



## Fuzheng Huayu recipe and vitamin E reverse renal interstitial fibrosis through counteracting TGF- $\beta$ 1-induced epithelial-to-mesenchymal transition

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### ABSTRACT

**Aim:** To investigate the mechanism of action of Fuzheng Huayu recipe (FZHY) and vitamin E (Vit E) against renal interstitial fibrosis related to transforming growth factor-beta1 (TGF- $\beta$ 1) mediated tubular epithelial-to-mesenchymal transition.

**Materials and methods:** Renal interstitial fibrosis was induced by administration of HgCl<sub>2</sub> at a dose of 8 mg/kg body weight once a day for 9 weeks. Rats were randomly divided into four groups: normal, model, FZHY, and Vit E group. Rats in the latter two groups were treated with the FZHY recipe and Vit E respectively. HK-2 cells were treated with TGF- $\beta$ 1 for 24 h, followed by incubation with either SB-431542 (a potent and specific inhibitor of T $\beta$ R-1 kinase) or FZHY drug-containing serum for another 24 h. Hyp content in rat kidney tissue was assayed with Jamall's method and collagen deposition in kidney was visualized using Masson stain. Protein expression of TGF- $\beta$ 1, T $\beta$ R-1, Smad2, p-Smad2, Smad3, and p-Smad3 was analyzed by Western blotting. Protein expression and the location of Smad3 in kidney was assayed by immunohistochemistry, E-cadherin, cytokeratin 18 (CK-18),  $\alpha$ -SMA and TGF- $\beta$ 1 by immunofluorescent stain.

**Results:** FZHY and Vit E inhibited renal collagen deposition and reduced Hyp content significantly. They upregulated E-cadherin protein expression and down-regulated the protein expression of  $\alpha$ -SMA, TGF- $\beta$ 1, p-Smad2, p-Smad3, and T $\beta$ R-1. Lastly, they inhibited the nuclear translocation of Smad3 in fibrotic kidney tissue. FZHY drug-containing serum significantly upregulated the expression of CK-18 and down-regulated the expression of  $\alpha$ -SMA, T $\beta$ R-1, p-Smad2/3 in TGF- $\beta$ 1 stimulated HK-2 cells.

**Conclusion:** The mechanism of action of FZHY and Vit E against renal interstitial fibrosis is related to the reversal of tubular EMT induced by TGF- $\beta$ 1.

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### 1. Introduction

Renal interstitial fibrosis (RIF) is the common pathway in progressive renal disease leading to functional deterioration and eventual loss of renal function, irrespective of the nature of the initial (Li and Liu, 2007). RIF is characterized by the accumulation of extracellular matrix (ECM) components and the loss of tubular architecture. One of the main effector cells that contributes to the development of progressive renal fibrosis in chronic renal disease is the tubulointerstitial fibroblast. Most notably, a large proportion of these cells are known to originate from tubular epithelial cells

through the process of epithelial-to-mesenchymal transition (EMT) during the progression to renal fibrosis (Iwano et al., 2002). Transforming growth factor-beta1 (TGF- $\beta$ 1) is proposed to be the major regulator in inducing EMT and renal fibrosis (Fan et al., 1999). In contrast to how EMT promotes disease progression, several studies have suggested that EMT of tubular epithelial cells can be reversible (Yang and Liu, 2003).

Fuzheng Huayu recipe (FZHY) is a SFDA-approved anti-fibrotic medicine in China (Zhao et al., 2006) made from the following six traditional Chinese herbs: Danshen (*Radix Salviae Miltiorrhizae*), Taoren (*Semen Persicae*), Wuweizi (*Fructus Schisandrae Chinensis*), Dongchongxiacao (*Fermentation Mycelium Powder*), Songhuafen (*Pollen Pini*) and Jiaogulan (*Gynostemma Pentaphyllum*). As far as Traditional Chinese Medicine (TCM) theory is concerned, this concoction functions to promote blood flow, dissolve blood stasis, tonify spirit, and nourish the liver. In our previous study, a rat renal interstitial fibrosis model was successfully established by the oral administration of mercuric chloride (HgCl<sub>2</sub>). It was characterized

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**Table 1**  
Antibodies used in the study.

Antibody	Isotype	Suppliers	Cat. no.	Dilution
E-cadherin	Mouse IgG <sub>2a</sub>	BD Biosciences	610182	0.25 µg/ml
α-SMA	Mouse IgG <sub>2a</sub>	Sigma	A2547	1:200
TGF-β1	Mouse IgG1	R&D Systems	MAB240	5 µg/ml
TβR-I	Rabbit IgG	Cell Signaling Technology	#3712	1:1000
Smad2	Mouse IgG1	Cell Signaling Technology	#3103	1:800
Smad3	Rabbit IgG	Zymed Laboratories	51-1500	1:200
p-Smad2	Rabbit IgG1	Cell Signaling Technology	#3101	1:200
p-Smad3	Rabbit IgG1	Cell Signaling Technology	#9520S	1:200
p-Smad2/3	Rabbit IgG	Santa Cruz Biotechnology	SC-11769-R	1:100
CK-18	Mouse IgG1	Chemicon International	MAB240	1:200

by obvious lipid peroxidation injury, activation of renal myofibroblasts, and the over-production and deposition of extracellular matrix (ECM) (Yuan et al., 2006, 2008). Moreover, we found that FZHY exhibited good effect against this renal interstitial fibrosis model. Its mechanism of action was associated with the inhibition of renal peroxidation (Jiang et al., 2004). Since vitamin E (Vit E) is a potential antioxidant and has been reported to prevent organ fibrosis (Huang et al., 2006; Di Sario et al., 2007; Hemmati et al., 2008), we also used Vit E in our previous study as a control drug and revealed that it could act against renal interstitial fibrosis induced by HgCl<sub>2</sub> through anti-lipid peroxidation injury, in rats (Wang et al., 2008). However, it is unknown whether the effects of FZHY and Vit E on rat renal interstitial fibrosis are related to the function of effector cells, i.e. EMT.

Therefore, in this study, we aimed to discover whether (1) FZHY and/or Vit E has inhibitory effects against EMT *in vivo* and *in vitro* (2). The effects of FZHY and/or Vit E on EMT are mediated by signaling molecules, including Smad2, Smad3, or phosphorylated Smad2/3 expression, which have been demonstrated to be the major signal pathways in TGF-β1-induced EMT (Phanish et al., 2006).

