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Baicalein ameliorates renal interstitial fibrosis by inducing myofibroblast apoptosis *in vivo* and *in vitro*

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Objective

To investigate the anti-fibrotic effects of baicalein and its influence on myofibroblasts *in vivo* and *in vitro*.

Materials and Methods

An *in vivo* unilateral ureteric obstruction (UUO) mouse model and an *in vitro* transforming growth factor β1 (TGF-β1) activated normal rat kidney (NRK)-49F cell model were established. Baicalein treatment was then investigated in these models to assess its anti-fibrotic effects and potential mechanisms of action.

Results

Baicalein attenuated renal fibrosis by ameliorating kidney injury, reducing deposition of fibronectin and collagen type 1, and inducing apoptosis in myofibroblasts in the UUO mouse model. Baicalein also induced apoptosis of TGF-β1-activated myofibroblasts *in vitro* in a dose-dependent manner. Furthermore, baicalein triggered a cascade of mitochondrion-associated apoptosis by upregulating cleaved-caspase-3, Bcl2-associated X protein (Bax), and cleaved-caspase-9 while downregulating the protein expression of B-cell lymphoma 2 (Bcl-2). Additionally, down-regulation of phosphorylated protein kinase B (pAkt) was found in the baicalein-induced pro-apoptotic components.

Conclusions

The present findings show that baicalein can ameliorate tubulointerstitial fibrosis by inducing myofibroblast apoptosis through the mitochondrion-associated intrinsic pathway, which may be mediated by the inhibition of the phosphoinositide-3-kinase/Akt (PI3k/Akt) pathway.

Keywords

baicalein, apoptosis, myofibroblast, kidney fibrosis, PI3K/AKT

Introduction

Renal fibrosis, which is a common manifestation of chronic kidney disease, is a complex process involving not only an imbalance in the synthesis and degradation of the extracellular matrix (ECM) but also cell infiltration, apoptosis, accumulation of activated myofibroblasts, and increased deposition of ECM [1]. Among the cells accumulating in the renal interstitium, myofibroblasts are major mediators in tubulointerstitial fibrosis, as they are the source of many cytokines and several components of the ECM contributing to renal damage [2]. Activated myofibroblasts, with α-smooth muscle actin (α-SMA)-positive hallmark, derive from resident fibroblasts, bone-marrow-derived fibrocytes and pericytes, and renal epithelial cells by a process called epithelial–mesenchymal transition [3]. The numbers of interstitial myofibroblasts and fibroblasts are closely related to the severity of renal fibrosis and attendant decline of kidney function [4]. Thus, the apoptosis of myofibroblasts in the injured kidney may attenuate renal fibrosis and thus yield a therapeutic option [5].

Baicalein (5,6,7-trihydroxyflavone) is a flavonoid that is mainly isolated from the roots of the Chinese herb *Scutellaria baicalensis* [6]. This compound exhibits many pharmacological benefits, such as cardiovascular protective properties [7], anti-inflammation [8], and antitumor activities [9–11]. Several studies have recently reported that baicalein is a potential anti-fibrotic agent. For example, baicalein prevented the progression of carbon tetrachloride-induced liver fibrosis *in vivo*. Baicalein also alleviated renal fibrosis by inhibiting TGF-β1 expression and reducing ECM production in a unilateral ureteric obstruction (UUO) model [12]. Moreover, baicalein can induce apoptosis in cancer cells and activated lymphocytes. However, no research has focused on whether baicalein affects fibroblasts or myofibroblasts. Thus,

we hypothesised that baicalein can induce apoptosis of activated myofibroblasts to accomplish its anti-fibrotic effect in a UUO model.

Methods and Materials

Mouse Model

C57/BL6 mice were purchased from the Wuhan University Centre for Animal Experiment (Wuhan, China). Animal studies were performed with an approved protocol by the Institutional Animal Care and Use Committee of the University of Wuhan, China. In all, 28 mice aged 6–8 weeks were used, and underwent UUO of the left kidney using an established procedure [13]. The mice were randomly allocated into four groups: sham; UUO plus vehicle; UUO plus 50 mg/kg body weight/day baicalein (Nanjing Dilger Medical Technology Co., Ltd., Nanjing, China); and UUO plus 100 mg/kg/day baicalein. The dosage of baicalein was derived from a previous study [14] and tested based on our preceding experiments to confirm whether the pathology staining in the UUO model was improved. Baicalein was dissolved in 0.1% dimethyl sulphoxide (DMSO; Sigma-Aldrich Company Ltd, Dorset, UK), and the mice received baicalein daily by oral gavage for 6 days subsequent to the day of surgery. The vehicle-treated UUO mice were administered with 300 μL PBS in DMSO. On day 7 after surgery, the mice were humanely killed and the obstructed kidneys were harvested.

