced Renal Pathological Changes

The results of H&E and Masson’s trichrome staining in renal tissue sections showed that the renal tubules in the sham group were complete and clear, and the epithelial cells of renal tubules were closely connected with each other and neatly arranged, without interstitial fibrosis. In UUO group, renal tubular lesions mainly showed tubular structure destruction, tubular dilatation or tubular atrophy, obvious fibrosis
The results showed that proteins in rat kidney tissue by immunohistochemistry staining. concentration showed a downward trend, but there was no statistically significant difference (Figure 2). Compared with UUO group, serum IL-6 in ACT + UUO group was increased in UUO and ACT + UUO group difference (Figure 2A), IL-18 concentration showed a downward trend, but there was no statistical difference (Figure 2B).

**ACT Reduced Serum Inflammation Cytokines**

Detected the expressions of cytokines IL-6 and IL-18 in serum by enzyme-linked immunosorbent assay (ELISA) and the results showed that the expressions of them were relatively low in sham group, while the concentration of cytokines was increased in UUO and ACT + UUO group difference (Figure 2). Compared with UUO group, serum IL-6 in ACT + UUO group was significantly decreased (Figure 2A), IL-18 concentration showed a downward trend, but there was no statistical difference (Figure 2B).

**ACT Attenuated Renal Fibrosis in UUO Rats**

Detected the expression of α-SMA, Collagen III and HSP47 proteins in rat kidney tissue by immunohistochemistry staining. The results showed that α-SMA, collagen III, and HSP47 proteins were almost not expressed in sham group. The protein levels of α-SMA, collagen III, and HSP47 in UUO and ACT + UUO group were increased (Figure 3A). The average optical densities of α-SMA, collagen III, and HSP47 proteins in ACT + UUO group were decreased and increased in UUO rats, and the expression of β-catenin and CTGF protein in the kidney tissues of ACT + UUO group was both increased and decreased (Figure 4). Immunohistochemical mean optical density statistics of β-catenin and CTGF showed that, compared with sham, the mean optical density of β-catenin and CTGF protein in the kidney tissues of UUO group was increased, P < .01, with significant statistical difference (Figure 4). Compared with UUO group, the average optical density of β-catenin and CTGF protein in the kidney tissue of ACT + UUO group was decreased, and P < .05, with statistical difference (Figure 4).

Correlation analysis showed that β-catenin was positively correlated with α-SMA and HSP47 protein expression, with correlation coefficients of 0.79 and 0.84, P < .001 (Figure 5). The protein expression of CTGF was positively correlated with α-SMA, collagen III, and HSP47, with correlation coefficients of 0.90, 0.79, and 0.97, P < .001 (Figure 5). Collagen III had no significant correlation with β-catenin, the correlation coefficients were 0.57, P < .05 (Figure 5).

**Discussion**

TCM plays an important role in the treatment of CKD, showing comprehensive treatment advantages in anti-inflammatory, anti-oxidant, reducing urinary protein, protecting renal function, and delaying renal fibrosis. ACT has a wide range of pharmacological activities, such as immune regulation, anti-oxidation, anti-apoptosis, anti-hypertension, renal function protection, and so on. Its antioxidant effect is mainly achieved through scavenging biological free radicals, chelating metal ions, inhibiting lipid per-oxidation and enhancing endogenous antioxidant defense of the body. At present, a number of studies have confirmed that ACT can improve renal function in diabetic nephropathy and improve renal disease.

Long-term chronic inflammation is the main influencing factor of renal tissue fibrosis in CKD. In this paper, the expression of inflammatory factors IL-6 and IL-18 was detected, and the results showed that the expression of IL-6 and IL-18 increased significantly in UUO rats, and the expression of IL-6 decreased after treatment with ACT. It was confirmed that ACT can reduce the expression of inflammatory factor IL-6, and Nam et al also confirmed that ACT can reduce the expression of IL-6 in LPS (lipopolysaccharide) induced THP-1 cells. However, there was no statistical difference in the effect of ACT on the expression of IL-18, which may be due to the small sample size, but the effect of ACT on the expression of IL-18 needs to be confirmed by more studies.

Although studies have shown that ACT can have a variety of functions, Khullar et al confirmed that ACT can inhibit liver fibrosis. ACT can effectively alleviate the pathological changes of diabetic nephropathy, such as glomerular hypertrophy, basal membrane thickening, extracellular matrix accumulation, and renal interstitial fibrosis. In our previous study, ACT alleviated renal fibrosis in CKD rats by inhibiting macrophage infiltration, macrophage infiltration related factors such as CD68+, F4/80+ cells, and suppressor of cytokine signaling-3 (SOCS3) were

| Table 1. Biochemical Indices of Renal Function in Rats. |
|-----------------|----------------|----------------|
| Group           | Urea nitrogen  | Urinary protein | Serum creatinine |
|                 | (mg/dL)        | (mg/mL)        | (mg/dL)         |
| Sham            | 48.95 ± 3.22   | 6.54 ± 0.38    | 0.55 ± 0.21     |
| UUO             | 95.78 ± 6.52   | 11.37 ± 1.04   | 2.68 ± 0.67     |
| ACT+UUO         | 77.42 ± 4.44   | 8.36 ± 0.57    | 2.23 ± 0.54     |