## 2. Materials and methods

### 2.1. Reagents

HgCl<sub>2</sub> was purchased from Tongren Chemical Reagent Factory, GuiZhou, China. Standard solution of hydroxyproline (Hyp) was from Dong Feng Japan Co., Ltd. Recombinant human TGF-β1 was obtained from R&D Systems, USA. SB-431542, a potent and specific inhibitor of TGF-β type I receptor (TβR-I) kinase, was purchased from TOCRIS Bioscience, USA. Primary antibodies used in the study are listed in Table 1. Horseradish peroxidase-conjugated rabbit anti-mouse Ig and peroxidase-conjugated goat anti-rabbit Ig were obtained from Chemicon, Temecula, CA, USA. BCA Protein Assay Kit and supersignal west pico chemiluminescent substrate (ECL) were purchased from Pierce Chemical Company, Rockford, USA. Cy3-labeled goat anti-mouse IgG (H+L) was provided by the Beyotime Institute of Biotechnology, Haimen, China. FITC-labeled goat anti-mouse IgG (H+L) was purchased from Invitrogen Corporation (Carlsbad, CA, USA).

### 2.2. Drugs

FZHY was prepared and provided by Shanghai Sundise Medicine Technology Development Co., Ltd., China (SFDA approval No: Z20050546) (Shanghai, China). The following crude herbs, 8.0 g of Danshen, 4.0 g of Dongchongxiacao, 2.0 g of Wuweizi, 2.0 g of Taoren, 2.0 g of Songhuafen, and 6.0 g of Jiaogulan, were made into FZHY extract powder. The quality control and preparing standardization of FZHY is established and enforced strictly by Shanghai Sundise Medicine Technology Development Co., Ltd. The content of major ingredients were measured on line during the manufacturing processes to provide adequate quality control

(Table 2). In this study, FZHY powder was suspended in distilled water.

Vitamin E (Vit E) was purchased from Shanghai Yanan Wangxing Pharmaceutical Co., Ltd., Shanghai, China.

### 2.3. *In vivo* experimental design

44 Sprague–Dawley male rats, weighing 120 ± 10 g (SPF, Certificate No. SCXK 2003-0003), were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The rats were randomly divided into four groups: normal (*n* = 8), model (*n* = 12), FZHY treatment (FZHY) (*n* = 12) and vitamin E treatment (Vit E) (*n* = 12). The renal interstitial fibrosis model was induced by oral administration of HgCl<sub>2</sub> at a dose of 8 mg/kg body weight once a day for 9 weeks (Yuan et al., 2006). Meanwhile, rats in the FZHY and Vit E groups were treated with FZHY powder at a dose of 4.0 g (crude drug)/kg body weight and Vit E at a dose of 100 mg/kg body weight respectively once a day for 9 weeks. Rats were sacrificed 9 weeks after treatment and the kidneys removed. A portion of each kidney was fixed in 10% phosphate-buffered formalin for histological and immunohistochemical studies after paraffin embedding. The remainder was snap-frozen in liquid nitrogen and stored at -80 °C for Hyp content determination and protein extractions. All experimental procedures were carried out in accordance with internationally accepted principles for laboratory and all animals received humane care during the study with unlimited access to chow and water.

### 2.4. Examination of Hyp content in renal tissues

Hyp content of kidney was assayed with HCl hydrolysis according to Jamall's methods (Jamall et al., 1981). Briefly, renal tissue samples weighing 100 mg were homogenized in 2.5 ml of ice-cold double-distilled water. After determining the total protein concentration in homogenates, 2 ml of homogenates were hydrolyzed with HCl (final concentration: 6 M) at 105 °C for 18 h. Hydrolysates were filtrated with 3 mm filter paper and dried at 40 °C. The samples were then incubated with Ehrlich's solution (25% (w/v) p-dimethylaminobenzaldehyde and 27.3% (v/v) perchloric acid in isopropanol) at 50 °C for 90 min and measured at A<sub>558</sub> nM. All results were normalized by

**Table 2**  
Quality control standard for Fuzheng Huayu recipe (FZHY).

Compounds (marker)	Quality criterion
Salvianolic acid B	Referred to Danshen, should not be <15.6 mg in 24 g of FZHY crug drug
Sodium Danshensu	Referred to Danshen, should not be <13.2 mg in 24 g of FZHY crug drug
Adenosine	Referred to Chongcaojunsi, should not be <4.8 mg in 24 g of FZHY crug drug
Schisandrin B	Referred to Wuweizi, should not be <2.28 mg in 24 g of FZHY crug drug

total protein concentration and calculated using a standard curve.

### 2.5. Histologic examination

The kidneys fixed in 10% phosphate-buffered formalin were embedded in paraffin and sectioned (3  $\mu$ m thickness). The sections were stained with Masson trichrome to assess collagen fiber deposition.

### 2.6. Immunohistochemical examinations

3  $\mu$ m-thick sections were used for immunohistochemical examinations. These sections were digested with pepsin at 37 °C for 20 min, followed by incubation with 0.1% BSA in PBS for 30 min, and then incubated again with primary antibodies against E-cadherin (1:50),  $\alpha$ -SMA (1:100), TGF- $\beta$ 1 (1:100), or Smad3 (1:100) at 37 °C for 1 h. For immunohistochemical analysis, the sections were incubated with horseradish-coupled secondary antibody at 37 °C for 1 h, and signals were detected by DAB. For immunofluorescence staining, the sections were incubated with FITC- or Cy3-labeled second antibodies at 37 °C for 1 h, and images were observed with a fluorescence microscope (Olympus).

### 2.7. Cell culture and treatment

Human proximal tubular epithelial cells (HK-2 cells) were obtained from The Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal bovine serum (FBS). HK-2 cells were seeded into 60-mm-diameter culture dishes to approximately 60–70% confluence in a complete medium containing 5% FBS for 16 h and then changed to serum-free medium after washing twice with PBS. TGF- $\beta$ 1 was added to the culture to produce a final concentration of 2.5 ng/ml and incubated for 24 h. Thereafter, the medium was changed, and the cells were incubated with (1) DMEM containing 10% normal rat serum, (2) DMEM containing 10% normal rat serum + 2.5 ng/ml TGF- $\beta$ 1, (3) DMEM containing 10% FZHY drug-containing serum + 2.5 ng/ml TGF- $\beta$ 1 and (4) DMEM containing 10% normal rat serum + 2.5 ng/ml TGF- $\beta$ 1 + 10  $\mu$ M SB-431542 for an additional 24 h. All experiments were repeated at least three times using independent cell cultures.

### 2.8. Preparation of FZHY-containing serum

FZHY powder was administered to the rats at a dose of 2 g (crude drug)/kg body weight by intragastric gavage twice a day for 3 days. The rats were given FZHY 1 h before being sacrificed on the 4th day. Blood samples were collected under anesthesia. The samples were placed at 4 °C for 3–4 h and then centrifuged at 3000 rpm for 20 min. The sera were inactivated at 56 °C for 30 min and then stored at –20 °C for further use.

