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• *BASIC RESEARCH* •

Tetrandrine inhibits activation of rat hepatic stellate cells *in vitro* via transforming growth factor-β signaling

Yuan-Wen Chen, Jian-Xin Wu, Ying-Wei Chen, Ding-Guo Li, Han-Ming Lu

Yuan-Wen Chen, Jian-Xin Wu, Ying-Wei Chen, Ding-Guo Li, Han-Ming Lu, Digestive Disease Laboratory, Xinhua Hospital, Shanghai Second Medical University, Shanghai 200092, China
Supported by the College Science and Technology Developing Foundation of Shanghai, No. 02BK14
Correspondence to: Dr. Jian-Xin Wu, Digestive Disease Laboratory, Xinhua Hospital, Shanghai Second Medical University, 1665 Kongjiang Road, Shanghai 200092, China. wjxgp@sh163.net
Telephone: +86-21-65790000-3362 Fax: +86-21-55571294
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Abstract

AIM: To investigate the effect of various concentrations of tetrandrine on activation of quiescent rat hepatic stellate cells (HSCs) and transforming growth factor-β (TGF-β) signaling *in vitro*.

METHODS: HSCs were isolated from rats by *in situ* perfusion of liver and 18% Nycodenz gradient centrifugation, and primarily cultured on uncoated plastic plates for 24 h with DMEM containing 20% fetal bovine serum (FBS/DMEM) before the culture medium was substituted with 2% FBS/DMEM for another 24 h. Then, the HSCs were cultured in 2% FBS/DMEM with tetrandrine (0.25, 0.5, 1, 2 mg/L, respectively). Cell morphological features were observed under an inverted microscope, smooth muscle-α-actin (α-SMA) was detected by immunocytochemistry and image analysis system, laminin (LN) and type III procollagen (PCIII) in supernatants were determined by radioimmunoassay. TGF-β_1 mRNA, Smad 7 mRNA and Smad 7 protein were analyzed with RT-PCR and Western blotting, respectively.

RESULTS: Tetrandrine at the concentrations of 0.25-2 mg/L prevented morphological transformation of HSC from the quiescent state to the activated one, while α-SMA, LN and PCIII expressions were inhibited. As estimated by gray values, the expression of α-SMA in tetrandrine groups (0.25, 0.5, 1, 2 mg/L) was reduced from 21.3% to 42.2% (control: 0.67, tetrandrine groups: 0.82, 0.85, 0.96, or 0.96, respectively, which were statistically different from the control, $P<0.01$), and the difference was more significant in tetrandrine at 1 and 2 mg/L. The content of LN in supernatants was significantly decreased in tetrandrine groups to 58.5%, 69.1%, 65.8% or 60.0% that of the control respectively, and that of PCIII to 84.6%, 81.5%, 75.7% or 80.7% respectively ($P<0.05$ *vs* control), with no significant difference among tetrandrine groups. RT-PCR showed that TGF-β_1 mRNA expression was reduced by tetrandrine treatments from 56.56% to 87.90% in comparison with the control, while Smad 7 mRNA was increased 1.4-4.8 times. The TGF-β_1 mRNA and Smad 7 mRNA expression was in a significant negative correlation ($r = -0.755$, $P<0.01$), and both were significantly correlated with α-SMA protein expression ($r = -0.938$, $P<0.01$; $r = 0.938$, $P<0.01$, respectively). The up-regulation of Smad 7 protein by tetrandrine (1 mg/L) was confirmed by Western blotting as well.

CONCLUSION: Tetrandrine has a direct inhibiting effect on the activation of rat HSCs in culture. It up-regulates the expression of Smad 7 which in turn blocks TGF-β_1 expression and signaling.

Key words: Tetrandrine; Hepatic stellate cell; Transforming growth factor-β; Smad 7; Liver fibrosis; Signal transduction

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INTRODUCTION

Hepatic stellate cells (HSCs) belonging to the nonparenchymal cell type in Disse space undergo morphological and biochemical transformation, which is quite a complex process of activation and evolution, into myofibroblasts in various liver injuries. The activated HSCs are the main source of extra cellular matrix (ECM). Many profibrogenic cytokines[1,2] can activate HSCs, and among them the transforming growth factor-β_1 (TGF-β_1) is the master factor for promoting HSC activation, ECM synthesis, and excretion of other profibrogenic factors[3-5]. Abnormally elevated TGF-β_1 level and altered intercellular signaling bear a close relation to persistent HSC activation and higher function[4-7].