Western Blot Analysis

Cells and tissues were treated as indicated and homogenised in a lysis buffer (Biyuntian, Haimen, China) with a polytron homogeniser (IKA GmbH, Königswinter, Germany) on ice. Samples were mixed with SDS, and supernatants were subsequently separated by SDS-PAGE. After electroblotting onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), the membranes were blocked and incubated with various primary antibodies, protein kinase B (Akt), phosphorylated Akt (pAkt; Cell Signaling Technology, Danvers, MA, USA), cleaved-caspase-3, B-cell lymphoma 2 (Bcl-2), Bcl2-associated X protein (Bax), and cleaved-caspase-9 (Abcam, Cambridge, MA, USA) overnight at 4 °C, with the dilutions specified by the preliminary experiment. Subsequently, the membranes were incubated with secondary antibody (LI-COR, Lincoln, NE, USA) and conjugated to horseradish peroxidase for 1 h. Finally, the membranes were scanned with a two-colour infrared imaging system (Odyssey; LI-COR).

Cell Culture and Treatments

Normal rat kidney (NRK)-49F fibroblasts and NRK-52E cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco, Grand Island, NY, USA) medium. After serum starvation for 24 h, the NRK-52E cells were treated with 20, 40, 80, and 160 μM baicalein, and the NRK-49F cells were treated with 0.5 ng/mL TGF-β1 (R&D) or (and) 20, 40 or 80 μM baicalein. Baicalein was dissolved in 0.1% DMSO, and cells were incubated for an additional 24 h. The survival percentage for each group was determined by Cell Counting Kit-8 method (Beyotime, Haimen, China) following the manufacturer's instructions. The absorbance (A) was measured at 450 nm, and the cell survival rate was calculated using the following formula: Cell survival rate (%) = (A of the experimental group/A of the control group) × 100.

Fluorescence Activated Cell Sorting (FACS) Analysis of Cell Apoptosis

The NRK-49F cells were treated as indicated above. All cell cultures were harvested 24 h after treatment. The medium was then collected and re-suspended at 1×10^6 in 300 μL binding buffer. Then, cells were stained with 10 μL propidium iodide (PI) and 5 μL of annexin V-fluorescein isothiocyanate (FITC) and incubated for 5 min. A total of 10 000 events were collected, and the proportion of apoptotic cells was analysed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose CA, USA).

Histological and Immunohistochemical Studies

The paraffin-embedded kidney tissues were sectioned at 4-μm thickness using an established procedure. Haematoxylin and eosin (H&E) and Masson's trichrome staining were used to assess interstitial collagen. The immunohistochemical studies were performed as follows. Tissue sections were dewaxed and hydrated in decreasing concentrations of ethanol and then antigen retrieved. After, endogenous peroxidase activity was quenched using 3% H_2O_2, and 5% BSA was used for blocking nonspecific binding for ≈1 h. Tissues were subsequently incubated with primary rabbit anti-mouse antibody (collagen type 1 [COL-1] and fibronectin [FN]; Abcam) overnight at 4 °C. The tissues were incubated with goat anti-rabbit secondary antibody for another 1 h. Peroxidase-streptavidin-biotin complex (Boshide Biotechnology Co., Ltd., Wuhan, China) and diaminobenzidine (Sigma, St. Louis, MO, USA) were used to visualise the proteins. In all, 10 random fields were selected (×200), which were photographed and measured by Image Pro-Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Real-Time PCR

Total RNA was extracted from the kidney tissues with Trizol reagent (Invitrogen, Grand Island, NY, USA) following the manufacturer's instructions. First-strand cDNA synthesis was performed using the PrimeScript™ reverse transcriptase (RT)

reagent kit (Takara, Otsu, Shiga, Japan). Subsequently, 2 μg of RNA isolated from tissues was used as a template to perform one-step RT-PCR, and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. All reactions were conducted in a 20-μL tube. Real-time PCR was performed on the resulting cDNA using the SYBR Green method and the AB7500 Real-time PCR system. The threshold cycle (Ct) values of each sample were used in the $2^{-\Delta\Delta CT}$ data analysis method.

