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Original Research Article

Catalpol attenuates EMT by inhibiting Wnt/β-catenin and TGF-β/Smads signaling to alleviate kidney fibrosis

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Abstract

Purpose: *To determine the anti-fibrosis effect and underlying mechanism of action of catalpol (CAT) in chronic kidney disease (CKD).*
Methods: *Forty (40) rats were randomly divided into a sham group (10 rats) and a unilateral ureteral obstruction (UUO) model group (30 rats) which was further randomly subdivided into three groups of ten (10) rats each: the UUO model group, UUO + CAT low-dose group, and UUO + CAT high-dose group. HK-2 cells were stimulated with TGF-β1 for in vitro studies. Renal injury and fibrotic lesions were determined by H&E and Masson's staining. Key proteins of TGF-β/Smads and Wnt/β-catenin signaling pathways involved in epithelial-mesenchymal transition (EMT) were determined by immunohistochemistry, immunofluorescence staining and Western blotting.*
Results: *Catalpol downregulated the expression of α-SMA ($p < 0.05$) and upregulated the expression of E-cadherin ($p < 0.05$) stimulated by TGF-β1 and LiCl in HK-2 cells, which is consistent with the role of DKK1 in vitro. CAT ameliorated renal fibrosis and repressed the expression of key proteins of TGF-β/Smads and Wnt/β-catenin pathways in UUO rats.*
Conclusion: *Catalpol inhibits EMT and alleviates kidney fibrosis by suppressing the hyperactivation of TGF-β/Smad and Wnt/β-catenin signaling pathways. Therefore, CAT is a promising therapeutic drug for renal fibrosis.*

Keywords: *Catalpol, Renal fibrosis, Wnt/β-catenin signaling pathway, Epithelial-mesenchymal transition (EMT), TGF-β/Smads signaling pathway*

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INTRODUCTION

The proportion of chronic kidney disease (CKD) is increasing worldwide, with 10.8 % of Chinese adults having some degree of CKD [1]. Renal fibrosis, a hallmark and common feature of all types of CKD, contributes to the deterioration of renal function ultimately resulting in end-stage

renal failure (ESRD) [2]. Accordingly, inhibiting the development of renal fibrosis is crucial for CKD patients and this has been taken as an effective way to delay the progression of CKD and reduce the high treatment costs paid for alternative therapies, such as renal dialysis and kidney transplantation. At present, existing drugs are unable to reverse the course of renal fibrosis; hence, it is necessary to find accessible and effective therapeutic methods to prevent renal fibrosis generation and CKD progression.

Catalpol, the first iridoid glycoside monomer isolated from *Rehmannia glutinosa (Gaertn.) DC.* (RG), is also the primary iridoid compound of the aqueous extracts of dried RG [3]. CAT has a variety of pharmacological actions, such as renal protection against adriamycin-induced nephropathy [4], diabetic nephropathy [5] and acute kidney failure [6]. Strikingly, CAT attenuated pulmonary fibrosis as well as liver fibrosis [7,8]. Preliminary studies revealed that dried RG has a higher CAT content and exhibits more antifibrotic action than steamed RG. In addition, it has been shown that the anti-fibrotic potential of CAT is involved in the regulation of Wnt/β-catenin signaling *in vitro* [9]. In this context, this study was designed to further elucidate the effect of CAT on renal fibrosis and determine its potential mechanism of action in order to provide a reference for the clinical application of CAT.

EXPERIMENTAL

Materials and reagents

Catalpol was purchased from PureChem-Standard Co. Ltd. (China). An immortalized HK-2 cell line (human kidney proximal tubule epithelial cell; ATCC® CRL-2190™) and K-SFM (Keratinocyte serum-free medium; Gibco 17005-042) were purchased from the Institute of Biochemistry and Cell Biology (China). Monoclonal anti-actin, α-smooth muscle (α-SMA, A5228), anti-β-catenin (C2206) and Snail (SAB5700796) antibodies were purchased from Sigma-Aldrich Chemical Co. (USA). Anti-E-cadherin (14472S), anti-Wnt3a (2721S) and anti-vimentin (D21H3) were purchased from Cell Signaling Technology (USA) and p-GSK3β (ab75745) antibodies were purchased from Abcam. A mouse monoclonal antibody against TGF-β1 (sc-130348) was purchased from Santa Cruz Biotechnology, Inc. (USA). Rabbit polyclonal antibodies against Smad2/3 (bs-3484R) were purchased from Bioss (China). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Zhongshan Golden Bridge (China).