Both experimental and clinical research have shown that tetrandrine, a bisbenzyl isoquinolone alkaloid derived from *Stephania tetrandra* S. Moore, a traditional Chinese herb medicine, could exert an anti-inflammation effect on injured liver, attenuate ECM deposition, and inhibit hepatic fibrosis. In order to further investigate the possible mechanism involved, we examined the effects of various dosages of tetrandrine on HSCs and also the expression of laminin (LN), type III procollagen (PCIII), smooth muscle-α-actin (α-SMA), TGF-β_1 and its downstream signal transduction

components, Smad 7, to elucidate the influence of tetrandrine on activation of HSCs and TGF-β_1 signaling.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 400-500 g were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. They received normal chow and water ad libitum.

Tetrandrine solution

Tetrandrine (molecular formula $C_{38}H_{42}N_2O_6$) purchased from Shanxi Huike Botanical Development Co., Ltd, was dissolved in ethanol at 1 mg/mL, diluted and added to the culture medium. The final concentration of ethanol was lower than 0.2%.

Main reagents

DMEM was purchased from Gibco. Nycodenz, pronase E, type IV collagenase and mouse α-SMA monoclonal antibody were purchased from Sigma. Two-step immunocytochemistry detection reagent was purchased from Antibody Diagnostica Inc. LN and PCIII radioimmunoassay kits were purchased from the Institute of Naval Medicine, Shanghai. TRIzol reagent, Moloney murine leukemia virus reverse transcriptase (M-MLV) and rabbit Smad 7 polyclonal antibody were purchased from Invitrogen, Promega, and Santa Cruz, respectively.

Isolation and cultivation of HSCs

HSCs were isolated and cultured as described by Friedman and Roll[8] with some modifications. Briefly, the rats were anesthetized with an intraperitoneal injection of pentobarbital. After cannulation into the portal vein, the liver was perfused with calcium-free balanced salt solution, salt solution containing 0.5 mg/mL collagenase, salt solution containing 0.5 mg/mL collagenase and 1 mg/mL pronase E in turn respectively. Then the liver was taken out, cut into small pieces and incubated in solution containing 0.5 mg/mL collagenase. After being washed by repeated suspension and centrifugation, HSCs were purified by density gradient centrifugation with 18% Nycodenz. HSCs were collected from the top layer, washed and suspended in DMEM supplemented with 20% fetal bovine serum (FBS) at a density of 1×10^6 cell/mL, and seeded on uncoated 24- and 6-well plastic plates at $1\times10^5/cm^2$ supplemented with 20% FBS/DMEM for 24 h. Then HSCs were subjected to tetrandrine treatment after cultivation in 2% FBS/DMEM for another 24 h. More than 90% of isolated HSCs were viable as assessed by tryphan blue exclusion and consisted of more than 90% of HSCs as determined by direct cell counting under a phase-contrast microscope and intrinsic vitamin A autofluorescence[9].

Immunocytochemistry analysis of α-SMA

HSCs were cultured on 24-well plates without or with tetrandrine (0.25, 0.5, 1, 2 mg/L). After being incubated for 3 d, the supernatant was collected and preserved at -20 ℃ for further assay. Cells were fixed with ethanol/acetic acid, and α-SMA antibody, horse radish peroxidase-conjugated secondary antibody and diaminobenzidine were added sequentially according to the standard protocol. Semi-quantitative assessment of protein expression was performed using a pathological image analysis system. The expression of α-SMA was estimated by gray value.