### 2.9. Immunocytochemical staining

Indirect immunofluorescence was the technique performed to achieve immunocytochemical staining. Briefly, HK-2 cells cultured on coverslips were washed with cold PBS twice and fixed with cold methanol:acetone (1:1) for 10 min on ice. After extensively washing three times with PBS, the cells were permeated with 0.05% saponin for 15 min. The cells were then blocked with 5% bovine serum albumin in PBS buffer for 30 min at room temperature before being incubated with the specific primary antibodies (Table 1). To visualize the primary antibodies, cells were stained with FITC-conjugated secondary antibodies. After washing, cells were doublestained with Hoechst 33258 to visualize the nuclei.

Stained cells were mounted with antifade mounting medium and viewed with a fluorescence microscope (Olympus).

### 2.10. Western blot analysis

Kidney tissues and HK-2 cells were homogenized in RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 $\times$  Roche complete mini protease inhibitor cocktail). The supernatants were collected after centrifugation at 10,000  $\times$  g at 4 °C for 15 min. Protein concentration was determined using a BCA Protein Assay Kit. Equal amounts of protein were separated by 10% SDS gel electrophoresis (SDS-PAGE) under denaturing and non-reducing conditions, and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST at room temperature for 1 h, and then incubated with primary antibody (Table 1) at 4 °C overnight. After washing in TBST, the blots were incubated with horseradish-coupled secondary antibody. The signals were visualized using the enhancement system (ECL).

### 2.11. Statistical analysis

The data were analyzed using a one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test. Values of  $P < 0.05$  were considered statistically significant.

## 3. Results

### 3.1. FZHY and Vit E ameliorated renal interstitial fibrosis induced by HgCl<sub>2</sub> in our rat model

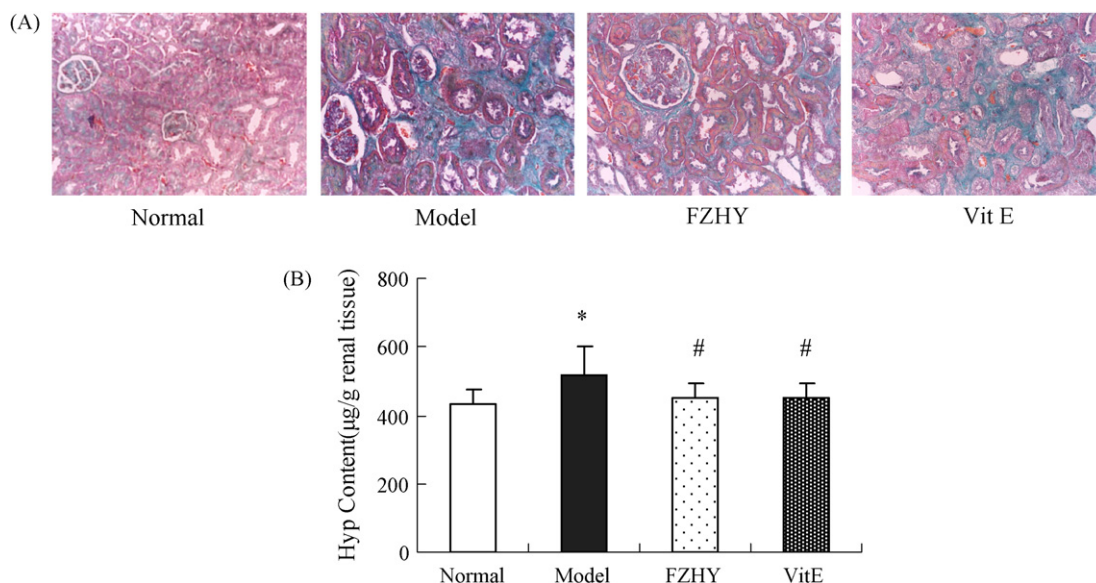
The excessive and disorganized deposition of collagens is a major pathogenic feature of fibrotic diseases. HgCl<sub>2</sub> induced an increased amount of collagen in the Masson's trichrome-stained kidney sections. In contrast, collagen was reduced in FZHY and Vit E-treated fibrotic kidneys (Fig. 1A).

Hyp content is a specific marker for collagen synthesis. The results showed that Hyp content in kidney was increased significantly in the model group when compared with the normal group. In contrast, FZHY and Vit E treatments significantly decreased kidney Hyp content of model rats, and there was no significant difference between FZHY and Vit E groups (Fig. 1B).

### 3.2. Effects of FZHY and Vit E on the expressions of $\alpha$ -SMA and E-cadherin in kidneys of model rats

Immunohistochemistry analysis revealed that the expression of  $\alpha$ -SMA was very weak in the renal interstium of normal kidneys, whereas it was prominent in model rats. On the other hand epithelial cell marker E-cadherin was markedly decreased in the tubular epithelium of model groups compared with normal group. Both FZHY and Vit E treatment attenuated the increased expression of  $\alpha$ -SMA in the fibrotic kidneys. Moreover, E-cadherin expression was maintained in response to FZHY and Vit E treatment (Fig. 2A). Therefore, the disruption of the tubular basement membrane might be ameliorated with FZHY and Vit E treatment. This observation could indicate that FZHY and Vit E treatment inhibit the process of EMT in the fibrotic kidneys, which is characterized by the loss of the epithelial phenotype and the appearance of the mesenchymal phenotype.

Consistent with the immunohistochemistry, Western blotting analysis revealed the increased  $\alpha$ -SMA and the decreased E-cadherin expression in the kidneys of rats of model group compared with normal group. In contrast, both FZHY and Vit E treatment significantly inhibited the up-regulation of  $\alpha$ -SMA, and the down-regulation of E-cadherin, but the effect of FZHY was more



**Fig. 1.** The Effect of FZHY and Vit E on RIF induced by  $\text{HgCl}_2$ . (A) Masson's trichrome staining for assessing renal interstitial fibrosis.  $\text{HgCl}_2$  induced an increase of collagen in kidney section. FZHY and Vit E treatment reduced collagen deposition. (B) Kidney Hyp content determined by Jamall's method. Hyp content of kidney was increased significantly in model group compared with normal group. In contrast FZHY and Vit E treatment significantly decreased kidney Hyp content.

pronounced than Vit E, with significant difference (Fig. 2B and C) but the effect of FZHY was more pronounced than Vit E.

### 3.3. FZHY and Vit E attenuated increase of TGF- $\beta$ 1 and T $\beta$ R-I in kidneys of model rats

Immunohistochemistry analysis revealed increased expression of TGF- $\beta$ 1 at the interstitial area in the kidneys of model rats compared with kidneys of normal rats, which was decreased by FZHY treatment and Vit E treatment (Fig. 3A).

Consistent with the immunohistochemistry, Western blotting analysis revealed the increased TGF- $\beta$ 1 and T $\beta$ R-I expression in the kidneys of rats in model group compared with the controls. In contrast, FZHY and Vit E treatment significantly inhibited the up-regulation of the TGF- $\beta$ 1 and T $\beta$ R-I, and there was no significant difference between FZHY and Vit E (Fig. 3B and C).

