Danshensu inhibits acetaldehyde-induced proliferation and activation of hepatic stellate cell-T6

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OBJECTIVE: To evaluate the effects of danshensu, the main component of the extract of Chinese medicine Salvia miltiorrhiza, on the proliferation and activation of hepatic stellate cells (HSCs).

METHODS: The activation of HSC-T6 was induced by exposure to acetaldehyde. In the meantime, different doses of danshensu were added to the culture medium. After 24 h of treatment with danshensu in acetaldehyde, the viability of HSC-T6 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, the cell cycle was determined through flow cytometry, and the gene transcription levels of plasminogen activator inhibitor-1 (PAI-1), transforming growth factor-β1 (TGF-β1), urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-2 (MMP-2) were analyzed by real-time quantitative polymerase chain reaction.

RESULTS: The proliferation of HSCs induced by 200 μmol/L acetaldehyde could be significantly inhibited by danshensu, and the percentage of HSCs in S phase was significantly increased as compared with the control cells (P<0.05), which were respectively evidenced by MTT assay and flow cytometry. Danshensu down-regulated the mRNA expression of TGF-β1 and PAI-1 and up-regulated the uPA transcription level (P<0.01), while the transcription level of MMP-2 was not significantly affected in HSC-T6.

CONCLUSION: Danshensu can inhibit the proliferation and activation of HSC-T6, as well as regulate some cytokines involved in extracellular matrix accumulation, which offers a potential therapeutic alternative for liver fibrosis.

KEYWORDS: danshensu; hepatocytes; cell proliferation; extracellular matrix; in vitro
Liver fibrosis is a wound-healing response to prolonged liver injury in chronic diseases including alcoholic liver disease (ALD). Without effective treatment, reversible liver fibrosis at an early stage can lead to irreversible cirrhosis\(^{11}\). Activation and proliferation of hepatic stellate cells (HSCs) is the key factor of fibrogenesis, which may induce more cytokine secretion leading to extracellular matrix (ECM) accumulation\(^{[2]}\). The rate of ECM synthesis and degradation, which closely correlated with liver fibrogenesis, is regulated by a series of cytokines such as plasminogen activator inhibitor-1 (PAI-1), transforming growth factor-β1 (TGF-β1), urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-2 (MMP-2)\(^{[3,4]}\).

Danshen, the major constituent purified from Salvia miltiorrhiza, is one of the most widely used traditional medicinal materials in China. The antioxidant and anti-inflammatory potential of danshen has been investigated in various studies\(^{[5,6]}\); however, the effects on liver fibrosis and the mechanisms involved require exploration. In the present study, the authors aimed to examine the antifibrosis effect of danshen in HSC-T6 activated by acetaldehyde, as well as the underlying mechanisms.

1 Materials and methods

1.1 Materials Danshen (C\textsubscript{9}H\textsubscript{10}O\textsubscript{3}, MW 198.17) was purchased from Shanghai Winherb Medical Sci & Tech Development Co. Ltd., Shanghai, China. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and acetaldehyde were purchased from Sigma Aldrich. TRIzol reagent was purchased from Invitrogen Co., Ltd. The reverse transcription kit was obtained from Fermentas, and SYBR Green was purchased from Applied Biosystems Company.

1.2 HSC culture and activation induction The HSC-T6 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% fetal bovine serum (Gibico) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in an incubator supplemented with 5% CO\textsubscript{2} at 37°C. To activate HSCs, cells were incubated in 2 mL medium with acetaldehyde of different concentrations (100, 200 and 300 μmol/L) for 24 h. In the meantime, when HSCs were exposed to the selected proper concentration of acetaldehyde, different doses of danshen (100, 125 and 150 μmol/L) were added to the medium and the cells were incubated for 24 h.

1.3 MTT assay Cells in 200 μL serum-free DMEM were seeded in 96-well plates. After treatment, MTT solution was added to each well at a final concentration of 1 mg/mL and the plates were incubated for another 4 h at 37°C. Each well was then washed with phosphatebuffer solution (PBS). Dimethyl sulfoxide was added to each well to dissolve formazan and the absorbance value was read at 570 and 630 nm using a spectrophotometric microplate reader. With this procedure, only viable cells with functioning mitochondria can oxidize MTT to a violet-red reaction product.

1.4 Flow cytometric analysis After danshen treatment for 24 h, HSC-T6 cells were trypsinized and resuspended in their original culture medium to avoid the loss of apoptotic and necrotic cells. Cell monolayer was fixed (70% ethanol in PBS) for 4 h and kept frozen at −20°C until analysis. Samples were stained with propidium iodide (PI), and then were analyzed in a Cytomics FC500 flow cytometer (Beckman Coulter in Brea, USA).