Measurement of TGF-β_1 and Smad 7 mRNA

HSCs were seeded on 6-well plates and treated as above. Total RNA was extracted with TRIzol reagent. For RT-PCR, 1 μg total RNA was reverse transcribed with M-MLV according to manufacturer's instructions. cDNAs were amplified using specific sets of primers for TGF-β_1 (ggactctccacctgcaagac, ccccagaaatcatcgagac) and Smad 7 (ctgtgttgctgtgaatcttac, gctgtaggccttttcatagt). The PCR procedure for TGF-β_1 consisted of 30 cycles of denaturation at 94 ℃ for 30 s, annealing at 53 ℃ for 30 s, extension at 72 ℃ for 30 s, with initial denaturation of sample cDNAs at 94 ℃ for 5 min before PCR and additional extension period of 10 min after the last cycle. A touch down PCR procedure was performed for Smad 7 cDNA amplification. After denaturation at 94 ℃ for 5 min, 10 cycles were run with an initial annealing temperature at 58 ℃, which was 0.5 ℃ lower after each cycle before 25 cycles of denaturation at 94 ℃ for 40 s, annealing at 53 ℃ for 40 s, extension at 72 ℃ for 40 s, and additional extension period of 10 min after the last cycle. In parallel, PCR reactions were performed with primers coding for the housekeeping gene, *GAPDH*, to control for equal amounts of template cDNAs. Five microliters of 20 μL total PCR reaction was analyzed in a 20 mg/g agarose gel with a 100-bp DNA marker. Densitometric analysis of PCR products was performed by the computer software, SmartViewer (Shanghai Furi Science and Technology), and standardized by the *GAPDH*.

Western blotting analysis of Smad 7

HSCs, which were cultured on 6-well plates with or without tetrandrine (1 mg/L) for 3 d, were washed twice with Hanks' balanced salt solution and lysed directly in SDS loading buffer. Cell lysates (5 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis on a 0.1 g/g gel and then transferred to a nitrocellulose membrane. The membrane was incubated with antibody to Smad 7 (diluted 1:500) at 4 ℃ for 12 h. After being vigorously washed, the membrane was incubated with horse radish peroxidase-conjugated secondary antibody (diluted 1:2 000). The membrane blot was developed with diaminobenzidine.

LN and PCIII content

LN and PCIII in supernatant were measured with radioimmunoassay kits according to manufacturer's instructions.

Statistical analysis

All data were expressed as mean±SD. Statistical significance for the difference between the groups was assessed using one-way ANOVA test. $P<0.05$ was considered statistically significant.