Cell culture and treatment

Human kidney proximal tubule epithelial (HK-2) cells were cultured in K-SFM supplemented with the K-SFM kit and maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. Cells stimulated with TGF-β1 (10 ng/mL) were co-cultured with CAT, LiCl or DKK1 for 24 h.

Animal protocol

Forty (40) male Sprague-Dawley rats (8 weeks old, 200 ± 20 g) were maintained at the animal care facility of the Chongqing Engineering Technical Research Center for the Characteristic Diagnosis and Treatment of Traditional Chinese Medicine (TCM) [10]. The rats were provided standard rodent chow and water *ad libitum*. The study was approved by the Animal Care and Use Committee of the Chongqing Hospital of TCM (approval no. 2018-DWKY-WQ) and followed international guidelines for animal studies [11].

Rat models were prepared using the unilateral ureteral obstruction (UUO) method, which was performed as described previously [12]. Sham-operated rats received identical surgical procedures except that the left ureter was simply manipulated without ligation. UUO model rats were further randomly divided into 3 groups (n = 10 per group) based on the treatments: vehicle only; Low dose CAT group (100 mg/kg) and High dose CAT group (200 mg/kg). Catalpol was dissolved in purified water and administered by oral gavage from post-UUO on days 1 to 2 or 14 days. The sham group was administered vehicle only. Five rats in each group were sacrificed on day 2 after treatment and the other 5 rats were sacrificed on day 14. Kidney tissues were collected for pathological examination and Western blot analysis.

Histological analysis

Kidney samples were collected from the rats under anesthesia. The samples were fixed in 4 % paraformaldehyde, embedded in paraffin and sectioned at 5μm thickness. Hematoxylin and eosin staining (H&E staining) and Masson's trichrome staining were performed according to the standard procedure while images were acquired with confocal microscopy (Leica, Germany).

Immunohistochemical studies

After deparaffinization, the sections were incubated in 3 % H_2O_2 deionized water and microwaved to retrieve the antigens. Next, the paraffin sections were incubated with primary

antibodies overnight at 4 °C after they were blocked with goat serum. Thereafter, they were treated with secondary antibody of streptavidin-HRP conjugation followed by diaminobenzidine (DAB) staining, hematoxylin counterstaining and gummy neutral balsam mounting. Finally, images were acquired with confocal microscopy (Leica, Germany).

Western blot

Kidney samples were minced and polished in RIPA buffer containing protease inhibitors on ice. HK-2 cells were lysed in RIPA buffer containing a protein inhibitor cocktail. The lysates were subjected to centrifugation at 13,000 × g for 15 minutes at 4 °C. The supernatant was collected into a new tube, protein concentration was determined by a BCA protein assay kit and the supernatants were analyzed by Western blot. In brief, the proteins were separated by SDS-PAGE in 10 % gels and transferred onto PVDF membranes (Millipore, USA). Then, the PVDF membranes were blocked with 5 % non-fat milk and individually incubated with primary antibodies against α-SMA (1:1000), vimentin (1:1000), Wnt3a (1:1000), β-catenin (1:1000), p-GSK3β (1:1000), Snail1 (1:1000), TGF-β1 (1:500), Smad2/3 (1:1000) and GAPDH (internal control) at 4 °C overnight. After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 hour at room temperature and protein bands were visualized with ECL (Beyotime Institute of Biotechnology, China) and determined by a ChemiDoc™ XRS+ imager with ImageLab™ software (Bio-Rad Laboratories, USA).

Statistical analysis

All data are reported as mean ± standard deviation (SD). Statistical analyses of the data were performed using GraphPad Prism software. Comparisons between groups were made using one-way ANOVA and statistical significance was set at $p < 0.05$.