Immunofluorescence Double Staining of α-SMA and Cleaved-Caspase-3

Briefly, tissue sections were dewaxed and hydrated in decreasing concentrations of ethanol and then antigen retrieved. After, the tissue slides were blocked for 30 min with BSA. Both α-SMA (Abcam) and cleaved-caspase-3 (Abcam) antibodies were then added to the sections, and the sections were incubated overnight. On the following day, the slides were incubated with labelled anti-mouse Cy3 (Santa Cruz, Dallas, TX, USA) and anti-rabbit FITC (Santa Cruz)-labelled secondary antibodies for 1 h. After three washes, the slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). The fields of each section were examined at ×200, and the α-SMA-positive cells and cleaved-caspase-3-positive cells were counted.

Statistical Analysis

All experiments were repeated thrice independently. Data are expressed as means (standard deviations, SD). Statistical analysis was performed using one-way ANOVA with SPSS 19.0 software (IBM, Armonk, NY, USA). A $P < 0.05$ was considered to indicate statistical significance.

Results

Baicalein Treatment Attenuates Tubular Injury and Interstitial Matrix Deposition

H&E stained micrographs showed that dilated renal tubules, interstitial expansion, and epithelial cell atrophy were ameliorated compared with the vehicle-treated group at 7 days after baicalein treatment. Masson's trichrome staining also indicated that UUO injury increased the collagen fibril accumulation (Fig. 1, green area) compared with the sham group (2.63 [0.57]% vs 17.53 [2.18]%, $P < 0.01$), and baicalein (50 and 100 mg/kg/day) attenuated collagen deposited in the renal interstitium. However, the anti-fibrotic effect in the 100 mg/kg/day baicalein group was greater than that in the 50 mg/kg/day baicalein group (5.57% [1.12]% vs 9.00% [1.92]%, $P < 0.05$; Fig. 1A,B). Furthermore, the immunohistochemical staining results indicated that the production of the ECM components, i.e. COL-1 and FN, decreased after baicalein treatment at 50 and 100 mg/kg/day (Fig. 1C–E) in a dose-dependent manner ($P < 0.05$). These observations were verified by real-time PCR, in which baicalein, especially at 100 mg/kg/day, markedly downregulated the expression of FN and COL-1 (Fig. 1F,G).

Baicalein Inhibited Accumulation of α-SMA by Promoting Apoptosis of α-SMA-Positive Myofibroblasts in Kidney Tissues