### 3.4. FZHY and Vit E treatment attenuated up-regulation of Smad2, Smad3 phosphorylation and Smad3 nuclear translocation in kidneys of model rats

Immunohistochemistry showed that the expression of Smad3 in kidney was almost in the cytoplasm of tubular cells in rats of normal group, whereas in model rats, the nucleus expression of Smad3 in kidney tissue was increased, FZHY and Vit E treatment inhibited the nucleus expression of Smad3 (Fig. 4A).

Western blotting analysis revealed unchanged expression of Smad2 in kidneys among four groups, whereas p-Smad2 and p-Smad3 expression was significantly increased in rats of model group compared with the controls. In contrast, FZHY and Vit E treatment significantly attenuated the up-regulation of p-Smad2 and p-Smad3 of model group, and the effect of FZHY was more pronounced better than Vit E, without statistical significance (Fig. 4B and C).

### 3.5. FZHY drug-containing serum and SB-431542 treatment attenuated TGF- $\beta$ 1-induced EMT in HK-2 cells

HK-2 cells were incubated with 2.5 ng/ml TGF- $\beta$ 1 to induce EMT. The results showed HK-2 cells were changed from a cuboidal

shape to a spindle shape in response to TGF- $\beta$ 1, whereas FZHY drug-containing serum and SB-431542 treatment blocked the morphologic transformation (Fig. 5A).

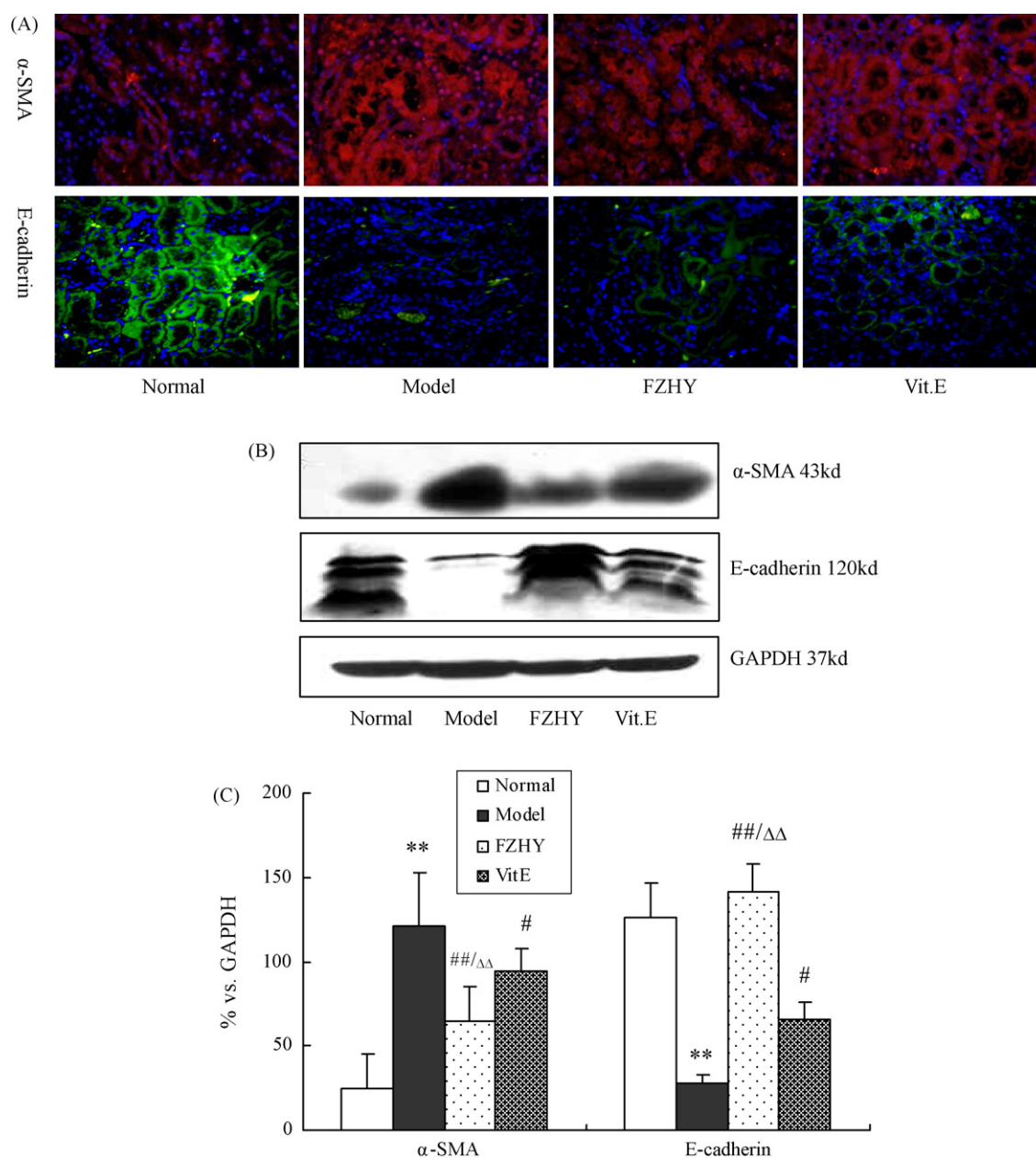
Immunofluorescence analyses showed the changes of cellular morphology were accompanied by increased labeling intensity of  $\alpha$ -SMA, and decreased labeling intensity of cytokeratin 18 (CK-18) after TGF- $\beta$ 1 treatment, while the increased labeling intensity of  $\alpha$ -SMA and decreased labeling intensity of CK-18 were reversed by FZHY drug-containing serum and SB-431542 treatment (Fig. 5A). Consistent with these findings, Western blotting revealed up-regulation of  $\alpha$ -SMA expression when the cells were exposed to TGF- $\beta$ 1, whereas FZHY drug-containing serum and SB-431542 treatment significantly attenuated the TGF- $\beta$ 1-induced up-regulation of  $\alpha$ -SMA (Fig. 5B). The decreased CK-18 expression after TGF- $\beta$ 1 exposure was significantly reversed by FZHY drug-containing serum and SB-431542 treatment (Fig. 5B). There was no significant difference between FZHY drug-containing serum and SB-431542 treatment.

### 3.6. FZHY drug-containing serum and SB-431542 treatment attenuated TGF- $\beta$ 1-induced Smad2/3 phosphorylation in HK-2 cells

As a mechanistic experiment for the effects of FZHY drug-containing serum, we examined the effects of FZHY drug-containing serum on TGF- $\beta$ 1-Smads signaling, which has been known to play a major role in the process of TGF- $\beta$ 1-induced EMT. The results showed that there was no significant change between each group in Smad2/3, but the expression of phosphorylated Smad2/3 was significantly increased after incubation with TGF- $\beta$ 1, whereas FZHY drug-containing serum and SB-431542 treatment significantly attenuated the TGF- $\beta$ 1-induced up-regulation of p-Smad2/3 (Fig. 6). The effect of SB-431542 was better than FZHY drug-containing serum, but without statistical significance.