RESULTS

CAT attenuates EMT in TGF-β1-stimulated HK-2 cells

Epithelial-mesenchymal transition (EMT) is a process characterized by both loss of epithelial traits and gain of mesenchymal characteristics in epithelial cells. This transition promotes excessive generation of fibroblast and myofibroblast and is accompanied by an increased expression of vimentin and α-SMA as well as a decreased expression of E-cadherin [13].

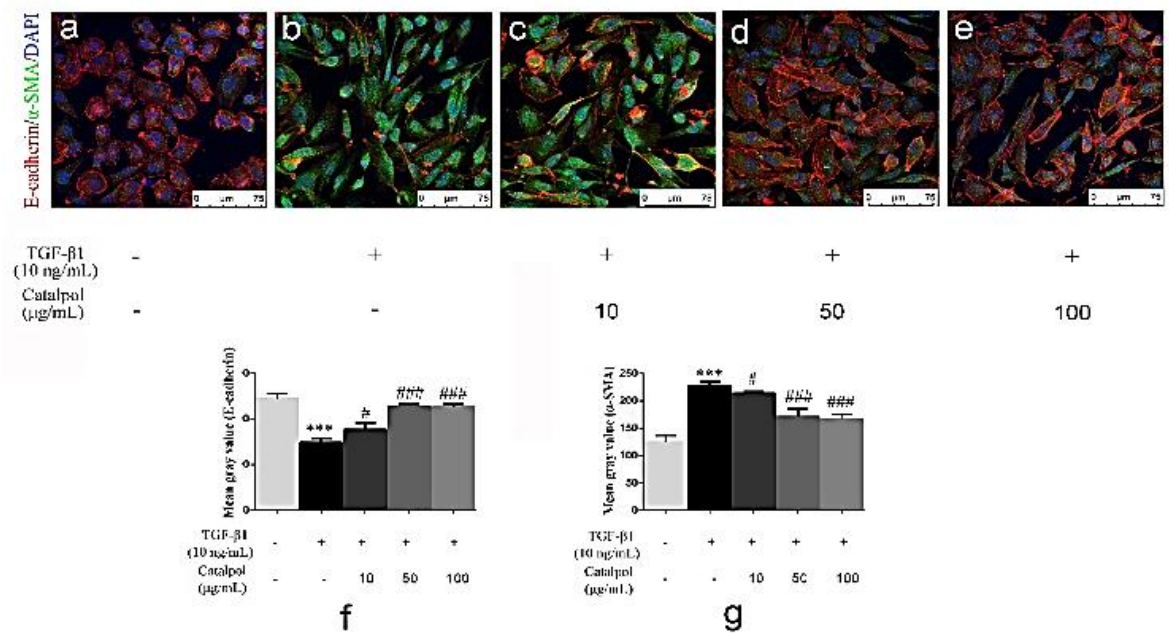

Figure 1: Catalpol inhibits EMT in HK-2 cells. The representative images of immunofluorescence staining of HK-2 cells (a) without the stimulation of TGF-β1, (b) TGF-β1-treated HK-2 cells, (c-e) TGF-β1-treated HK-2 cells with CAT (20X). The relative intensity of immunofluorescence evaluated using ImageJ (f-g). *$P < 0.05$ vs. control group, #$p < 0.05$ vs. TGF-β1-treated group

Adding TGF-β1 stimulation to HK-2 cells is a classical model for studies related to EMT. In this study, therefore, the intensities of α-SMA and E-cadherin immunofluorescence were determined to estimate the inhibitory action of CAT on EMT induced by TGF-β1 in HK-2 cells. Compared with HK-2 cells without TGF-β1 stimulation, TGF-β1-stimulated HK-2 cells had increased expression of α-SMA ($p < 0.05$), and decreased expression of E-cadherin ($p < 0.05$; Figure 1). However, compared with TGF-β1-induced HK-2 cells, CAT significantly depressed the expression of α-SMA ($p < 0.05$) and upregulated the expression of E-cadherin ($p < 0.05$) in a dose-dependent manner (Figure 1), suggesting that CAT represses EMT progression stimulated by the TGF-β1.