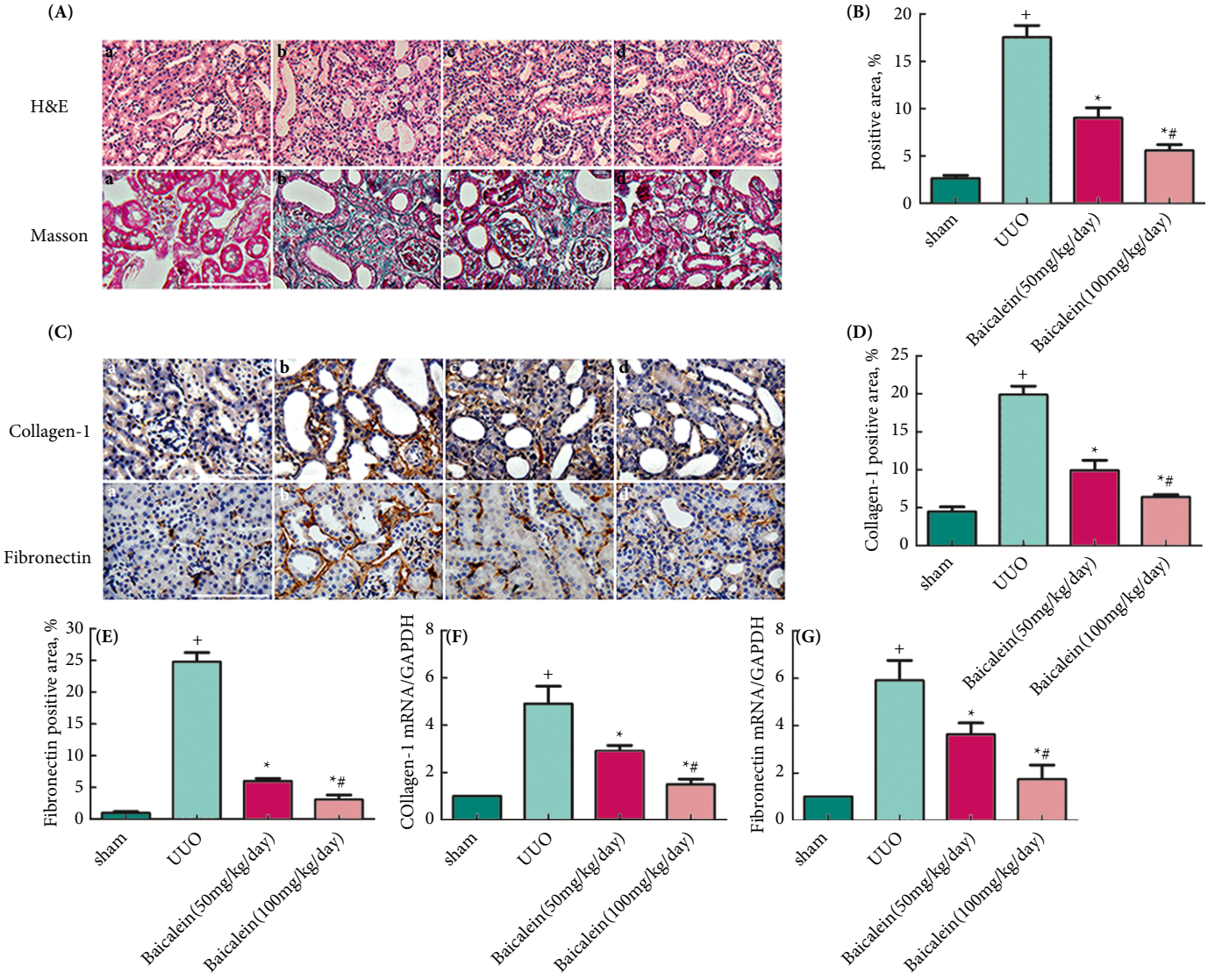
To investigate whether baicalein inhibits the accumulation of myofibroblasts, the expression of α-SMA in the obstructed kidney was detected using Western blot and real-time PCR (Fig. 2A–C). The UUO markedly upregulated the α-SMA expression in the UUO plus vehicle group compared with the sham group ($P < 0.01$). Compared with the UUO plus vehicle group, the 50 and 100 mg/kg/day baicalein groups showed significant downregulation in α-SMA expression in the kidney tissues ($P < 0.01$). Double staining of α-SMA and cleaved-caspase-3 in the kidney tissue sections also showed that the number of cleaved-caspase-3-positive cells increased after baicalein treatment, mainly in the α-SMA-positive myofibroblasts (Fig. 2D). The apoptotic index (AI) is the percentage of α-SMA-positive cells in the interstitium. The AI of the myofibroblasts in the 50 mg/kg/day baicalein group was significantly lower than that of the 100 mg/kg/day baicalein group, at 18.33% (4.04)% vs 23.67% (4.93)% ($P < 0.05$; Fig. 2E).

Baicalein Showed Cytotoxic and Inhibitory Effects

To measure the cytotoxic effects of baicalein, we detected the cell viability of the NRK-52E cells after baicalein treatment. As shown in Fig. 3A, 20–80 μM baicalein did not inhibit cell viability of NRK-52E cells after it was added 24 h later, but cell viability was <85% when the concentration reached 160 μM. This result indicated that baicalein had cytotoxic effects on renal epithelial cells at high concentration. By contrast, baicalein significantly inhibited myofibroblasts, which transformed from NRK-49F cells activated by TGF-β1 at 20–80 μM baicalein, with statistical differences between the different concentrations of baicalein ($P < 0.05$, Fig. 3B).

In fibrotic kidneys, interstitial fibroblasts are often activated by TGF-β1 and transformed into myofibroblasts expressing α-SMA, fibroblast-specific protein 1 (FSP-1), or vimentin [15]. After the NRK-49F cells were treated with 0.5 ng/mL TGF-β1 for 24 h to induce myofibroblast activation, the apoptotic effect of baicalein on myofibroblasts was analysed by flow cytometry. Compared with the control group (0.7 [0.4%]), baicalein concentrations at 20 (11.67 [2.53%]), 40 (18.33 [3.33%]), and 80 μM (26.66 [3.82%]) resulted in significant apoptosis of myofibroblasts in a dose-dependent manner ($P < 0.01$). The 80 μM baicalein group showed the