## 4. Discussion

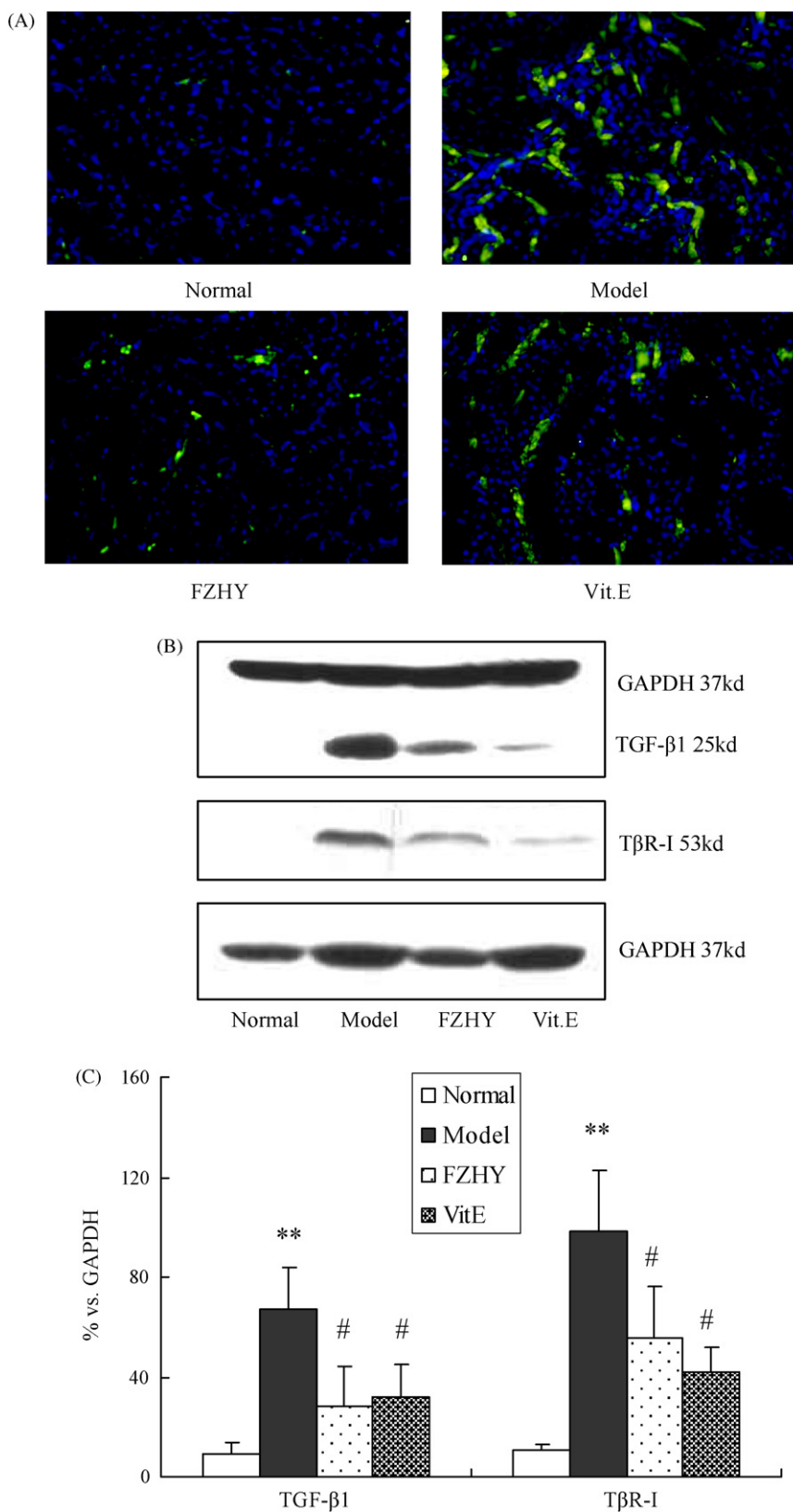
The pathophysiology of chronic kidney disease is characterized by a progressive loss of renal function and deposition of extracellular matrix leading to widespread tissue fibrosis. RIF is particularly