CAT inhibits hyperactivation of Wnt/β-catenin pathway

When HK-2 cells were stimulated with TGF-β1 and LiCl (the activator of Wnt/β-catenin signaling), the expression levels of α-SMA were found to increase significantly. In contrast, E-cadherin was found to significantly decrease ($p < 0.05$) when compared with the pre-treatment levels. However, CAT significantly reversed this observation in HK-2 cells ($p < 0.05$; Figure 2 a - e). Furthermore, CAT and DKK1 (the inhibitor of Wnt/β-catenin signaling pathway) both significantly reduced α-SMA expression and enhanced E-cadherin expression in TGF-β1-induced HK-2 cells.

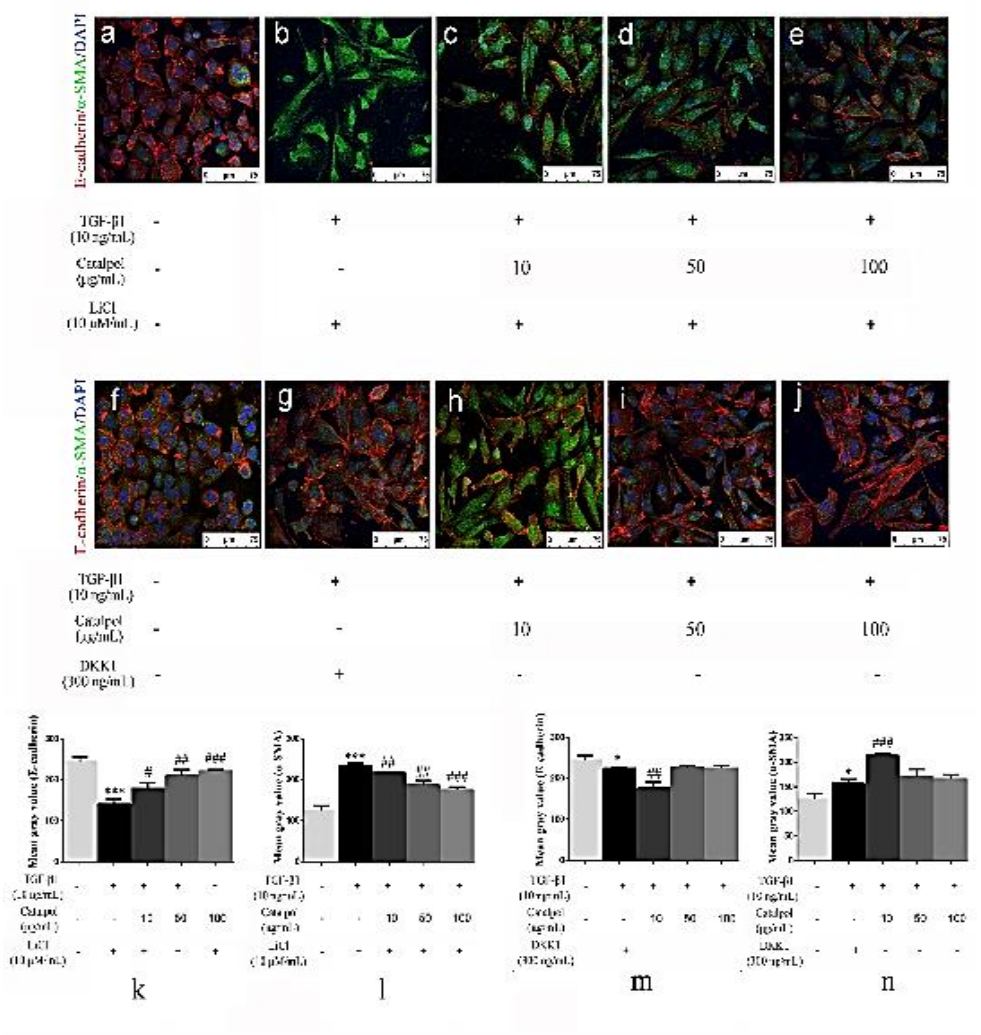

Figure 2: Catalpol inhibits EMT in TGF-β1-treated HK-2 cells by blocking Wnt pathway. The representative images of immunofluorescence staining of HK-2 cells without the stimulation of TGF-β1 (a - f), TGF-β1-treated HK-2 cells co-incubated with LiCl (b), TGF-β1-treated HK-2 cells co-incubated with DKK1 (g), TGF-β1-treated HK-2 cells co-incubated with LiCl and CAT (c-e), TGF-β1-treated HK-2 cells co-incubated with CAT (h-j) (20X). The relative intensity of immunofluorescence evaluated using ImageJ (k-n). *$P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. TGF-β1 and LiCl-treated group

