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Safflower Extract: A Novel Renal Fibrosis Antagonist That Functions by Suppressing Autocrine TGF-Beta

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Abstract Progressive renal disease is characterized by the accumulation of extracellular matrix proteins in the renal interstitium. Hence, developing agents that antagonize fibrogenic signals is a critical issue facing researchers. The present study investigated the blood-circulation-promoting Chinese herb, safflower, on fibrosis status in NRK-49F cells, a normal rat kidney interstitial fibroblast, to evaluate the underlying signal transduction mechanism of transforming growth factor-beta (TGF-β), a potent fibrogenic growth factor. Safflower was characterized and extracted using water. Renal fibrosis model was established both in vitro with fibroblast cells treated with β-hydroxybutyrate and in vivo using rats undergone unilateral ureteral obstruction (UUO). Western blotting was used to examine protein expression in TGF-β-related signal proteins such as type I and type II TGF-β receptor, Smads2/3, pSmad2/3, Smads4, and Smads7. ELISA was used to analyze bioactive TGF-β1 and fibronectin levels in the culture media. Safflower extract (SE) significantly inhibited β-HB-induced fibrosis in NRK cells concomitantly with dose-dependent inhibition of the type I TGF-β1 receptor and its down-stream signals (i.e., Smad). Moreover, SE dose-dependently enhanced inhibitory Smad7. Thus, SE can suppress renal cellular fibrosis by inhibiting the TGF-β autocrine loop. Moreover, remarkably lower levels of tissue collagen were noted in the nephron and serum TGF-β1 of UUO rats receiving oral SE (0.15 g/3 ml/0.25 kg/day) compared with the untreated controls. Hence, SE is a potential inhibitor of renal fibrosis. We suggest that safflower is a novel renal fibrosis antagonist that functions by down-regulating TGF-β signals. J. Cell. Biochem. 104: 908–919, 2008.

Key words: TGF-β; fibrosis; fibronectin; safflower; Smad; Chinese herb

Abbreviations used: ELISA, enzyme-linked immunosorbent assay; FCS, fetal-calf serum; NRK, normal rat kidney cells; RT-PCR, reverse-transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; TGF-β1, transforming growth factor-β1; TGF-β RI, type I TGF-β receptor; TGF-β RII, type II TGF-β receptor; β-HB, β-hydroxybutyrate.

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Chronic renal failure is characterized by renal fibrosis, regardless of the cause of the disease. One of the main effector cells that contributes to the development of progressive renal fibrosis in chronic renal diseases is the tubulointerstitial fibroblast. As renal scarring leads to impairment of kidney function, slowing or retarding the progression to renal fibrosis is the biggest challenge facing nephrologists today.

Natural products of plant origin are still a major part of traditional medicinal systems in many countries, however, herbal medicine is often categorized as a form of complimentary or alternative treatment because the lack of scientific and supportive evidence excludes it from mainstream therapy [Cassileth, 1999]. It is well known that the herb, Carthamus tinctorius L, commonly known as safflower, has anti-inflammatory properties [Kim et al., 1999]. According to ancient Chinese medicinal and herbal literature, the dried flower of Carthamus tinctorius L. promotes blood circulation by removing blood stasis, and it has been used in China as an effective biological-response modifier, augmenting host homeostasis in body circulation [Shi, 1983]. However, literature survey showed no scientific evidence supporting the therapeutic effects of Carthamus tinctorius L. in treatment of renal fibrosis. Hence, the present study was prompted by the urgent requirement for natural, safe and effective anti-fibrosis treatments, and the lack of any real scientific data to support the claims made in the ancient literature. TGF-β is widely recognized as an essential fibrogenic cytokine that plays an important role in the pathogenesis of renal fibrosis [Klahr and Ichikawa, 1988; Hostetter, 1991; Young et al., 1995; Huang et al., 2005]. Further, extensive investigations over the past decade have proven the significance of TGF-β in terms of the initiation and progression of chronic kidney disease (CKD). Induction of TGF-β expression is found in many types of CKD [Hostetter, 1991; Young et al., 1995; Yang et al., 1998]. In vitro, TGF-β1 stimulates activation of interstitial fibroblasts [Guh et al., 1996; Liu et al., 2006] and promotes tubular epithelial-to-mesenchymal transition [Lee et al., 2004, 2005]. Our previous studies have also demonstrated that TGF-β plays an essential role in fibrosis induction in diabetic nephropathy [Guh et al., 1996, 2003; Yang et al., 1998, 2004; Chuang et al., 2003; Lee et al., 2004, 2005; Huang et al., 2005; Liu et al., 2006].

Inhibition of TGF-β using various strategies, including antisense inhibition of TGF-β expression and blockade of TGF-β action by neutralizing antibodies or soluble receptors, attenuates renal fibrosis and ameliorates progressive loss of kidney function in animal models [Isaka et al., 1999, 2000; Ziyadeh et al., 2000; Ma et al., 2004]. However, systemic blockade of TGF-β or other non-native approaches may produce unexpected results. Thus, exploring natural approaches to desensitize responsiveness to TGF-β or antagonize its signal transduction pathway has become an urgent issue for nephropathologists.

TGF-β signaling is transduced from the cell surface to the nucleus through transmembrane type I and type II serine/threonine/tyrosine kinase receptors and their downstream mediators (known as Smads) [Young et al., 1995; Massague, 2000]. On stimulation of TGF-β, Smad2, and Smad3 undergo phosphorylation, which triggers their interaction with Smad4. This Smad complex is then translocated into the nucleus, binding to the specific cis-acting element in the regulatory region of the TGF-β target genes (e.g., fibronectin) and directing their transactivation.

In this study, we investigated the mechanism underlying the attenuation of renal fibrosis produced by the Chinese herb, safflower. We have demonstrated that the crude extract of safflower may contain some natural compounds with potential therapeutic effects for renal fibrosis that are associated with antagonization of TGF-β-induced fibrogenic signals (i.e., Smad pathways) in interstitial fibroblast cells. This finding is essential for the development of a novel agent against TGF-β signaling and renal interstitial fibrosis.