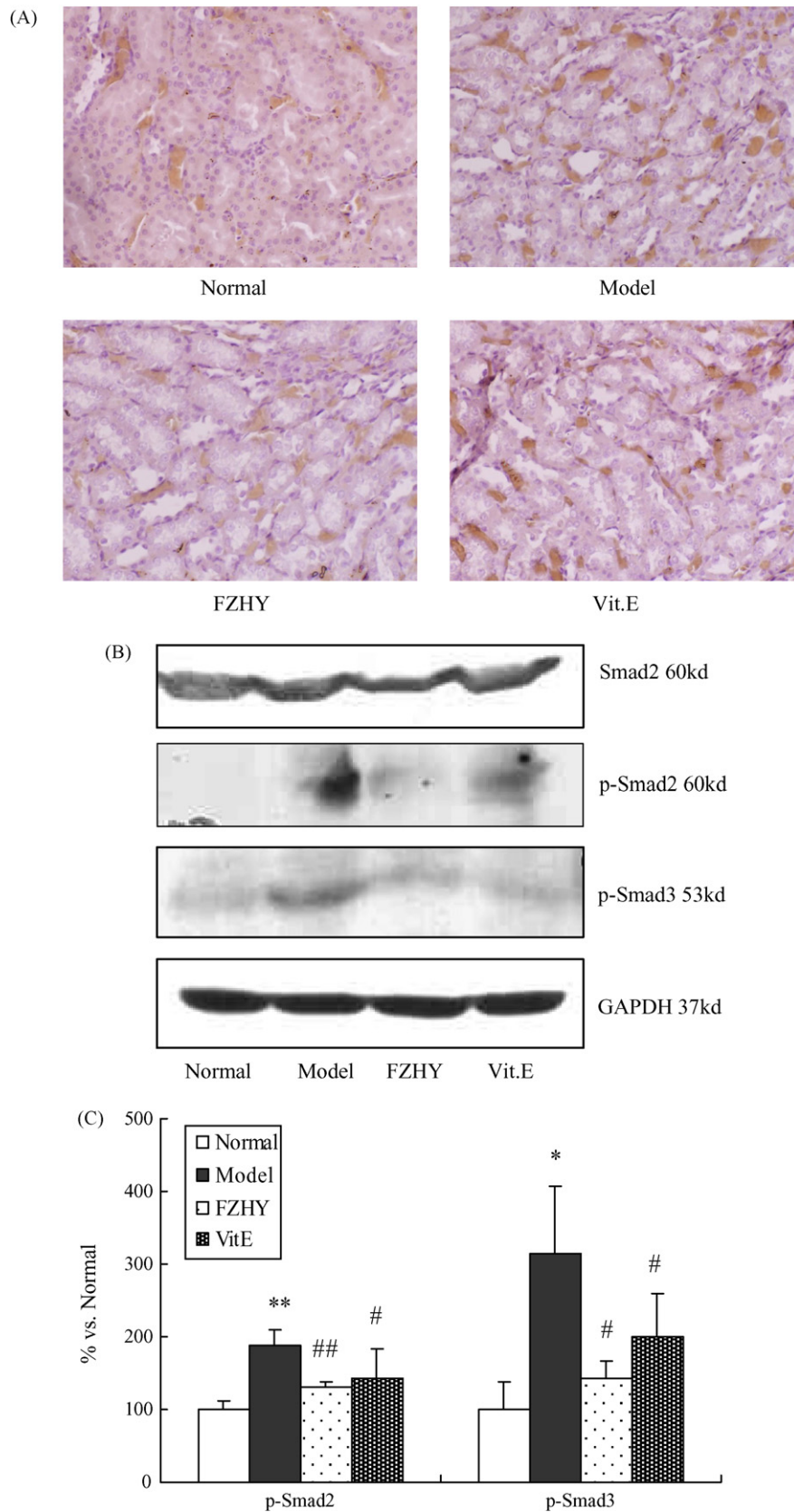
**Fig. 2.** The Effect of FZHY and Vit E on  $\alpha$ -SMA and E-cadherin expression in kidneys of model rats. (A) Immunofluorescence staining of  $\alpha$ -SMA and E-cadherin in kidneys. Increased labeling of  $\alpha$ -SMA at the interstitium was observed in model rats, which was decreased by both FZHY and Vit E treatment. The E-cadherin labeling intensity was decreased in model rats, whereas the labeling was maintained in FZHY treatment group and Vit E treatment group. (B) Western blotting analyses for  $\alpha$ -SMA and E-cadherin expression in renal tissues. Significantly increased  $\alpha$ -SMA expression and decreased E-cadherin expression were observed in rats of model group. FZHY and Vit E treatment attenuated HgCl<sub>2</sub>-induced up-regulation of  $\alpha$ -SMA expression. Moreover, both FZHY and Vit E treatment significantly inhibited the HgCl<sub>2</sub>-induced decrease of E-cadherin expression. (C) Graphical presentation of the relative expression of  $\alpha$ -SMA and E-cadherin. The values are represented as the density of  $\alpha$ -SMA or E-cadherin vs GAPDH (%). \*\* $P < 0.01$  vs normal; # $P < 0.05$  vs model; ## $P < 0.01$  vs model;  $\Delta\Delta P < 0.01$  vs Vit E.

important because deterioration of renal function is mainly determined by the extent and severity of interstitial lesions in many forms of chronic kidney disease. Regardless of the initial cause, RIF is characterized by interstitial fibroblast activation that is believed to play a central role in the pathogenesis of RIF. Emerging evidence suggests that under pathologic conditions, myofibroblasts derived from tubular epithelial cells through EMT (Iwano et al., 2002). During the process of EMT, tubular epithelial cells lose their epithelial phenotype but acquire the mesenchymal phenotype and the tubular basement membranes are disrupted (Yang and Liu, 2001; Kalluri and Neilson, 2003). Consequently, this process induces renal tubular destruction and accumulation of myofibroblasts.

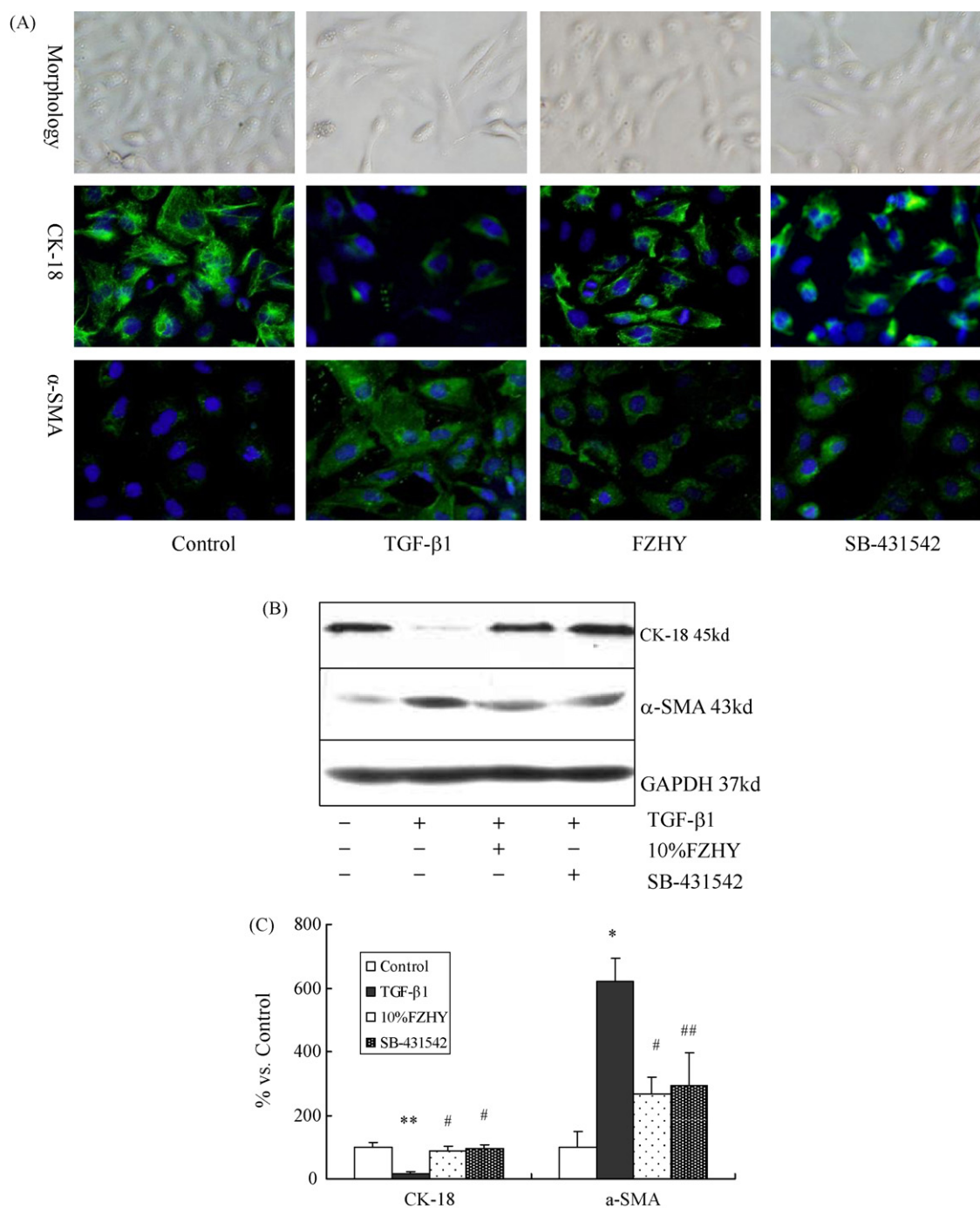
Many efforts have been made to find anti-renal fibrosis treatment. Although angiotensin II (Ang II) blockade is rapidly becoming standard anti-fibrotic therapy in renal diseases, current data suggest that Ang II blockade alone cannot stop fibrotic disease effectively (Yu et al., 2004). Of an increasing number of therapies showing efficacy in animal studies, antibodies to TGF- $\beta$  are the most thoroughly studied and are likely to be effective in human clinical trials. However, hints exist in the literature suggest that no single agent will effectively halt renal fibrosis and that combinations of agents will be required (Yu et al., 2002). More recently, research and drug development have shown that drugs that may bind to many different molecular targets or receptors, and so tend to have a wide range of effects and