A similar inhibition pattern/trend/intensity was observed between CAT treatment (50 and 100 µg/mL) and DDK1. Treatment of stimulated HK-2 cells with DDK1 presented similar inhibition as treatment with 50 and 100 µg/mL CAT (Figure 2 f - j). The above data showed that CAT had bioactivity in preventing EMT in TGF-β1-treated HK-2 cells, and the mechanism is related to neutralizing the activation of Wnt/β-catenin and TGF-β1 signaling pathways.

CAT alleviates kidney fibrosis

To confirm the antifibrotic activity of CAT on renal fibrosis *in vivo*, a UUO model which is a typical interstitial fibrosis model, was utilized in this study [12]. The results of H&E staining demonstrated that UUO resulted in pathological changes, such as tubule simplification, swelling and vacuolization, inflammatory cell infiltration and tubular cell death by apoptosis and necrosis. The presence of mild tubular abnormalities was observed on day 2 and severe tubular injury with interstitial matrix accumulation was observed on day 14 after surgery (Figure 3 a). Furthermore, compared with the 2-day UUO rats, the 14-day UUO rats had increased tubular interstitial fibrosis with severe collagen deposition, as assessed by Masson staining (Figure 3 b). In addition, compared with the UUO group, CAT alleviated tubular injury, including tubular expansion, vacuolization and dilation, tubular interstitial fibrosis and collagen deposition in the 2-day and 14-day CAT-treated UUO groups (Figure 3 a and b). Moreover, the ameliorative effect on interstitial fibrosis and collagen deposition occurred strongly in a dose-related manner in the 2-day CAT-treated UUO group (Figure 3 b). These data indicate that CAT has a notable antifibrotic effect, especially at the early stages of kidney injury, and delays the development and progression of renal fibrosis.

CAT inhibits UUO-induced EMT

EMT is a key promoter of fibrosis development and progression [14]. To study the effect of CAT on EMT in UUO rat kidneys, α-SMA and vimentin expression were determined by immunohistochemistry and Western blot. The results indicated that compared with the sham group, the UUO group had positive expression for α-SMA and vimentin, whereas treatment with CAT decreased this positive expression after surgery at 2 days and 2 weeks, in a dose-dependent manner (Figure 4). These results indicate that CAT prevents UUO-induced EMT progression during chronic injury.

CAT inhibits the hyperactivation of TGF-β/Smads and Wnt/β-catenin signaling pathways

Based on the *in vitro* results, the expression levels of Wnt3a, p-GSK3β, β-catenin, TGF-β1 and Smad2/3 were examined by Western blot to verify further the inhibitory effects of CAT on the TGF-β/Smad and Wnt/β-catenin signaling pathways in UUO rats. Compared with the sham group, the UUO rats had upregulated expression levels of Wnt3a, p-GSK3β, β-catenin, TGF-β1 and Smad2/3 at day 2 and 14 post-injury (Figure 5 a - b), which revealed hyperactivation of TGF-β/Smad and Wnt/β-catenin signaling pathways in UUO rats. In contrast to the hyperactivation induced by UUO, CAT significantly downregulated the increased expression of Wnt3a, p-GSK3β, β-catenin, TGF-β1 and Smad2/3 in UUO rats in a dose-dependent manner at day 2 and 14 (Figure 5 c - g).

Snail1, an essential transcription factor for the EMT process, is a downstream target gene of the TGF-β/Smad and Wnt/β-catenin signaling pathways [15]. Compared with the sham group, Snail1 was significantly overexpressed in the UUO group. Still, CAT administration reversed the Snail1 overexpression induced by UUO in a concentration-dependent manner at days 2 and 14, which is similar to the trend seen in the expression patterns of Wnt3a, p-GSK3β, β-catenin, TGF-β1 and Smad2/3 (Figure 5 h). Therefore, CAT blocked the aberrant activation of the TGF-β/Smad and Wnt/β-catenin signaling pathways simultaneously and then downregulated the expression of Snail1, hence preventing the development of EMT, and eventually alleviated renal fibrosis.