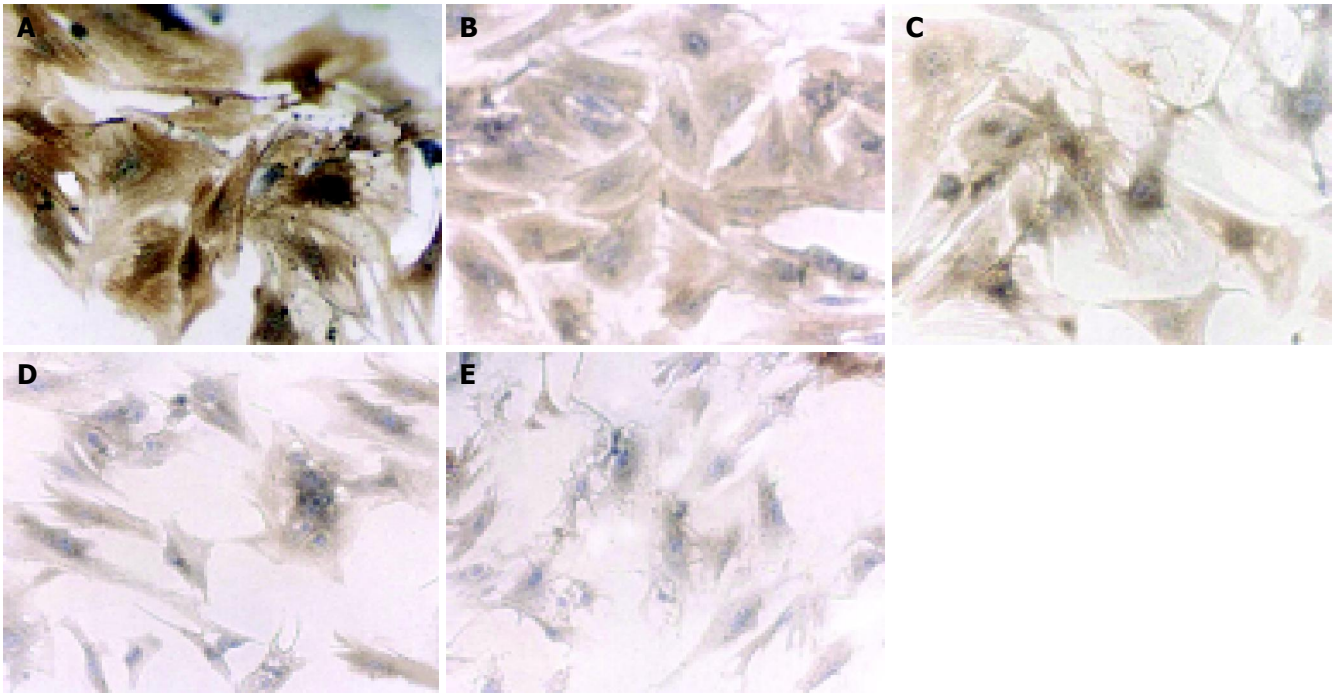

Figure 1 Immunocytochemistry analysis of α-SMA of HSCs subjected to different treatment of tetrandrine (magnification ×200). **A**: control; **B**: tetrandrine 0.25 mg/L; **C**: tetrandrine 0.5 mg/L; **D**: tetrandrine 1 mg/L; **E**: tetrandrine 2 mg/L.

RESULTS

Changes of HSC morphology

HSCs cultured for 5 d exhibited flattened and membranous processes, representing a myofibroblastic morphology (Figure 1 A), while those treated with tetrandrine (0.25, 0.5, 1, 2 mg/L) showed a more slender, spindle stellate cell shape (Figures 1B-E), which was similar to the appearance of quiescent HSCs.

α-SMA expression of culture-activated HSCs

In the control group, HSCs cultured for 5 d were strongly positive for α-SMA (Figure 1A), which was weaker in tetrandrine-treated groups in varying degrees according to the doses used (Figures 1B-E). Image analysis showed a statistical difference between the control and tetrandrine groups ($P<0.01$), and the difference was more significant in tetrandrine at 1 and 2 mg/L (Figure 2).

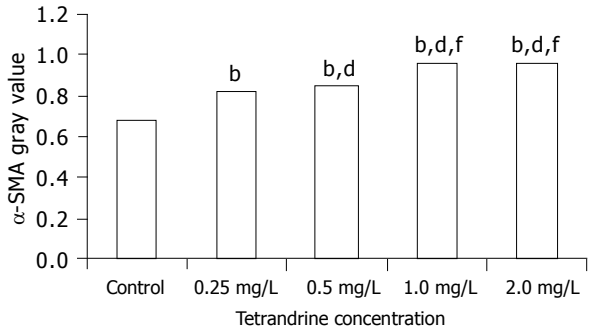

Figure 2 Expression of α-SMA in culture-activated HSCs. [b]$P<0.01$ *vs* control group. [d]$P<0.01$ *vs* tetrandrine 0.25 mg/L group. [f]$P<0.01$ *vs* tetrandrine 0.25, 0.5 mg/L groups.

TGF-β$_1$ and Smad 7 mRNA expression

TGF-β_1 mRNA expression of HSCs was suppressed from 56.56% to 87.90% in tetrandrine groups (0.25, 0.5, 1, 2 mg/L) when compared with that of the control ($P<0.01$), while the up-regulated Smad 7 mRNA expression in tetrandrine groups reached 2.4-5.8 times that of the control. A dosage-dependent effect was observed at 0.25-1 mg/L ($P<0.01$), but no difference at 1 and 2 mg/L was demonstrated ($F = 0.394$, $P = 0.564$) (Table 1 and Figure 3). TGF-β_1 and Smad 7 mRNA expressions showed a clear negative correlation ($r = -0.755$, $P<0.01$), and both were significantly correlated with α-SMA protein expression ($r = -0.938$, $P<0.01$; $r = 0.938$, $P<0.01$, respectively).

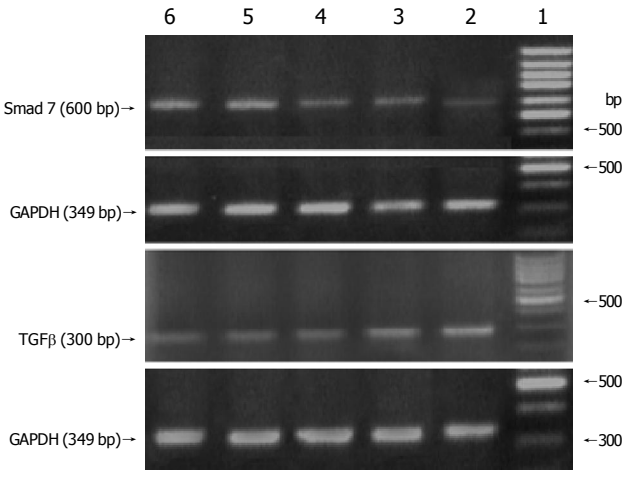

Figure 3 RT-PCR determination of Smad 7 and TGF-β_1 mRNA expressions. Lane 1: 100-bp marker, lane 2: control group, lanes 3-6: treatment with tetrandrine at 0.25, 0.5, 1.0, 2.0 mg/L, respectively.