1.5 Real-time quantitative polymerase chain reaction analysis Total RNA was isolated using TRIzol reagents, and the RNA concentration was determined by spectrophotometric readings at 260 and 280 nm. And 2 μg of RNA was reverse-transcribed into cDNA with RevertAid TM First Strand cDNA Synthesis Kit. Real-time quantitative polymerase chain reaction (RT-PCR) was then performed on an ABI Step One Plus instrument (Applied Biosystems), using SYBR Green PCR Master Mix. The primers of PAI-1, TGF-β1, uPA and MMP-2 are listed in Table 1. Volume of the reaction was 20 μL, which contained 2 μL of 10 μmol/L of cDNA, 1 μL of 10 μmol/L of primer and 10 μL of Power SYBR Green PCR Master Mix. The amplification reaction program was set at the following steps: 50°C for 2 min, 95°C for 10 min, and 40 cycles of

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94°C for 15 s and 60°C for 1 min. Duplicates were performed for each sample. The cycle threshold (CT) value was defined automatically by the instrument.

<table>
<thead>
<tr>
<th>Primer</th>
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| PAI-1  | Forward 5’-GTGGTGGCAATTTCAACAGAG-3’  
|        | Reverse 5’-GCAATGGGACGATACAGGCTG-3’ |
| uPA    | Forward 5’-AAGGCTTGGATATGCGAGG-3’  
|        | Reverse 5’-CAGGATATCTCCCTGGACCC-3’ |
| TGF-β1 | Forward 5’-GGCCCTGCACACAAAATTCG-3’  
|        | Reverse 5’-GCTGCATCTTGGACAGGCTG-3’ |
| MMP-2  | Forward 5’-ACTCCACATGATGCAGAC-3’  
|        | Reverse 5’-GAAGAGGAAAGGGAACTTG-3’ |
| GAPDH  | Forward 5’-GGTGCGAGTCAAGCGATTTG-3’  
|        | Reverse 5’-ATGACCCCCGAGCTTCTCCA-3’ |

PCR: polymerase chain reaction; PAI-1: plasminogen activator inhibitor-1; uPA: urokinase-type plasminogen activator; TGF-β1: transforming growth factor-β1; MMP-2: matrix metalloproteinase-2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

1.6 Statistical analysis Data were presented as mean ± standard error of mean. Statistical analysis was performed by one-way analysis of variance followed by Fisher’s multiple-comparison test using SPSS 18.0 and GraphPad 5.0 software. When P < 0.05, it was considered as statistically significant.

2 Results

2.1 HSC-T6 proliferation with acetaldehyde or danshensu Exposing to acetaldehyde resulted in HSC-T6 proliferation in a dose-dependent manner, namely, the 100, 200 and 300 μmol/L of acetaldehyde promoted HSC proliferation respectively to 105%, 120% and 122% of the basal line. See Figure 1A. Therefore, the authors chose 200 μmol/L of concentration to stimulate HSC-T6 for further study. MTT assay found that the half maximal inhibitory concentration (IC₅₀) of danshensu in HSC-T6 is 615 μmol/L. See Figure 1B.

2.2 Danshensu regulated cell cycle of HSC-T6 Figure 2 shows that cells were rich in G₀ and S phase, and less in G₁ phase with acetaldehyde stimulation, indicating that the proliferation in HSC-T6 was significantly enhanced as compared with control cells (P < 0.05). The percentage of danshensu-interfered HSCs was significantly decreased in G₂ phase in a dose-dependent manner, while the percentage in S phase was significantly increased when compared with the cells merely exposed to acetaldehyde (P < 0.05).

2.3 Danshensu regulates ECM-related gene expression The transcription levels of PAI-1 and TGF-β1 were significantly enhanced in cells exposed to 200 μmol/L acetaldehyde. When incubating with the mixture of danshensu (100, 125, and 150 μmol/L) and acetaldehyde, PAI-1 mRNA expression was significantly reduced in a dose-dependent manner (P < 0.01), and the TGF-β1 mRNA expression was markedly decreased in cells with 150 μmol/L of danshensu as compared to that of the model group (P < 0.01). See Figures 3a and 3b. uPA and MMP-2 are the cytokines that promote ECM degradation. Results showed that uPA and MMP-2 expression was significantly decreased in cells with acetaldehyde stimulation (P < 0.01), while danshensu up-regulated uPA transcription in a dose-dependant manner (P < 0.01). See Figures 3c and 3d.