Fig. 1 Kidney fibrosis induced by UUO for 7 days. (**A**) Sham-operated (**B**) UUO in vehicle-treated (**C**), UUO in 50 mg/kg/day baicalein-treated (**D**), and UUO in 100 mg/kg/day baicalein-treated groups. **A**. Representative photomicrographs of H&E and Masson's trichrome staining, original magnification ×200; **B**. Image analysis for the positive area of collagen in the different groups with Masson's trichrome staining; **C**. Immunohistochemical images for the expression of COL-I and FN, original magnification ×200; **D**, **E**. Image analysis for COL-1-positive and FN-positive areas in the different groups; **F**, **G** mRNA expression of COL-1 and FN in the different groups. $^{+}P < 0.01$ vs sham group, $^{*}P < 0.01$ vs UUO vehicle-treated group, $^{\#}P < 0.05$ vs UUO 50 mg/kg/day baicalein-treated group. Scale bars, 200 μm.



highest apoptotic rate (26.66 [3.82%]) compared with the 40 and 20 μM baicalein groups ($P < 0.01$; Fig. 3C,D).

Mechanism of Baicalein-Induced Apoptosis on Myofibroblasts was Explored

To explore the apoptotic mechanism of the myofibroblast response to baicalein, we measured the protein expression of cleaved-caspase-3, Bcl-2, Bax, and cleaved-caspase-9 after baicalein treatment of TGF-β1-activated NRK-49F cells. The results indicated that baicalein activated cleaved-caspase-3, cleaved-caspase-9, and Bax in TGF-β1-activated myofibroblasts but downregulated the expression of Bcl-2 (Fig. 4A,B). Furthermore, the ratio of pAkt/Akt increased after activation of TGF-β1 but was attenuated by baicalein treatment (Fig. 4C,D).

Discussion

In nature, interstitial fibroblasts are limited in number and often quiescent in normal kidneys. After chronic injury, these cells are activated by TGF-β1 and express α-SMA,

Fig. 2 Kidney fibrosis induced by UUO for 7 days. **A**. Western blot gel for α-SMA; **B**. Semi-quantitative histogram analysis for expression of α-SMA in the different groups; **C**. α-SMA mRNA expression in the different groups; **D**. Kidney sections were dually stained with α-SMA (red) and cleaved-caspase-3 (green). Double-stained positive cells (yellow) in the renal interstitium indicate myofibroblast apoptosis, and the negative control groups were stained without primary antibody. Original magnification ×200; **E**. Double-stained positive cells were counted in the stained sections at ×400 under a microscope by using at least 10 randomly selected microscopic fields per section. $^{+}P < 0.01$ vs sham group, $^{*}P < 0.01$ vs vehicle-treated group, T $P < 0.01$ vs 50 mg/kg/day baicalein-treated group. $^{\#}P < 0.05$ vs 50 mg/kg/day baicalein-treated group. Scale bars, 200 μm.