DISCUSSION

Renal injury leads to the production and secretion of a series of profibrotic factors such as TGF-β, Wnt and hedgehog ligands, inducing epithelial cells to transform into fibroblasts and myofibroblasts, leading to ECM accumulation, and ultimately to renal fibrosis and the progression of CKD [16]. Numerous studies have demonstrated that the TGF-β and Wnt signaling pathways work synergistically in mesenchymal-derived cells of different organs to accelerate fibroblast activation, proliferation and ECM accumulation [17]. The TGF-β and Wnt/β-catenin signaling pathways interconnect and converge at β-catenin activation, leading to EMT transcriptional program activation. TGF-β plays a crucial role in progressive renal fibrosis.

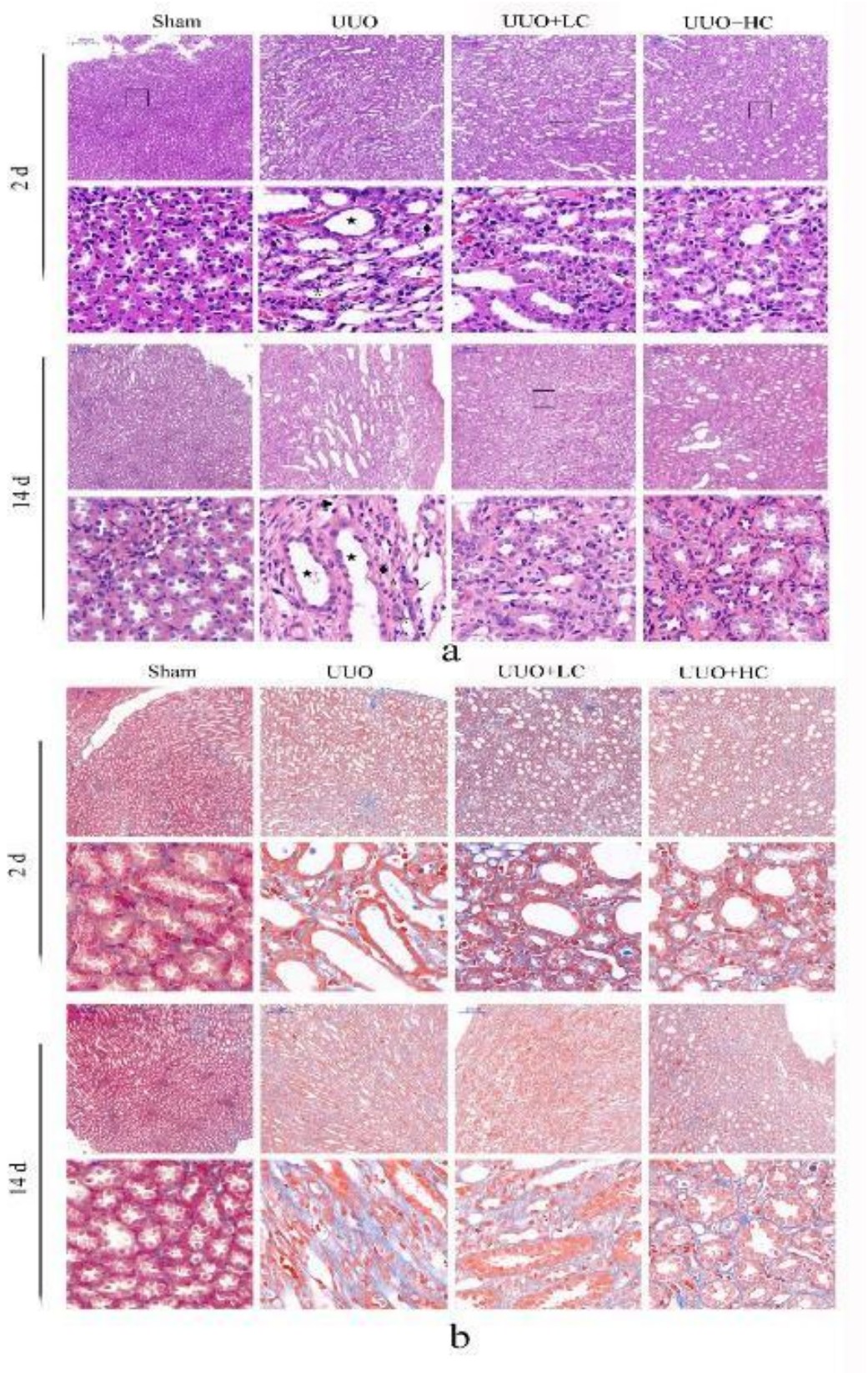

Figure 3: CAT ameliorated renal fibrosis induced by UUO. Representative H&E-stained (a) and Masson trichrome-stained (b) pathological images of the renal medulla from UUO rats treated with vehicle or CAT for 2 days or 2 weeks (5x, 40x for both). UUO + LC: UUO rats treated with CAT at a low dosage (100 mg/kg), UUO+HC: UUO rats treated with CAT at a high dosage (200 mg/kg). ►: simplification, ∗: apoptosis, ↑: inflammatory infiltration, →: necrosis, ♦: vacuolization, ★: swelling