**Fig. 3.** The effect of FZHY and Vit E on TGF-β1 and TβR-I expression in kidneys. (A) Immunofluorescence staining of TGF-β1. Increased labeling of TGF-β1 at the interstitium was observed in model rats, which was decreased by FZHY and Vit E treatment. (B) Western blotting analysis for TGF-β1 and TβR-I expression. The results showed significantly increased TGF-β1 and TβR-I expression were observed in rats of model group. In contrast, FZHY and Vit E treatment attenuated HgCl<sub>2</sub>-induced up-regulation of TGF-β1 and TβR-I expression. (C) Graphical presentation of the relative expression of TGF-β1 and TβR-I. The values are represented as the density of TGF-β1 or TβR-I vs GAPDH (%). \*\**P*<0.01 vs normal; #*P*<0.05 vs model.



**Fig. 4.** The effect of FZHY and Vit E on the expression of Smad2, p-Smad2, Smad3, p-Smad3 in kidney. (A) Immunohistochemistry analysis of Smad3. The expression of Smad3 in kidney was almost in the cytoplasm of tubular cells in rats of normal group, whereas in model rats, the nuclear expression of Smad3 in kidney tissue was increased, FZHY and Vit E treatment inhibited the nuclear expression of Smad3. (B) Western blotting analysis for Smad2, p-Smad2 and p-Smad3 expression. Unchanged expression of Smad2 in kidneys among four groups, whereas p-Smad2 and p-Smad3 expression was significantly increased in rats of model group compared with the controls. In contrast, FZHY and Vit E treatment significantly attenuated the up-regulation of p-Smad2 and p-Smad3 of model group. (C) Graphical presentation of the relative expression of p-Smad2 and p-Smad3. The values are represented as 100% vs. normal. \*\* $P < 0.01$  vs. normal; #  $P < 0.05$  vs. model; ##  $P < 0.01$  vs. model.

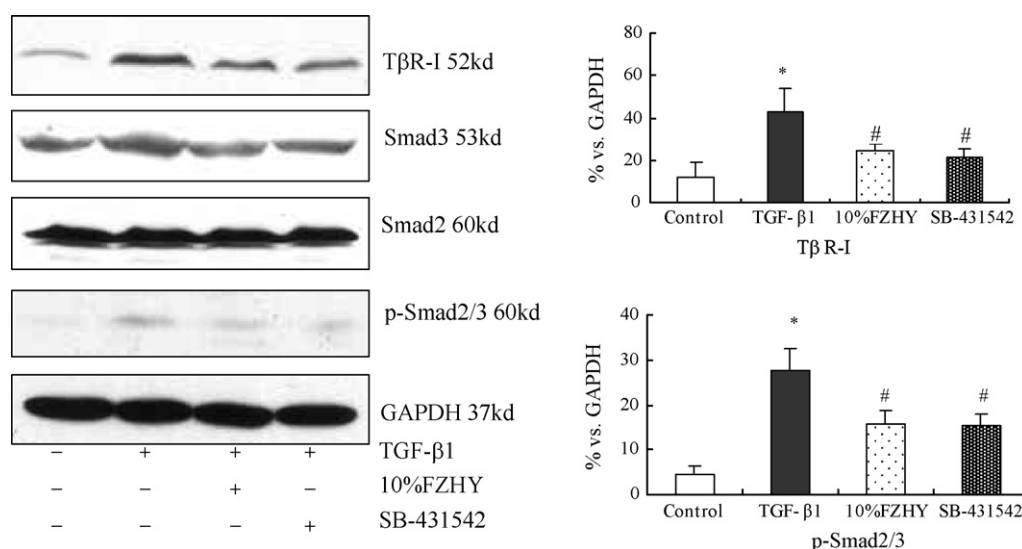


**Fig. 5.** The effects of FZHY drug-containing serum and SB-431542 on HK-2 cell morphology,  $\alpha$ -SMA and CK-18 expression. HK-2 cells were incubated without (control) or with 2.5 ng/ml TGF- $\beta$ 1 for 24 h, then the medium was changed and the cells were incubated with (1) DMEM containing 10% normal rat serum, (2) DMEM containing 10% normal rat serum + 2.5 ng/ml TGF- $\beta$ 1, (3) DMEM containing 10% FZHY drug-containing serum + 2.5 ng/ml TGF- $\beta$ 1 (4) DMEM containing 10% normal rat serum + 2.5 ng/ml TGF- $\beta$ 1 + 10  $\mu$ M SB-431542 for an additional 24 h. (A) The effects of FZHY drug-containing serum on morphology, CK-18 and  $\alpha$ -SMA of HK-2 cells. HK-2 cells were changed from a cuboidal shape to a spindle shape in response to TGF- $\beta$ 1, whereas FZHY drug-containing serum and SB-431542 treatment blocked the morphologic transformation. Immunofluorescence staining showed the decreased immunolabeling intensity of CK-18 and increased labeling intensity of  $\alpha$ -SMA was observed after TGF- $\beta$ 1 treatment, while the decreased CK-18 and increased  $\alpha$ -SMA was reversed by FZHY drug-containing serum and SB-431542 treatment. The blue staining is nuclear counterstaining with Hoechst 33258. (B) Western blotting analysis for CK-18 and  $\alpha$ -SMA.  $\alpha$ -SMA expression was increased and CK-18 expression was decreased when the cells were exposed to TGF- $\beta$ 1, whereas FZHY drug-containing serum and SB-431542 treatment significantly attenuated the TGF- $\beta$ 1-induced up-regulation of  $\alpha$ -SMA and down-regulation of CK-18. (C) Graphical presentation of the relative expression of CK-18 and  $\alpha$ -SMA. The values are represented as 100% vs control. \*\* $P$  < 0.01 vs control; \* $P$  < 0.05 vs TGF- $\beta$ 1, ## $P$  < 0.01 vs TGF- $\beta$ 1.

possibly negative side effects – dirty drugs – may be better options for complex diseases (Frantz, 2005). Traditional Chinese Medicine (TCM) and its prescriptions are composed of groups of active substances, which enter into compatibility and combination according to certain requirements and exert their functions

through multi-targets and various ways. FZHY is a TCM prescription used for treatment of liver fibrosis in China (Zhao et al., 2006); and our previous study found that FZHY had good effect against renal interstitial fibrosis induced by HgCl<sub>2</sub> in rats (Jiang et al., 2004).