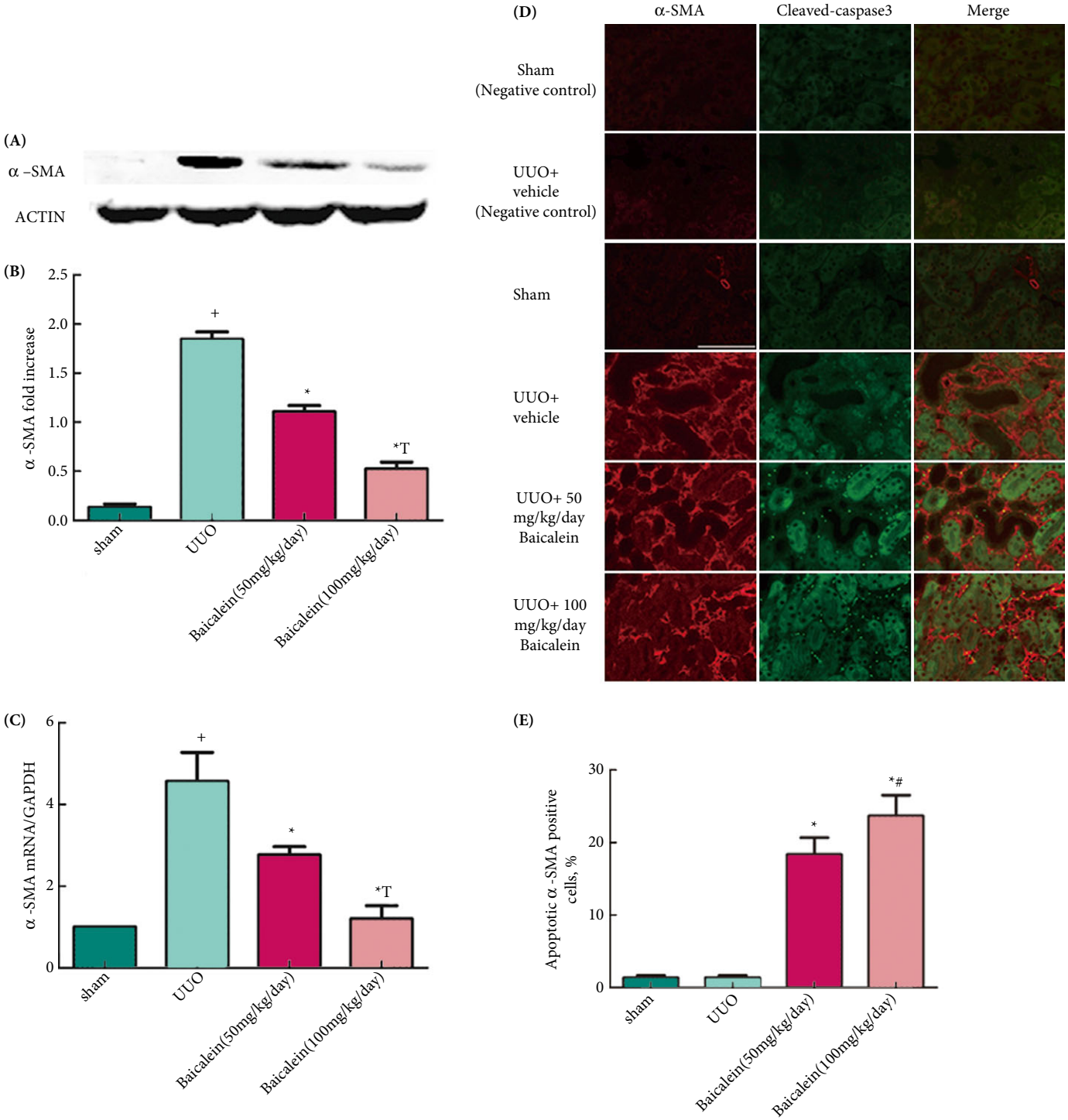


Fig. 3 **A**. Effects of baicalein on cell viability of NRK-52E measured using the Cell Counting Kit-8 method. $^{\#}P > 0.05$ vs sham group; **B**. Effects of baicalein on the cell viability of TGF-β1-activated NRK-49F detected using the Cell Counting Kit-8 method; **C**. Apoptotic rate was measured by FACS using PI and annexin V-FITC double staining; **D**. Quantitation of apoptotic cells in 10 000 cells. Data are representative of three similar experiments. $^{*}P < 0.01$ vs 0.5 ng/mL TGF-β1-treated group, $^{\star}P < 0.05$ vs the former group, $^{\#}P > 0.05$ vs the former group. $^{+}P < 0.05$ vs 20 μM baicalein group, T $P < 0.01$ vs 40 μM baicalein group. All data are expressed as means ± SD from three independent experiments.