EXPERIMENTAL METHODS AND MATERIALS

Extraction and Isolation of Safflower

Safflower was purchased and verified by Kaiser Pharmaceutical Company (Tainan, Taiwan). One hundred grams of the dried flower was immersed in 1,000 ml of distilled water and boiled at 100°C for 20 min. The particulate fraction was filtered using 325-mesh sieve (Kuang Yang), and the flow-through mixture concentrated to 100 ml at 40°C and further lyophilized (Kingmech, FD-4.5-12P).

The powder extract was then collected and used in the subsequent experiments.

Animal Study

Seven-month-old Sprague–Dawley male rats (body weight about 200 g) were allocated randomly into three groups (five per group) after confirmation that the animals were free of specific pathogens. The left ureter of each rodent was ligated under pentobarbital anesthesia. Unilateral ureteral obstruction (UUO) was performed in each animal to provoke renal fibrosis (Fig. 6). The non-ligated right kidneys of the experimental and sham-operated rats were used as controls.

Each negative control (untreated UUO animal) rat was treated orally with distilled water daily for 2 weeks (3 ml/0.25 kg/day). This group provided pathological data about renal fibrosis. The positive controls (treated UUO animals) were given daily captopril (2.5 mg/3 ml/0.25 kg/day for 2 weeks), an angiotensin converting enzyme inhibitor (ACEI) which has been widely used in the treatment of nephropathy. The treated group provided therapeutic data about renal fibrosis. In the test group, each rat was fed with the aqueous extract of safflower (0.15 g/3 ml/0.25 kg/day for 2 weeks). During the investigative period, the blood and urine samples were collected at three time points (before, and at 1 and 2 weeks after UUO) for routine analysis. The blood samples were subjected to clinical biochemical analysis. Serum TGF-β1 was determined using the ELISA kit (Quantikine™; R&D, Minneapolis, MN).

Histopathological Examination

At the end of the experiments, all rats were sacrificed and pathological analysis (i.e., biopsy) of the kidney was performed. The paraffin-embedded kidney tissue was sectioned for hematoxylin-eosin (HE) and Masson staining, and the frozen tissue was then stored for immunohistochemistry. Kidney specimens were preserved in 4% buffered paraformaldehyde and dehydrated in a graded alcohol series, then embedded in paraffin and sliced into 5-μm sections which were placed on plain glass slides. The sections were then stained with HE and Masson trichome to evaluate collagens. Light microscopy (100×) was used for blinded qualitative and quantitative histological analyses, with computer image analysis applied to calculate the ratio of collagen to renal tissue. The degree of fibrosis was calculated as the ratio of the areas of collagen to renal tissue, with the mean of 10 different fields per slide used for the result.

Cell Culture

NRK-49F (CRL-1570, ATCC), a normal Rattusnorvegicus kidney cell line, was cultured in Dulbecco's modified Eagle's medium (Hyclone Labs, UT) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Hyclone Labs) at 37°C in 5% CO_2. The cells were trypsinized using 0.025% trypsin-EDTA (Hyclone Labs).

Western Blotting

Western blot assay was utilized to evaluate the protein expression for the TGF-β receptors (RII and RI) and their downstream signal transducers (e.g., Smad2/3, Smad4, and Smad7). In brief, cells were lyzed using lysis buffer (10 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM Na3VO4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM dithiothreitol and 50 μM PMSF). The crude protein lysate was resolved by 7.5%, 10%, or 12.5% SDS–PAGE. After protein transfer to a polyvinylidene difluoride (PVDF) membrane using an electrotransfer unit, the PVDF membrane was blocked with 10% (w/v) de-fat milk in Tris-buffered saline (TBS-T) for 2 h at 37°C. The blots were probed with a 1:1,000 (v/v) dilution of polyclonal antibodies, all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). After hybridization at 37°C, the blots were washed and hybridized with 1:6,000 (v/v) dilutions of goat anti-rabbit IgG or horseradish peroxidase-conjugated secondary antibody (Calbiochem, Germany) or donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.). TBS-T buffer with 5% defat milk was used for blocking. The signal for respective proteins was generated by adding enhanced chemiluminescent reagent, with β-actin used as an internal control.

LDH Assay for Cytotoxicity

Cells were maintained and passaged as described above. The cells were seeded in six-well plates at a density of 2×10^5 cells/well in complete medium and incubated at 37°C in 5% CO_2 overnight. Supernatant from the conditioned cells was collected and stored. Supernatant from maintained cells treated with 1% Triton X-100 was regarded as a positive control for maximum lactate dehydrogenase

(LDH) release. After 24 h incubation at 37°C in 5% CO2, the supernatants were collected and centrifuged at 4,500*g* for 5 min to remove contaminating cells, and the level of LDH measured in duplicate using a cytotoxicity detection kit (Roche, Mannheim, Germany) in a 96-well plates. The cytotoxicity (%) was calculated using the formula:

$$100 \times \frac{\text{experimental value} - \text{low control}}{\text{high control} - \text{low control}}$$

Note that supernatants from the maintained and 1% Triton X-100-treated cells were considered low and high controls, respectively.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to evaluate the expression of secreted fibronectin. Conditioned culture media from NRK-49F cells were collected and centrifuged at 1,200 rpm for 5 min to remove particulate. The clear supernatant was then collected and concentrated and finally stored at −80°C for further use. A commercial sandwich enzyme-linked immunosorbent assay kit was utilized for detection of extracellular fibronectin (Tarkara Bio, Inc., Shiga, Japan) or TGF-β1 (Quantikine™; R&D systems). The procedure was performed according to the manufacturer's instruction manual. The absorbance (450 nm) for each sample was analyzed using an ELISA reader, and the concentration of each sample determined by interpolation with the standard curve, which was generated using an exogeneous fibronectin (12.5, 25, 50, 100, 200, 400, 800 μg/ml) or TGF-β1 (0–2,000 pg/ml) as the standard.

Statistical Analysis

Results are expressed as mean ± SEM. Unpaired Student's *t* tests were used for between-group comparison. A *P*-value of <0.05 was considered statistically significant for all tests.