Table 1 Densitometric analysis of PCR products of each group (mean±SD)

Group	A_S/A_G	A_T/A_G
Control	11.5±0.5	78.5±0.4
Tet-0.25	27.3±0.3[a]	34.1±0.3[a]
Tet-0.5	25.9±0.2[a,c]	12.6±0.3[a,c]
Tet-1.0	67.0±1.0[a,c,e]	9.5±0.3[a,c,e]
Tet-2.0	67.4±0.2[a,c,e]	9.5±0.1[a,c,e]

[a]P<0.05 *vs* control group. [c]P<0.05 *vs* Tet-0.25 group. [e]P<0.05 *vs* Tet-0.25, Tet-0.5 groups. A_S/A_G: Ratio of the densities of Smad 7 to that of *GAPDH* (%). A_T/A_G: Ratio of the densities of TGF-β_1 to that of *GAPDH* (%).

LN and PCIII excretion of culture-activated HSCs

Compared with the control group, the content of LN in HSC culture medium was reduced in tetrandrine groups (0.25, 0.5, 1, 2 mg/L) to 58.5%, 69.1%, 65.8% or 60.0%, respectively (P<0.05), and that of PCIII to 84.6%, 81.5%, 75.7% or 80.7%, respectively (P<0.05), whereas there was no difference among the groups with various concentrations of tetrandrine (Figure 4).

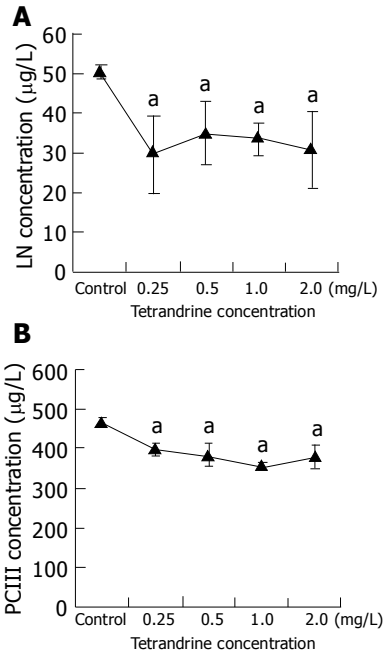

Figure 4 Radioimmunoassay of LN and PCIII. [a]P<0.05 *vs* control group.

Expression of Smad 7 of culture-activated HSCs

Immunoblot analysis indicated that HSCs after 5 d of culture expressed Smad 7, which was markedly increased by tetrandrine (1 mg/L) treatment. This was consistent with RT-PCR results (Figure 5).

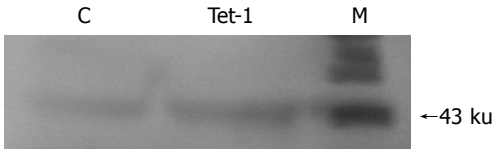

Figure 5 Immunoblot analysis for Smad 7. C: control group, Tet-1: treatment with tetrandrine at 1 mg/L, M: protein marker.