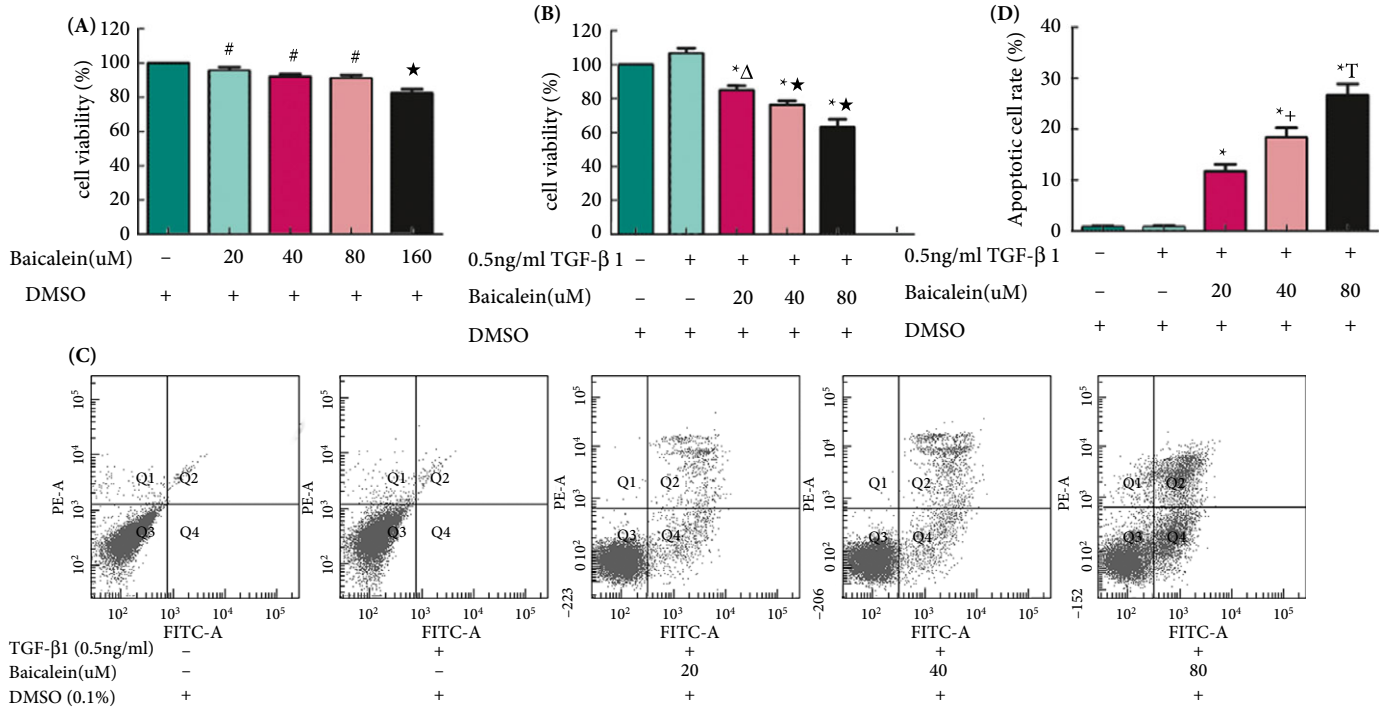
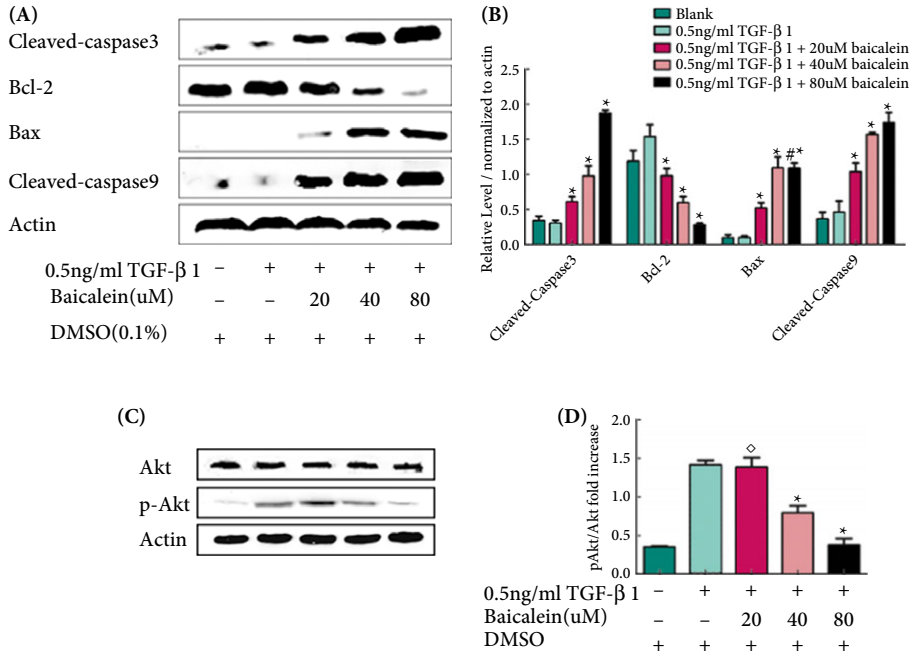


Fig. 4 **A**. NRK-49F cells were treated with 0.5 ng/mL TGF-β1 and the indicated concentrations of baicalein for 24 h, and then subjected to Western blot to detect apoptosis-related proteins; **B**. and **D**., Statistical analysis of the relative expression of the different proteins after treatment; **C**. Western blot analysis for pAkt and Akt. $^{*}P < 0.01$ vs 0.5 ng/mL TGF-β1 group, $^{\#}P > 0.05$ vs 40 μM baicalein group. $^{\diamond}P > 0.05$ vs 0.5 ng/mL TGF-β1 group.