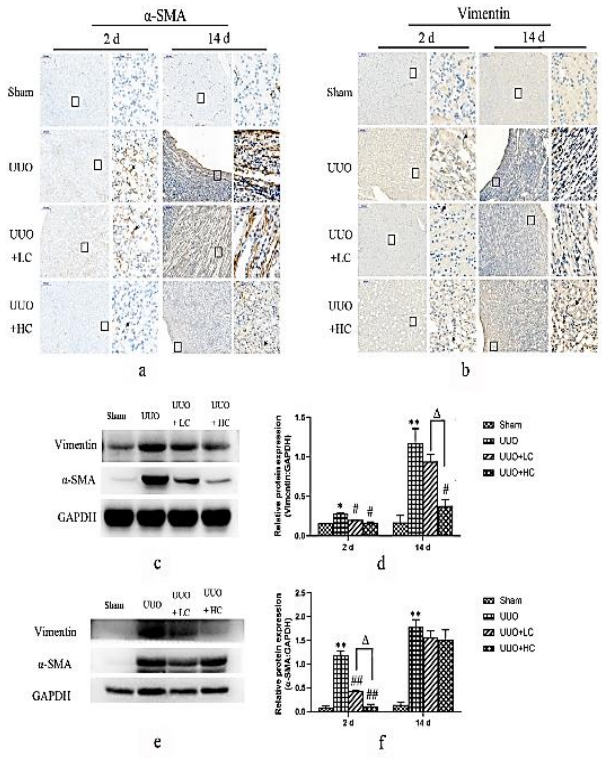

Figure 4: CAT delayed the progression of UUO-induced EMT. Immunohistochemistry detection of the myofibroblast and mesenchymal cell markers α-SMA (a) and vimentin (b) in the medulla from UUO rats with or without CAT treatment for 2 and 14 days (5x, 40x). Vimentin and α-SMA protein expression was determined by Western blot, with GAPDH expression as a control (c-f). UUO + LC: UUO rats treated with CAT at a low dosage (100 mg/kg), UUO + HC: UUO rats treated with CAT at a high dosage (200 mg/kg). The arrow indicates positive α-SMA and vimentin staining. The data are expressed as the means ± SD from 3 independent experiments. $^{**}P < 0.05$, $^{*}p < 0.01$, compared with the sham group. $^{\#}P < 0.05$, $^{\#\#}p < 0.01$, compared with the UUO group. $^{\triangle}P < 0.05$, compared with the CAT group (100 mg/kg)

Activated TGF-β1 binds to cell membrane-embedded signal receptors and initiates Smad signaling pathways, which induce the transcription of target gene Snail1 [18,19].