**Fig. 6.** The effects of FZHY drug-containing serum and SB-431542 on T $\beta$ R-I, Smad2, Smad3 and p-Smad2/3 expression in HK-2 cells. HK-2 cells were incubated with or without 2.5 ng/ml TGF- $\beta$ 1 for 24 h, then the medium was changed and the cells were incubated with (1) DMEM containing 10% normal rat serum, (2) DMEM containing 10% normal rat serum + 2.5 ng/ml TGF- $\beta$ 1, (3) DMEM containing 10% FZHY drug-containing serum + 2.5 ng/ml TGF- $\beta$ 1 (4) DMEM containing 10% normal rat serum + 2.5 ng/ml TGF- $\beta$ 1 + 10  $\mu$ M SB-431542 for an additional 24 h. The expression of T $\beta$ R-I, Smad2, Smad3 and p-Smad2/3 was detected by Western blots. There was no significant change between each group in Smad2/3, but the expression of phosphorylated Smad2/3 and T $\beta$ R-I expression was significantly increased after incubation with TGF- $\beta$ 1, whereas FZHY drug-containing serum and SB-431542 treatment significantly attenuated the TGF- $\beta$ 1-induced up-regulation of p-Smad2/3 and T $\beta$ R-I. The values are represented as the density of p-Smad2/3 or T $\beta$ R-I vs GAPDH (%). \* $P$  < 0.01 vs control; # $P$  < 0.05 vs TGF- $\beta$ 1, ## $P$  < 0.01 vs TGF- $\beta$ 1.

HgCl<sub>2</sub>-induced rat renal interstitial fibrosis was successfully established in our lab, which was characterized by activation of renal fibroblasts, over-production and deposition of extracellular matrix and renal lipid peroxidation injury (Yuan et al., 2008). Since renal lipid peroxidation injury is the main character of this model, and it has been reported that the powerful antioxidant agent – Vit E has the effect against organ fibrosis (Huang et al., 2006; Di Sario et al., 2007; Hemmati et al., 2008), we presume that Vit E also has effect against renal interstitial fibrosis. Moreover, our previous study has confirmed that Vit E had good effect on this renal interstitial fibrosis model.

In this study, we investigated the effect of FZHY and Vit E on renal tubular EMT using this model. Phenotypic features of renal tubular EMT decrease expression of the cell adhesion receptor E-cadherin and de novo expression of  $\alpha$ -SMA (Wheelock and Johnson, 2003; Liu, 2004). In this study, we observed that the expression of  $\alpha$ -SMA in rats administrated with HgCl<sub>2</sub> was increased, while the expression of E-cadherin was decreased, with either FZHY or Vit E treatment, the increased expression of  $\alpha$ -SMA was attenuated, and the loss of E-cadherin expression was maintained. These results indicated that FZHY and Vit E treatment could inhibit the process of EMT in the fibrotic kidneys. The results also show that the effect of FZHY was more pronounced than Vit E, therefore we focused on the effects of FZHY on EMT in our *in vitro* experiments.

EMT is regulated by numerous growth factors, cytokines, hormones, and extracellular cues in different ways, among which, TGF- $\beta$ 1 is the most potent inducer that is capable of initiating and completing the entire EMT course (Fan et al., 1999). TGF- $\beta$ 1 signals are transduced by transmembrane serine/threonine kinase type I and type II receptors and intracellular mediators known as Smads (Massague, 2000; Bottinger and Bitzer, 2002; Schnaper et al., 2003). Upon TGF- $\beta$ 1 stimulation, Smad2 and Smad 3 are phosphorylated at serine residues in the carboxyl termini by the type I receptor. Such phosphorylation of Smad2/3 induces their association with common partner Smad4 and subsequently translocate into the nuclei, where they con-

trol the transcription of TGF- $\beta$ 1 responsive genes (Li et al., 2002). In this study, we observed that TGF- $\beta$ 1, T $\beta$ R-I, Smad2 phosphorylation and Smad3 phosphorylation expression in kidneys of model group were upregulated, and nucleus expression of Smad3 was increased. FZHY and Vit E treatment significantly inhibited the up-regulation of the TGF- $\beta$ 1, T $\beta$ R-I, Smad2 phosphorylation, Smad3 phosphorylation and Smad3 nucleus expression. These results indicated that FZHY may inhibit TGF- $\beta$ /Smads signal transduction, thus reverse EMT in renal interstitial fibrosis.

The above findings were further confirmed by our *in vitro* experiment. In this experiment, the sera were collected from rats which took FZHY and was used as medicine for incubating the cells in order to investigate the effects of FZHY on cultured cells. This is called serum pharmacological method, which was first introduced by Japanese for herbal medicine research *in vitro* (Iwama et al., 1987), and have been used widely and successively in recent years (Shen et al., 2007; Wang et al., 2009), with the advantages of mimicking real action of herbal medicine *in vivo* and of avoiding non-pharmacological noise caused by contaminants. In addition, SB-431542 (Inman et al., 2002), a potent and specific inhibitor of T $\beta$ R-I kinase was used as active control. The results revealed that FZHY drug-containing serum, as well as SB-431542 significantly upregulated expression of CK-18, meanwhile, down-regulated expression of  $\alpha$ -SMA, T $\beta$ R-I, p-Smad2/3 in TGF- $\beta$ 1 stimulated HK-2 cells. These results demonstrated that FZHY drug-containing drug could reverse EMT induced by TGF- $\beta$ 1 in HK-2 cells through TGF- $\beta$ /Smads signaling pathway, which were consistent with results observed *in vivo*.

## 5. Conclusions

FZHY and Vit E have an inhibitory effect on renal fibrosis by reversing tubular EMT in renal interstitial fibrosis induced by HgCl<sub>2</sub>. Moreover, FZHY directly attenuated TGF- $\beta$ 1-induced EMT in HK-2 cells; FZHY is likely to counteract TGF- $\beta$ 1-induced EMT, at least partly, by decreasing Smad2/3 phosphorylation.

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