FSP-1 or vimentin, with phenotypic transition to myofibroblasts. On one hand, the activation of myofibroblasts is a feature of the progression of renal fibrosis; on the other hand, myofibroblasts are key for the production of ECM components, such as COL-1 and FN [16]. Over accumulation of ECM squeezes the nearby vessels of the renal tubules, thus decreasing the supplement of and diffusion of oxygen to the kidney tissues. Recently, increasing evidence has shown that apoptosis of myofibroblasts exerts a notable anti-fibrotic effect [5,17]. Therefore, the reduction of activated myofibroblasts may provide a therapeutic option to prevent progression of renal fibrosis. Baicalein is a natural active agent that has been recognised to exert a potent anti-fibrotic effect. After oral administration, baicalein presents as a free form at high concentration in the kidneys [18]. Given that baicalein can ameliorate fibrosis and induce apoptosis, we hypothesised that baicalein could induce apoptosis of myofibroblasts and thus improve renal fibrosis. In our present study, Masson's trichrome and immunohistochemical staining with image analysis confirmed that baicalein inhibited the accumulation of the ECM components FN and COL-1 deposited in an established UUO model. Consistent with our assumption, the double staining of α-SMA and cleaved-caspase-3 indicated that baicalein blocked renal fibrosis by inducing apoptosis of α-SMA-positive myofibroblasts, which are activated by chronic injury. In addition, 80 mM baicalein resulted in significant cytotoxic effects on TGF-β1-activated myofibroblasts *in vitro*. Furthermore, PI/annexin V-FITC double staining of activated fibroblasts detected by flow cytometry suggests a notable dose-dependent apoptotic effect of baicalein. The present study has shown for the first time that baicalein achieves its anti-fibrotic action by inducing myofibroblast apoptosis. Thus, our present study has clearly found a novel and previously unrecognised function for baicalein in the control of fibrosis in obstructed kidneys.

To elucidate the mechanisms underlying the apoptosis induced by baicalein, we analysed some components in the mitochondrial pathway and the phosphoinositide-3-kinase/Akt (PI3K/Akt) signal pathway that contribute to cell apoptosis. The PI3K/Akt pathway has been found to play an essential role in baicalein-induced apoptosis in oesophageal squamous cell carcinoma [19]. In addition, activated Akt acts to phosphorylate Bcl-2-associated death promoter (Bad), Bax, and caspase-9 or activate the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway to promote the resistance of cells to apoptosis. Our present data showed that pAkt was activated in fibroblasts after TGF-β1 treatment, but this phenomenon was reversed after baicalein administration. Moreover, the protein levels of Bax, cleavedcaspase-3, and cleaved-caspase-9 were significantly upregulated, while Bcl-2 was downregulated after baicalein treatment. Thus, Akt phosphorylation decreased when activated myofibroblasts were exposed to baicalein, which contributed to the dephosphorylation of intrinsic pro-apoptotic proteins Bax and caspase-9. Homo-oligomerisation of Bax results in a permeabilised mitochondrial membrane and subsequent release of cytochrome C to activate apoptosis [20]. The release of cytochrome C triggers the activation of cleaved-caspase-9 and subsequently upregulates cleaved-caspase-3 to induce myofibroblast apoptosis [21], which contributes to a decrease in the interstitial matrix to alleviate fibrosis after UUO. Some studies have also reported that baicalein inhibits the receptor activator of NF-κB ligand to block the activation of Akt to induce apoptosis in mature osteoclasts [22]. This phenomenon is being evaluated in our ongoing studies of how baicalein affects the Akt signal pathway to induce apoptosis.

In conclusion, the present study has shown that baicalein has an anti-fibrotic effect by inducing apoptosis of myofibroblasts *in vivo* and *in vitro*, which may be achieved via downregulation of the PI3K/Akt signal. Our present study provides theoretical evidence supporting the use of baicalein as a therapeutic agent for treating renal fibrosis, but well-designed prospective clinical studies are required before its use can be warranted in clinical practice.

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Conflict of Interest

None declared.

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Abbreviations: AI, apoptotic index; (p)Akt, protein kinase B; α-SMA, α-smooth muscle actin; Bad, Bcl-2-associated death promoter; Bax, Bcl2-associated X protein; Bcl-2, B-cell lymphoma 2; COL-1, collagen type 1; DMSO, dimethyl sulphoxide; ECM, extracellular matrix; FACS, fluorescence activated cell sorting; FITC, fluorescein isothiocyanate; FN, fibronectin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; FSP-1, fibroblast-specific protein 1; H&E, haematoxylin and eosin; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NRK, normal rat kidney; PI3K, phosphoinositide-3-kinase; RT, reverse transcriptase; UUO, unilateral ureteric obstruction.