RESULTS

Safflower is used as a traditional Chinese medicine. Figure 1 clearly shows that the aqueous extract of safflower does not affect the viability of cultured fibroblasts. As diabetes is usually concomitant with ketonuria and end-stage renal fibrosis, β-HB (the major form of ketone body in humans) was used to induce renal cellular fibrosis in this study. As shown in Figures 2 and 3 10 mM of β-HB induced a significant twofold increase in extracellular fibronectin and bioactive TGF-β1 level. More importantly, the water extract of Carthamus tinctorius L. dose-dependently (25, 50, 100, and 200 μg/ml) and dramatically suppressed β-HB-induced increases in both fibronectin and bioactive TGF-β1 levels. TGF-β1 is the most potent fibrosis inducer for renal fibroblasts. It appears reasonable to suggest that safflower extract may contain active compounds with fibrosis-inhibition potential.

The expression of two types of TGF-β receptor (i.e., type I and type II TGF-β) was determined under the treatment of β-HB in NRK cells as the presence of these receptors are reportedly strongly related to the susceptibility to cellular fibrosis. As shown in Figure 4, β-HB induced a significant increase in the level of type I TGF-β receptors. Most importantly, statistically significant reduction of protein expression of type I TGF-β receptors instead of type II receptor was demonstrated for the aqueous extract of Carthamus tinctorius L. from Western blot analysis. These results (see also Fig. 3) suggest that safflower extract may have the potential to retard β-HB-induced cellular fibrosis through down-regulation of the TGF-β autocrine loop (i.e., decreasing both TGF-β secretion and receptor expression).

Smads-related signal molecules were examined under the treatment of safflower because the Smad family is the most important mediator for post-receptor signaling of TGF-β The β-hydroxybutyrate (10 mM) significantly increased pSmad2/3, Smad2/3, and Smad4 (Fig. 5B–E). Intriguingly, the water extract of Carthamus tinctorius L. dose-dependently (25, 50, 100, and 200 μg/ml) and dramatically suppressed β-HB-induced increases in pSmad2/3, Smad2/3, and Smad4. These observations show that safflower may reverse β-HB-induced cellular fibrosis by suppressing TGF-β down-stream signals. On the other hand, the Smad-7, an inhibitory Smad, has also been examined. We showed that 10 mM of β-HB significantly induced a decrease in Smad7; however, the aqueous extract of Carthamus tinctorius L. dose-dependently (25, 50, 100, 200 μg/ml) and dramatically reversed the β-HB-induced decrease in Smad7. Actually, 200 μg/ml of safflower extract induced a sevenfold increase in the level of Smad7 (Fig. 5F). In other words, safflower extract may ameliorate renal cellular fibrosis by inducing an increase in the inhibitory

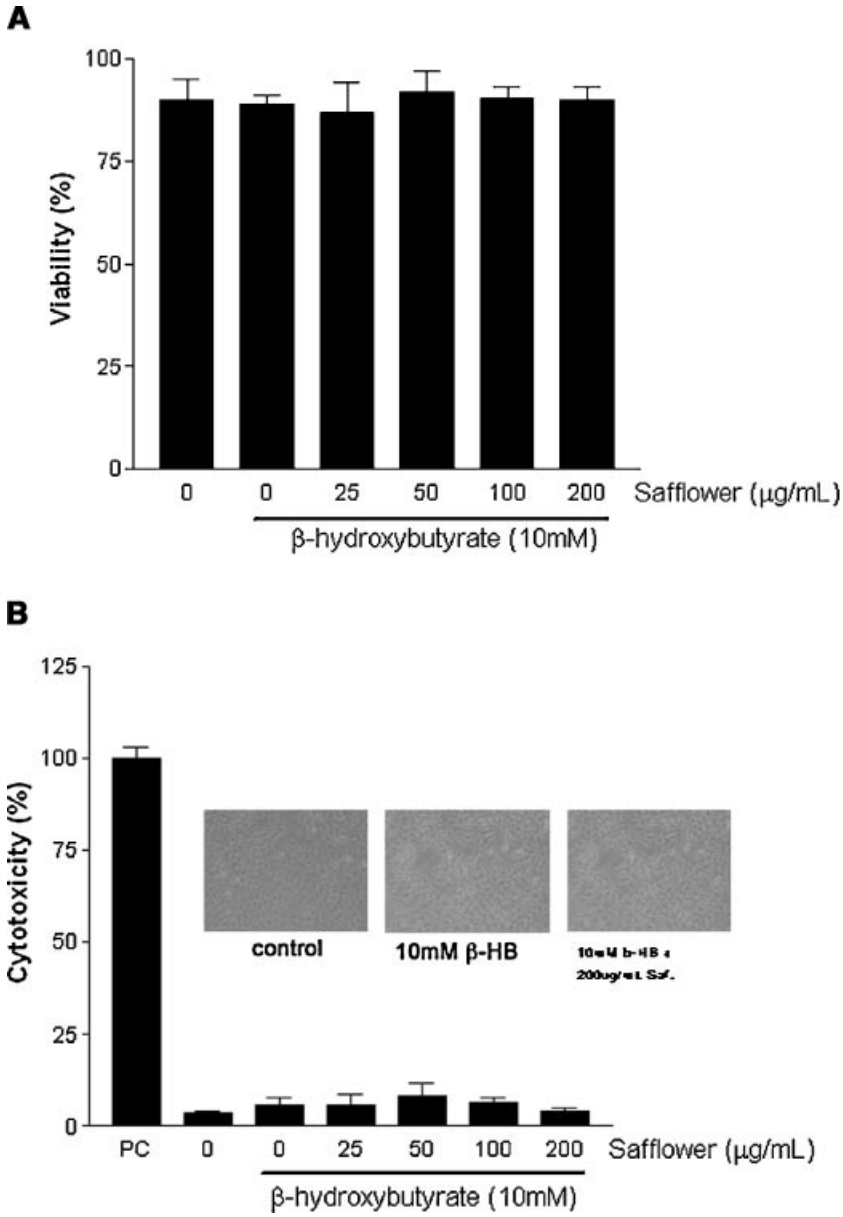

Fig. 1. Effects of safflower extract on cellular viability and cytotoxicity. **A**: Cells were treated with 10 mM β-hydroxybutyrate in 10% FCS for 48 h, followed by treatment with water extract of safflower (0, 25, 50, 100, and 200 μg/ml) for another 24 h. The supernatant was then collected and subjected to LDH assay. The remaining cell layer was subjected to trypan blue exclusion assay. The cytotoxicity (%) was expressed as: 100× (experimental value−low control)/(high control−low control). Supernatants from the maintained and 1% Triton X-100 treated cells (designated PC) were considered as low and high controls, respectively. Results were expressed as mean ± SEM of three independent experiments performed in duplicate. It is evident that safflower extract does not affect the viability of culture cells. **B**: 10 mM β-HB +200 μg/ml safflower.