*p < .05 versus sham; **P < .05 versus UUO.*
significantly down-regulated after ACT treatment.\textsuperscript{19} In this study, H&E and Masson staining results showed that ACT could reduce inflammatory cell infiltration and interstitial fibrosis in the renal tissues of UUO rats after treatment with it. This result supports the conclusion of Tao et al’s study on ACT in diabetic nephropathy, ACT can reduce renal fibrosis in CKD.\textsuperscript{20} In this study, the expressions of $\alpha$-SMA, collagen III, and HSP47 in the kidney tissues of UUO rats were up-regulated, and the expressions of $\alpha$-SMA, Collagen III and HSP47 in kidney tissues of UUO rats were down-regulated by ACT. These results further confirmed the therapeutic effect of ACT on renal fibrosis in UUO rats. At present, the author did not find any studies that confirmed the effects of ACT on the expression of $\alpha$-SMA, collagen III, and HSP47, but some studies confirmed that ACT can reduce the expression of TGF-$\beta_1$, a renal fibrosis regulator.

Although the role of ACT in renal fibrosis has been confirmed, the mechanism needs further study. The $\beta$-catenin signaling pathway is involved in almost every aspect of embryonic development and also controls the balance of self-renewal in many tissues.\textsuperscript{21} Recently, new evidence has been developed suggesting that sustained $\beta$-catenin pathway overactivation is associated with the pathogenesis of fibrotic diseases, such as pulmonary fibrosis, liver fibrosis, skin fibrosis, and renal fibrosis.\textsuperscript{8} In addition, CTGF is a member of the CCN protein family and has been found to be highly expressed in many chronic kidney diseases, which is closely related to renal fibrosis in UUO. Some studies have confirmed that CTGF, as a promoter of renal fibrosis, is directly regulated by the $\beta$-catenin signaling pathway. In our study, the expression of $\beta$-catenin/CTGF was significantly increased in the renal tissues of UUO rats, and the expression of

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Effect of acteoside on renal histopathology of UUO rats. H&E staining (×400), Masson’s trichrome staining (×400)}
\end{figure}

\begin{figure}
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\caption{Effect of acteoside on renal serum inflammatory factors in UUO rats. Serum cytokines was detected by ELISA. A—\textsuperscript{a}: P < .01 versus sham group; b: P < .01 versus UUO group. B—\textsuperscript{a}: P < .05 versus sham group; b: P > .05 versus UUO group.}
\end{figure}
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β-catenin/CTGF was significantly decreased after treatment with ACT. Jia et al. also confirmed that ACT can block the β-catenin signaling pathway of glioblastoma, inhibit the invasion, migration and proliferation of glioblastoma cells, and promote their apoptosis and autophagy, thus achieving anti-tumor effects. β-catenin signaling can promote the expression of α-SMA, collagen III, and HSP47 in fibroblasts. In this study, ACT can down-regulate the expression of α-SMA, collagen III, and HSP47 in the renal tissues of UUO rats and reduce renal fibrosis in UUO rats.

**Figure 3.** Effect of acteoside on renal fibrosis in UUO rats. α-SMA, collagen III, and HSP47 protein levels in the kidney of different experimental groups. A—Expression of α-SMA, collagen III, and HSP47 in the renal tissues (immunohistochemical staining, ×400). B—Average optical density analysis of α-SMA immunohistochemical staining—a: P < .01 versus SHAM group; b: P < .01 versus UUO group. C—collagen III immunohistochemistry analysis of—a: P < .01 versus sham group; b: P < .05 versus UUO group. D—Average optical density analysis of HSP47 immunohistochemical staining—a: P < .01 versus sham group; b: P < .01 versus UUO group.

**Figure 4.** Effects of acteoside on β-catenin/CTGF signaling pathway in the renal tissues of UUO rats. A—Expression of β-catenin and CTGF in renal tissue (immunohistochemical staining, ×400). B—Average optical density analysis of β-catenin immunohistochemical staining—a: P < .01 versus sham group; b: P < .05 versus UUO group. C—Average optical density analysis of CTGF immunohistochemical staining—a: P < .01 versus sham group; b: P < .05 versus UUO group.
There may be some potential limitations in this study. Firstly, we only evaluated the proinflammatory cytokines IL-6 and IL-18, didn’t test their anti-inflammatory counterparts, this weakens the argument for the anti-inflammatory effects of ACT. Secondly, the study focused on fibrin-related proteins, fibrosis downstream signaling pathways were not shown. Therefore, the protective mechanism of ACT against CKD remains to be further studied.

Conclusion

In this study, we found that ACT improved the parameters of renal function in UUO rats, including decreased creatinine and urea nitrogen, and declined urinary protein. Pathological analysis suggested that ACT improved the conditions of renal tubule lesion (including structure destruction, atrophy, and lumen obstruction), renal interstitial fibrosis, and inflammatory cell infiltration in UUO rats. It also down-regulated the expressions of fibrin-related proteins β-catenin, CTGF, α-SMA, collagen III, and HSP47. Correlation analysis found that β-catenin and CTGF were correlated with the expressions of α-SMA, collagen III, and HSP47. ACT could alleviate renal fibrosis in UUO rats probably via inhibiting β-catenin/CTGF signaling pathway.

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Declaration of Conflicting Interests

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