This study revealed that TGF-β1 promoted the expression of α-SMA and decreased the expression of E-cadherin *in vitro*. The Wnt/β-catenin signaling pathway is activated and GSK-3β is phosphorylated. Meanwhile, active β-catenin induced the activation of target gene Snail1. Notably, Snail1 is a transcription factor expressed during renal embryogenesis and is maintained in a silent state in renal epithelial cell precursors during adulthood. The reactivation of Snail1 promotes renal fibrosis [15]. In this experiment, the results show that after UUO *in vivo*, the expression of α-SMA and vimentin and collagen deposition increased in rat kidney tissue, which indicates renal tissue fibrosis. Simultaneously, the study showed that the expression levels of key protein factors increased in the TGF-β and Wnt/β-catenin signaling pathways. Therefore, inhibiting the common target genes in these two signaling pathways would be an underlying therapeutic strategy for improving renal fibrosis.

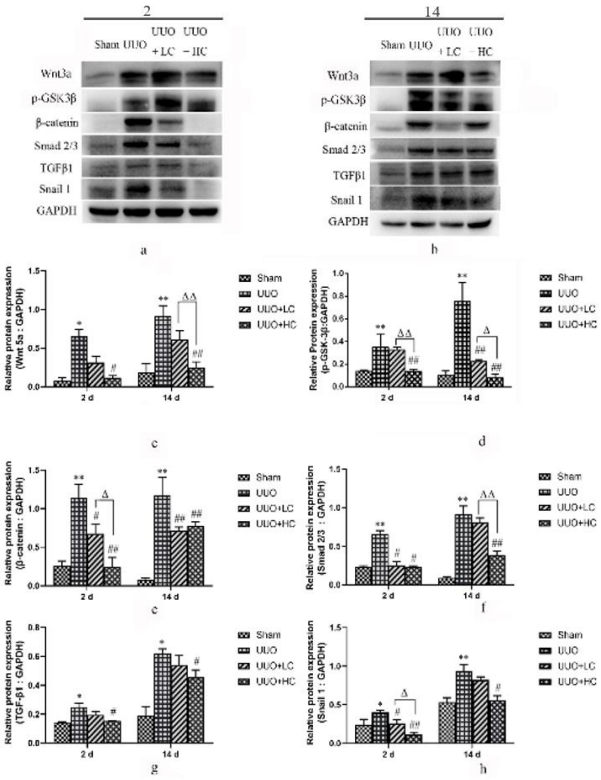

Figure 5: CAT inhibited the aberrant activation of the Wnt/β-catenin and TGF-β1/smads pathways in UUO rats. Kidney injury induced the activation of Wnt/β-catenin pathway at days 2 (a) and 14 (b). CAT reversed the upregulation of Wnt3a (c), β-catenin (d), p-GSK3β (e), Smad2/3 (f), TGF-β1 (g) and Snail1 (h) expression in a dose-dependent manner at days 2 and 14 after UUO surgery. $*P < 0.05$, vs. sham group. $^{\#}P < 0.05$, vs. UUO group. $^{\triangle}P < 0.05$, vs. CAT group (100 mg/kg). UUO + LC: UUO rats treated with CAT at a low dosage (100 mg/kg), UUO+HC: UUO rats treated with CAT at a high dosage (200 mg/kg)

In this study, CAT effectively inhibited the EMT hyperactivated by TGF-β1 in a dose-dependent manner, alleviated tubular injury, interstitial fibrosis and collagen accumulation, and significantly downregulated the overexpression levels of α-SMA and vimentin in UUO rats at days 2 and 14 after the obstruction in a dose-dependent manner. In UUO rats, CAT inhibited the increased expression levels of Wnt3a, β-catenin, p-GSK-3β and Snail1 induced by ureteral obstruction in a dose-dependent manner at 2 and 14 days, meaning that CAT prevented EMT progression and alleviated renal fibrosis by

repressing the hyperactivation of Wnt/β-catenin signaling pathway induced by obstructive injury. Meanwhile, CAT downregulated the overexpression of TGF-β1 and Smad2/3 induced by obstructive injury, suggesting that CAT has excellent antifibrotic effects in UUO rats, which is also associated with deactivating the TGF-β/Smads signaling pathway.

CONCLUSION

This study reveals that catalpol has anti-fibrotic pharmacological action, effectively prevents EMT and relieves renal fibrosis. The underlying mechanisms are related to repressing the hyperactivation of the TGF-β/Smad and Wnt/β-catenin signaling pathways. Therefore, catalpol is a promising candidate for ameliorating renal fibrosis.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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