Smad-7, a powerful intracellular TGF-β antagonist. Note that 200 μg/ml of safflower extract also dramatically decreased Smad4, Smad2/3, and pSmad2/3. Thus, this study was the first to demonstrate that an aqueous safflower extract is a natural Chinese herb with strong TGF-β antagonist activity.

According to Li and Che [1998] Carthamus tinctorius L. is one of the most important drugs in traditional Chinese medicine. To further demonstrate the fibrosis-antagonizing effects of safflower extract in vivo, we established an animal model of renal fibrosis using unilateral ureteral obstruction. According to our

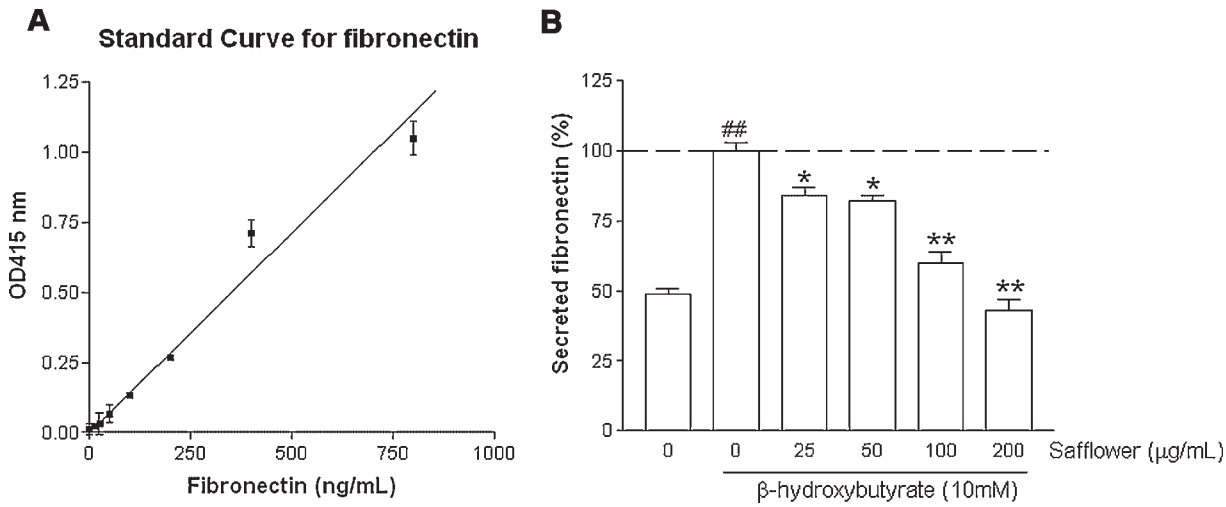

Fig. 2. Antagonizing effects of safflower extract on β-hydroxybutyrate-induced fibronectin expression in NRK cells. **A**: Standard curves were generated using known concentrations of fibronectin (12.5, 25, 50, 100, 200, 400, and 800 μg/ml). ELISA was performed as the manufacturer's instructions. The absorbance (415 nm) of each sample was then analyzed by an ELISA reader. **B**: Cells were treated with 10 mM β-hydroxybutyrate in 10% FCS for 48 h, followed by treatment with water extract of safflower for another 24 h. Supernatant was collected and subjected to fibronectin ELISA analysis. Fibronectin level was determined by interpolation with the standard curve. The fibronectin level of each condition was normalized to the cell number of each well. It is evident that β-hydroxybutyrate induced a significant increase in fibronectin levels. In addition, the safflower extracts dose-dependently attenuated β-hydroxybutyrate-induced increase in fibronectin level. $^{*}P < 0.05$, $^{**}P < 0.01$ versus β-hydroxybutyrate control (10 mM β-hydroxybutyrate in the absence of safflower), $^{\#\#}P < 0.01$ versus control (0 mM β-hydroxybutyrate).

unpublished observations, the plasma TGF-β1 levels in rats 2 weeks after UUO was significantly higher relative to controls (i.e., non-UUO rats). This finding is in accordance with previous studies showing that renal TGF-β1 was dramatically increased in both obstructive renal disease and fibrosis [Kaneto et al., 1999; Klahr and Morrissey, 2002]. In addition, the right kidneys from all animals subjected to UUO showed no significant evidence of renal