DISCUSSION

Tetrandrine is a bisbenzyl isoquinolone alkaloid extracted from *Stephania tetrandra* S. Moore, a traditional Chinese herb medicine. With the progression of basic researches and clinical experiments in recent years, tetrandrine has been demonstrated to be effective in anti-inflammation, immunomodulation, reversion of cardiac and vascular remodeling, inhibition of pulmonary vessels and airway smooth muscle contraction, suppression of tumor proliferation and multidrug resistance, and so on[10-12]. Anti-fibrogenesis have long been an important field. Over the years, more experimental animal and clinical studies have shown that tetrandrine has anti-fibrogenic actions, such as inhibition of inflammatory reaction of injured liver, attenuation of ECM deposition, decrease in serum PCIII and LN levels, and inhibiting proliferation and collagen synthesis of fibroblasts or HSCs induced by platelet-derived growth factor. Some earlier studies showed a direct inhibiting effect of tetrandrine on the proliferation of vascular smooth muscle cells and pulmonary fibroblasts[13,14]. More recently, Lee *et al*[15], showed that tetrandrine inhibited tissue inhibitor of metalloproteinase expression and thus attenuate liver fibrosis, while the study of Park *et al*[16], revealed that α-SMA, the marker of activated HSCs, was significantly reduced when primary cultured HSCs were treated with tetrandrine (10 mg/L), and much higher concentrations of tetrandrine induced apoptosis in HSCs[17]. However, little is known how tetrandrine affects HSC activation, the key process in the progression of liver fibrosis, and the possible mechanism involved. It is true when it comes to a lower concentration of tetrandrine of less than 10 mg/L. This seems to be more important when realizing its cytotoxicity. In this study, using the most popular cell model of HSC activation[2], we disclosed that tetrandrine could suppress the morphological transformation of HSCs from the quiescent type to activated one, and decrease the expression of α-SMA. Likewise, the production of LN and PCIII and expression of TGF-β_1 mRNA were diminished. Our results strongly demonstrated the direct inhibiting effect of tetrandrine on HSC activation and transformation to myofibroblast. Additionally, our data revealed the influence of tetrandrine on inhibitory signaling molecule (Smad 7) for TGF-β. All the findings enriched our knowledge of the anti-fibrogenic mechanism of tetrandrine.

In the course of liver fibrosis, activated HSCs are the most important source of TGF-β_1, which in turn induces and accelerates the activation of quiescent HSCs in an autocrine and paracrine manner, promotes ECM production, and results in progression of liver fibrosis. It has been widely accepted that TGF-β_1 is the most potent profibrogenic mediator in liver fibrosis[2-4], and thus to inhibit TGF-β_1 function is of great importance and effective in anti-fibrogenic strategy[4,18-20]. The signals of TGF-β mainly go through its receptors and downstream Smad proteins to HSCs[21]. Smads, including Smad 1-Smad 8, are the intercellular components of the signal pathway of TGF-β superfamily. Smads interact with type I and type II TGF-β receptors and directly or indirectly transduce or modulate TGF-β signals into nuclei[5,6]. It has been reported that the balance between Smads may be the key issue in maintaining

normal TGF-β functions[5,21], and Smad 7 is the main negative feedback regulator of TGF-β signaling in HSCs[6]. However, in activated HSCs, TGF-β-induced Smad 7 expression was diminished in an unknown way and this was thought to be strongly associated with the overwhelming profibrogenic action of TGF-β following liver injury. Therefore, an attempt to up-regulate Smad 7 expression might be a promising way to reduce TGF-β excretion, restore normal signal regulation, and inhibit HSC activation with resulting abnormal functions.

Our study indicated that the activation of HSCs was significantly inhibited by tetrandrine, as shown by the delayed morphologic transformation, diminution of α-SMA and TGF-β_1 mRNA expression, decrease in LN and PCIII production, increase in Smad 7 expression. Statistical analysis showed a close correlation among the changes of α-SMA, TGF-β_1 mRNA and Smad 7. These data suggested that tetrandrine inhibited activation of HSCs via down-regulation of TGF-β_1 expression. However, this may not be the only mechanism, for it might be the result of inhibition of HSC activation via some other pathways instead of TGF-β_1 signaling[22]. Smad 7, both at mRNA and protein levels, was remarkably increased in comparison with lowered TGF-β_1, suggesting that Smad 7 plays an important role in HSC inactivation, in addition to the result of diminution of TGF-β_1. Tetrandrine may inhibit activation of HSCs by up-regulation of Smad 7 with resulting blockage of TGF-β gene transcription. This view is supported by recently reported findings that up-regulation of Smad 7 or blockage of TGF-β_1 signaling resulted in inhibition of HSC activation[23,24].

HSC activation is a complicated process, which is even true when it comes to *in vivo* studies. The reasons why the feedback regulation of TGF-β signaling in normal liver cell is maintained and why such a regulation is lost in the pathological process of liver fibrosis are still not fully understood at present. Although in this study we revealed that tetrandrine could influence TGF-β_1 and its signaling through Smads, more efforts should be made to further investigate the mechanisms involved and the interaction with other factors in their signal transduction pathways.

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