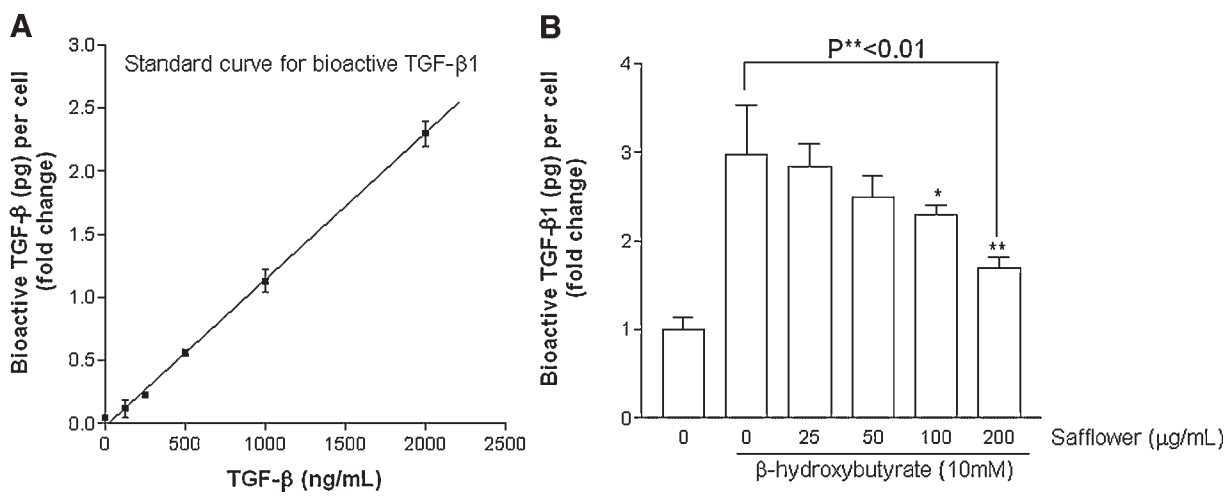

Fig. 3. Effects of safflower extract on the level of secreted bioactive TGFβ1. **A**: To establish the standard curve for TGF-β1 ELISA assay, exogeneous TGF-β1 (from 0 to 2,000 pg/ml) was used as a standard. The absorbance (450 nm) of each sample was correlated with the concentration of TGF-β1. Linear regression was performed as above. **B**: Cells were treated with 10 mM β-hydroxybutyrate in 10% FCS for 48 h, followed by treatment with water extract of safflower for another 24 h. The supernatant was then collected and subjected to TGF-β1 ELISA analysis. The secreted level for each experimental condition was normalized according to cell number. Measurements were repeated twice and similar results obtained. It is evident that safflower extract dose-dependently induced a significant decrease in TGF-β1 secretion in NRK cells. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (0 mM β-hydroxybutyrate).

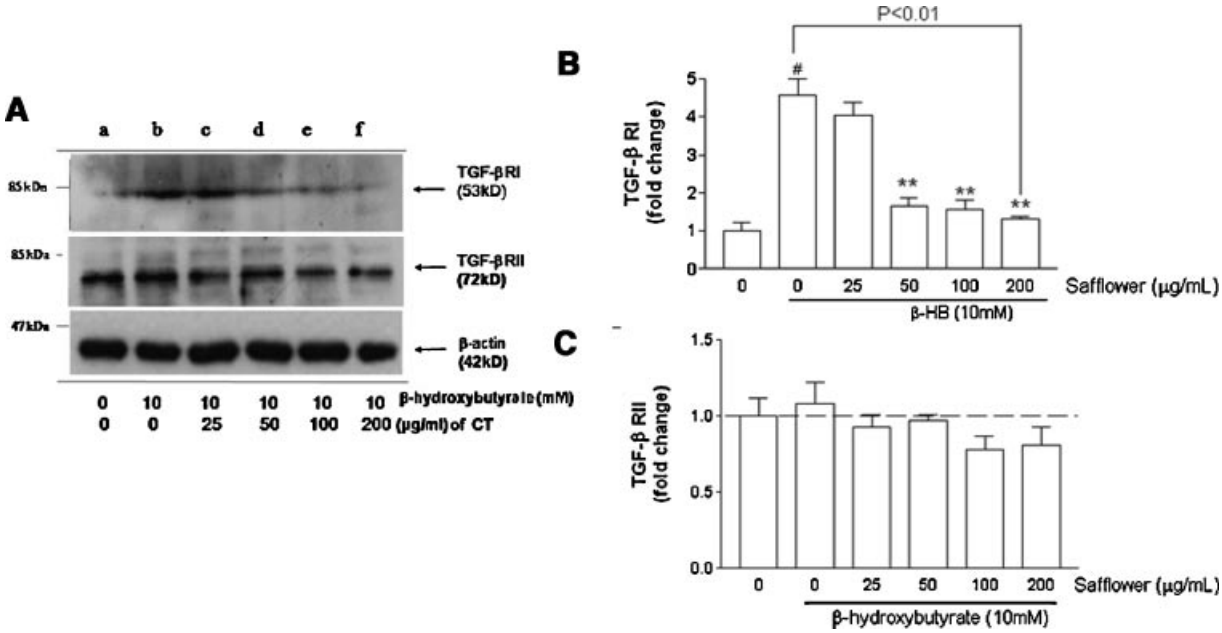

Fig. 4. Effects of safflower extract on β-hydroxybutyrate-induced TGF-β receptor expression in NRK cells. **A**: NRK Cells were treated with 10 mM β-hydroxybutyrate in 10% FCS for 48 h, followed by treatment with water extract of safflower for another 24 h. Protein was extracted and 10 μg of protein lysate was subjected to 10% SDS–PAGE analysis. After protein transfer to nitrocellulose paper, the blots were probed with 1:1,000 (v/v) dilution of polyclonal anti-TGF-βRI or TGF-βRII primary antibody. After hybridization, the blots were washed and hybridized with 1:2,000 (v/v) dilution of goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody. β-actin was used as a loading control. The expression of TGF-β RI (**B**) and TGF-β RII (**C**) proteins was normalized to that of β-actin. Results were expressed as mean ± SEM of three observations. It is evident that safflower extract significantly reduced β-hydroxybutyrate-induced increase in the expression level of type I TGF-β receptors. $^{**}P < 0.01$ versus control (0 mM of safflower extract). $^{\#}P < 0.05$ versus control (0 mM of β-hydroxybutyrate).

fibrosis or structural abnormality from Masson staining for extracellular collagen (Fig. 6A–C). These observations demonstrate that the right kidney appears to operate normally and compensate for the loss of function caused by the UUO. By contrast, the obstructed left kidneys exhibited serious structural abnormalities and significant fibrosis around the glomerulus and tubular interstitial connective tissues (Fig. 6D). More interestingly, continuous oral administration of safflower extract for 2 weeks after the UUO (Fig. 6F) was associated with significant amelioration of tubular and interstitial fibrosis in the experimental animals compared with the controls (Fig. 6D). It appears reasonable to suggest, therefore, that the beneficial effects of safflower extract in UUO-induced renal interstitial fibrosis may be due to inhibition of TGF-β autocrine loop (Figs. 2–5).

TGF-β1 is a relatively ubiquitous cytokine that functions in an autocrine or paracrine fashion to elicit a multiplicity of effects principally related to the accumulation of extracellular matrix. Further, it has been demonstrated that TGF-β1 plays an important role in renal fibrosis. Thus, the plasma TGF-β1 was also evaluated in this study. Figure 7 shows a significant decrease in TGF-β1 plasma level in UUO rats treated with safflower extract (0.15 g/3 ml/0.25 kg/day) (ameliorated) compared with the negative controls. Hence, safflower extract has the potential to antagonize renal fibrosis through down-regulation of TGF-β1 and its down-stream signal transducers. To sum up, this study provides important and pivotal evidence with respect to the anti-fibrogenic role of Carthamus tinctorius L. Thus, it appears reasonable to suggest that safflower extract may be an effective therapeutic supplement for treatment of renal interstitial fibrosis.

DISCUSSION

Safflower is used as a traditional Chinese medicine that has antioxidant and anti-inflammatory effects. The results of the present study demonstrate that safflower extract may play a protective role for renal fibrosis. Although several reports show that safflower

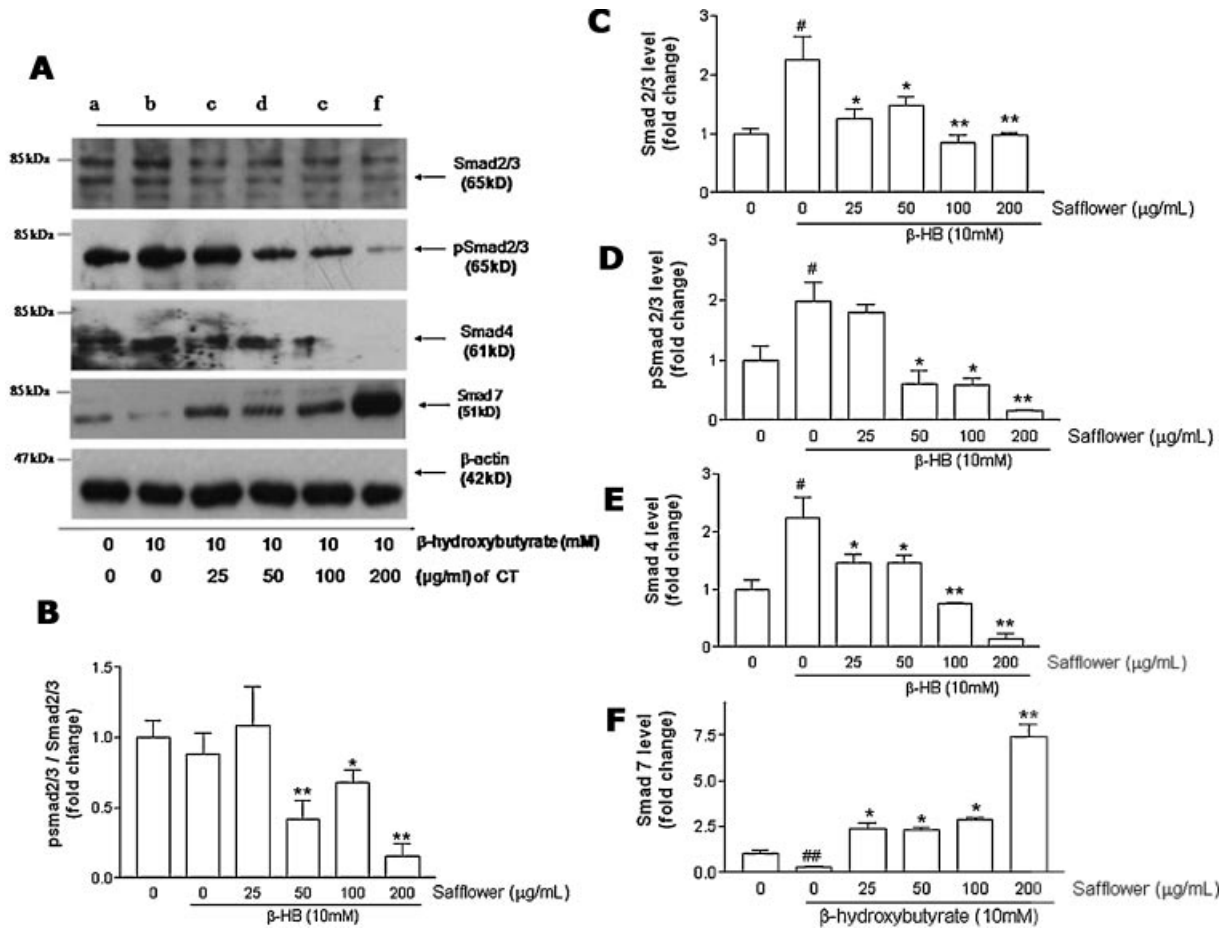

Fig. 5. Antagonizing effects of safflower extract on β-hydroxybutyrate-induced expression of TGF-beta downstream signals in NRK cells. **A**: NRK Cells were treated with 10 mM β-hydroxybutyrate in 10% FCS for 48 h, followed by treatment with water extract of safflower (Carthamus tinctorius *L.*, designated as ''CT'') for another 24 h. Protein was extracted and 10 μg of protein lysate was subjected to 10% SDS–PAGE analysis. After protein transfer to nitrocellulose paper, the blots were probed with 1:1,000 (v/v) dilution of antibodies against Smads proteins, including Smad2/3 (**C**), pSmad2/3 (**D**), Smad4 (**E**), and Smad7 (**F**), with β-actin as a loading control. **B**: Level of pSmad2/3 expressed as fold change of total Smad. It is evident that safflower extract not only induces a decrease in Smad2/3 but it also suppressed the level of pSmad2/3 relative to total Smad. In addition, it is evident that β-hydroxybutyrate induced activation of Smads pathway (increasing Smad2/3, pSmad2/3 and Smad4, and decreasing inhibitory Smad7). By contrast, safflower extract dramatically attenuated the effects of β-hydroxybutyrate (i.e., decreasing Smad2/3, pSmad2/3 and Smad4, and increasing inhibitory Smad7). The experiment was duplicated with similar results observed. $^{**}P < 0.01$, $^{*}P < 0.05$ versus control (0 mM safflower extract). $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus control (0 mM β-hydroxybutyrate).

contains active fractions which can antagonize against oxidative and inflammatory stress [Nakamura et al., 1973; Bolte et al., 2002; Kanehira et al., 2003], the functional significance of renal protection in, and mechanism of action against, renal fibrosis remain unclear. This study provides convincing evidence that the aqueous extract of safflower is a novel fibrosis antagonist that functions by suppressing TGF-β signals.

Progressive renal disease, characterized histologically by tubular atrophy and the accumulation of extracellular matrix proteins in the renal interstitium, is associated with declining renal function [Eddy, 1996, 2000; Placier et al., 2006; Rastaldi, 2006]. Thus, it has been suggested that fibrotic activity in renal interstitial fibroblasts is an early indicator of nephropathy. In this study, β-hydroxybutyrate was used to establish a fibrosis model in fibroblasts as β-hydroxybutyrate (<10 mM) is the major ketone body in serum during diabetic ketoacidosis. Blood ketone-body concentrations can reach 7–8 mM following starvation, and can exceed 20 mM during ketoacidosis. In the present study, β-hydroxybutyrate treatment

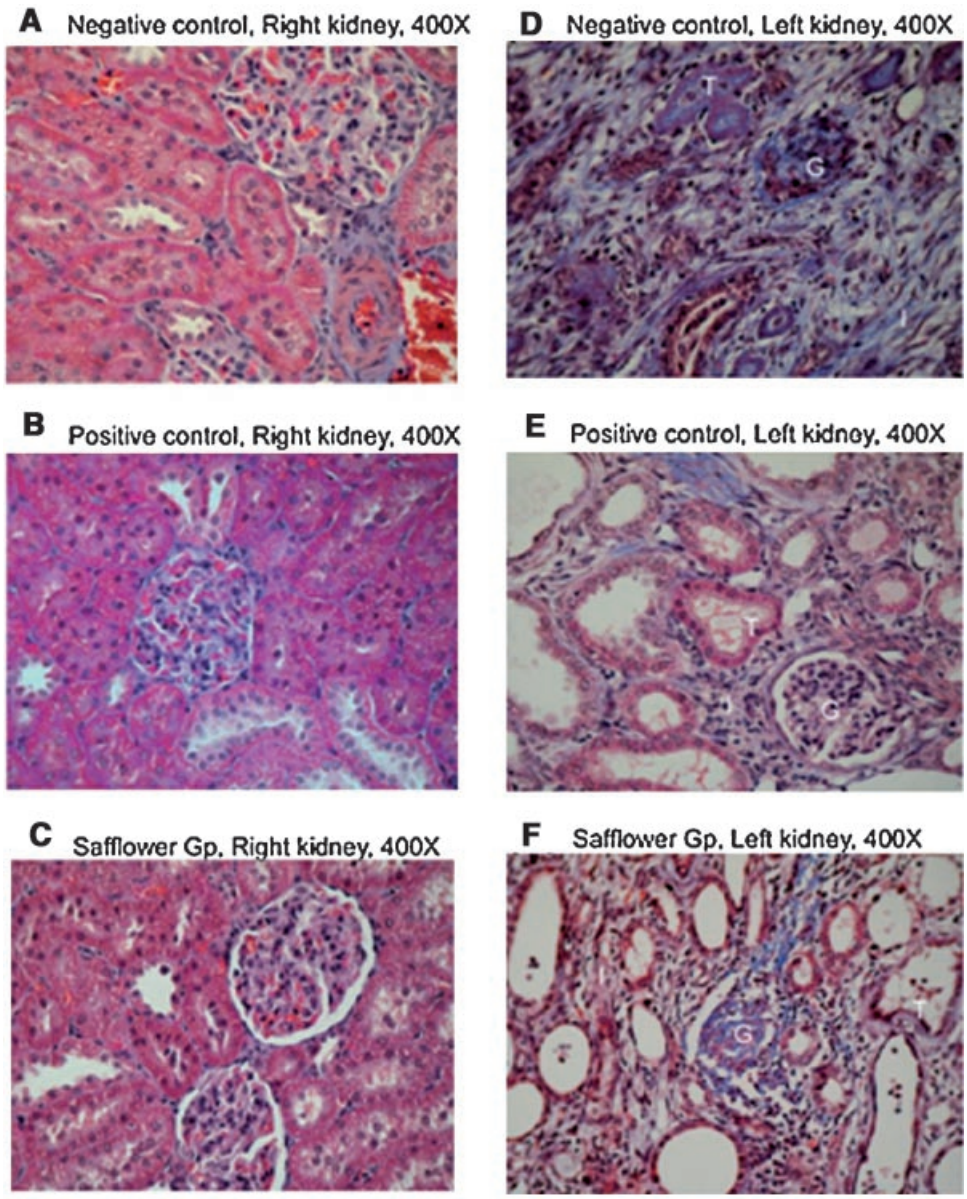

Fig. 6. Nephron fibrosis severity. UUO rats were allocated into three groups (n = 5 in each). The negative control group (**A**,**D**; un-therapeutic group) consisted of rats fed with distilled water daily for 2 weeks (3 ml/0.25 kg/day). This group was used to provide pathological data about renal fibrosis. The positive control group (**B**,**E**; therapeutic group) consisted of rats given captopril (2.5 mg/3 ml/0.25 kg/day) for 2 weeks. The experimental group (**C**,**F**) consisted of rats orally given water extract of safflower (0.15 g/3 ml/0.25 kg/day) for 2 weeks. Kidney sections were stained with Masson trichrome, and the proportion of aniline blue-stained area (%) measured. It is evident that there was no evidence of fibrosis in the right kidney in any group (A–C). However, significant fibrosis (blue color) is evident in the left kidneys, which are subjected to ureteral ligation, around the glomerulus and tubular interstitial connective tissues (D). More importantly, amelioration of tubular and interstitial fibrosis was demonstrated for the animals treated with captopril (E) or safflower extract (F) relative to the negative controls. (Masson staining, original magnification 400×; G″ glomerulus; T, tubular; I, interstitial tissue).

(10 mM) induced significant expression of fibronectin in NRK cells (renal interstitial fibroblasts) (Fig. 2) concomitantly with the induction of bioactive TGF-β1 (Fig. 3). In addition, expression of Smads 2, 3, and 4 was increased with treatment of β-hydroxybutyrate. Simultaneously, Smad 7 was dramatically suppressed (Fig. 5). This observation is totally consistent with our previous findings showing that β-hydroxybutyrate-induced collagen production in proximal tubule cells is dependent on TGF-β and Smad3 [Guh et al., 2003]. Thus, this

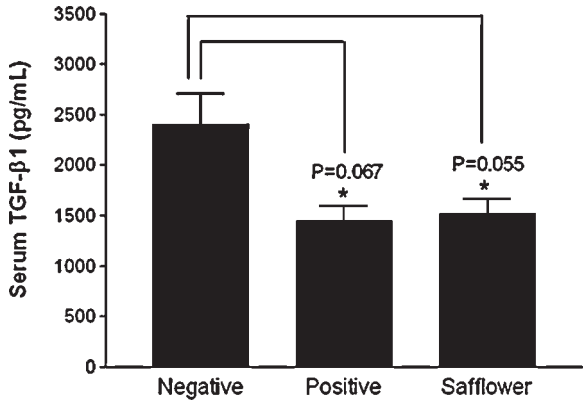

Fig. 7. Effects of safflower extract on serum level of fibrogenic growth factor, TGF-β1. Rats were subjected to unilateral ureteral obstruction and then allocated into a negative or (untreated) positive control group (captopril) or an experimental group (safflower extract) (n = 5 each) as described in Methods Section. After 2 weeks, a blood sample was collected from each animal and serum TGF-β1 was assayed using an ELISA kit. It is evident that both captopril (2.5 mg/3 ml/0.25 kg/day) and safflower extract (0.15 g/3 ml/0.25 kg/day) significantly decreased serum TGF-β1 compared with the untreated animals (n = 5). $*P < 0.05$ versus negative control.

study clarifies the mechanism underlying β-hydroxybutyrate-induced renal fibrosis.

Induction of oxidative stress may be one of the mechanisms underlying the pathogenesis of renal fibrosis. Antioxidant therapy may help prevent the development of renal fibrosis [Yan et al., 2007]. Further, many reports have shown that antioxidant treatment attenuates these pathological changes and prevents renal fibrosis [Guh et al., 2003; Sugiyama et al., 2005]. The present study is the first to demonstrate that safflower can dose-dependently and significantly inhibit renal cellular fibrosis. Further, β-hydroxybutyrate induced a significant increase in the secretion of both TGF-β and fibronectin (Figs. 2B and Fig.33B). More importantly, we demonstrated that the aqueous extract of safflower dose-dependently attenuates β-hydroxybutyrate-induced increases in the levels of secreted TGF-β and fibronectin. We suggest that this is partially due to the antioxidant compounds contained in safflower extract as significant free-radical scavenging properties have previously been demonstrated [Nakamura et al., 1973; Bolte et al., 2002; Kanehira et al., 2003]. Further, many researchers have shown that this antioxidative potential protects cells against oxidative stress [Lee et al., 2002]. Taken together with our unpublished observations showing that aqueous safflower extract induces a significant decrease in cellular oxidative stress (from cytometry assay), we hypothesize that the antioxidants in safflower may partly account for our results (Figs. 2 and 3).

TGF-β is known to have powerful fibrogenic properties resulting from both stimulation of matrix synthesis and inhibition of matrix degradation [Border and Noble, 1994; Wolf and Ziyadeh, 1999]. Thus, developing agents that antagonize TGF-β post-receptor signaling has become a critical issue facing nephropathologists. In the present study, β-hydroxybutyrate enhanced the expression of type I TGF-β receptors (Fig. 4) and induced activation of the Smads pathway (i.e., increase Smad2/3, pSmad2/3, Smad4, and decrease inhibitory Smad7) (Fig. 5). Further, water extract (hydrophilic fractions) of safflower dramatically attenuated β-hydroxybutyrate-induced cellular fibrosis by decreasing type I TGF-β receptors and the downstream signaling (including Smad2/3, pSmad2/3, and Smad4). Thus far, few inhibitors for type I TGF-β receptor that show clear antifibrotic effects have been identified. At the time of writing, this study was the first to demonstrate the potential medical efficacy of safflower as an inhibitor for the type I TGF-β receptor for treatment of renal fibrosis. However, due to the pleiotropic effects of TGF-β, the beneficial antifibrotic effects of type I TGF-β-receptor inhibition should be carefully balanced against the potential risk of unwanted outcomes stemming from chronic treatment.

In China, safflower is grown almost exclusively for its flowers, which are used in the treatment of many diseases. The main active ingredient in safflower medicines is water-soluble safflower yellow; however, alcohol extracts are used in some preparations. The results of numerous clinical and laboratory studies confirm the efficacy of safflower for menstrual problems, cardiovascular disease, and the pain and swelling associated with trauma. Other research has shown that safflower contains ingredients with anti-inflammatory properties [Romano et al., 1993; Takii et al., 2003]. The presence of these compounds may partially account for the fibrosis-antagonizing effects of safflower as anti-inflammatory agents might play a role in the control of cellular fibrosis.

In the UUO model, the ligated kidney is characterized by increased fibrosis and apoptosis, along with decreased renal function. TGF-β,

a proapoptotic and profibrotic cytokine, has been implicated in these changes. It has been shown that both a TGF-β antibody and antisense nucleotides directed toward TGF-β protect the obstructed kidney and ameliorate renal damage [Miyajima et al., 2000]. Hence, it appears reasonable to speculate that the water extract of safflower, a potential inhibitor of the TGF-β autocrine loop in renal fibroblasts (Figs. 2–5), has the potential to ameliorate renal fibrosis in vivo. As shown in Figure 6, oral administration of safflower extract significantly ameliorates interstitial renal fibrosis in comparison to controls. This may reflect the in vitro effects of renal fibroblasts treated with safflower extract. In addition, significant therapeutic efficacy was also shown for the captopril-treated (positive) controls, a finding which is consistent with that of Klahr and Morrissey [1997] who showed that renal fibrosis can be ameliorated by angiotensin-converting enzyme (ACE) inhibitors or an angiotensin II receptor antagonist.

In view of the results of the biochemical analysis from UUO rats (treated/untreated control groups), most serological results, including aspartate aminotransferase, alanine aminotransferase, glucose, total protein, albumin, creatinine, alkaline phosphatase, triglyceride, cholesterol, amylase, and serum ion (Ca^{++}, Na^{+}, K^{+}, phosphorus) were almost in the normal range (data not shown). These observations demonstrate the near-total compensation for lost renal function by the non-ligated right kidney.

At the time of writing, there were no reports in the literature demonstrating safflower's potential for amelioration of nephropathy. Therefore, we explored the mechanisms underlying safflower's retardation of renal fibrogenesis via blockade of the TGF-β autocrine pathway. The effectiveness of the short-term intervention on established fibrosis suggests that similar applications could be used in chronic cases to avoid long-term systemic inhibition of TGF-β.

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