Figure 1 Viability of HSC-T6 cells affected by different doses of acetaldehyde and danshensu A: line graph showing the percentage of viable cells subjected to acetaldehyde (0, 100, 200, and 300 μmol/L); B: line graph showing the percentage of viable cells exposed to danshensu (0, 150, 250, 615 and 1000 μmol/L) respectively. Values were measured in three independent experiments done in triplicate and mean ± standard error was plotted. HSC: hepatic stellate cell.
3 Discussion

Alcohol use disorders and alcohol dependency affects millions of individuals worldwide. Acute and chronic exposure to ethanol promotes ALD. Liver stenosis is the first morphological change in the liver in ALD, followed by liver fibrosis and cirrhosis. Activation and proliferation of HSCs are the key drivers of fibrogenesis, and ECM accumulation is a key feature of fibrosis. Currently, antiproliferative agents that inhibit HSC activation are considered to be effective for treating fibrosis.  

The therapeutic efficacy of currently well-known antifibrosis agents in treating human liver fibrosis, such as vitamin E and superoxide dismutase are generally unimpressive. Natural plant products represent a major group of promising therapeutic agents for liver fibrosis. S. miltiorrhiza has long been used in treating liver diseases as a single herb or as a component of recipes, and its main constituent danshensu (Figure 4) possesses protective action on hepatic injury. In addition, danshensu has also attracted considerable attention for preventing oxidative stress-related diseases including cancers, cardiovascular diseases and diabetes mellitus. The toxicity study indicated that danshensu is a safe regimen even at high doses. 

Acetaldehyde is the oxidative metabolite of alcohol, whose effect on HSC activation has been proved. Culturing quiescent HSC with acetaldehyde causes spontaneous activation and changes into a myofibroblast-like shape, mimicking the process in vivo. This provides a simple and useful model...
for studying the activation of these cells. In the present study, the authors exposed HSCs to 200 μmol/L of acetaldehyde. Cell percentage in G1 and S phases was significantly increased, indicating that HSCs were activated and proliferated. HSC viability and percentage of cells in G1 phase decreased obviously when simultaneously exposed to danshensu. This indicated the anti-activation and antiproliferation effects of danshensu on HSCs, which were consistent with previous reports.[10]

To explore the potential mechanisms underlying the effects of danshensu, the authors examined several cytokines that regulate ECM formation and degradation. TGF-β1 and PAI-1 are the most potent fibrogenic cytokines in HSCs, and uPA and MMP are the cytokines involved in ECM degradation. Once HSCs are activated, they transdifferentiate into myofibroblast-like cells, increasing the expression of TGF-β1 and PAI-1 and down-regulating the expression and activity of uPA and MMP, thus the ECM production surpasses its degradation and results in fibrosis. It has been suggested that regulating the levels of the cytokines mentioned above to suitable states can be proposed to antagonize the process of liver fibrosis. By silencing TGF-β1 with siRNA and shRNA, Cheng et al.[20] successfully blocked the secretion of inflammatory cytokines in HSC-T6. PAI-1 could increase the incidence of injuries in liver diseases, while the PAI-1(-/-) mice had less fibrosis than the wild type mice in cholestatic liver fibrosis[21]. The role of the fibrinolysis system in fibrosis is still controversial. Some researchers reported that the increasing uPA and MMP2 expression correlated with an increase in proteolytic activity that promotes the ECM degradation[22], while others believed that MMP-2 could induce the HSC proliferation and collagen synthesis in reactive oxygen species-mediated pathway[23]. In the present study, expression of PAI-1 and TGF-β1 in HSC-T6, the cytokines that promote ECM production, was up-regulated by acetaldehyde stimulation and decreased in response to danshensu. Expression of uPA, the cytokine enhancing ECM degradation, was down-regulated when incubated with acetaldehyde, and increased by danshensu treatment in HSC-T6.

In summary, danshensu can inhibit HSC activation and proliferation induced by acetaldehyde, and inhibit the ECM formation via down-regulating the TGF-β1 and PAI-1 expression and up-regulating the uPA transcription. The effect on activation and proliferation of HSC can be considered as one of the beneficial feature of danshensu to be a potential antifibrosis agent in ALD.

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5 Competing interests

The authors declare that they have no competing interests.

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丹参素抑制乙醛诱发的肝星状细胞 T6 活化与增殖的研究

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目的：研究中药丹参的主要成分丹参素对肝星状细胞（hepatic stellate cell，HSC）活化与增殖的影响。

方法：乙醛与 HSC-T6 共同孵育诱发细胞活化，再分别应用不同浓度的丹参素进行干预。四甲基偶氮唑盐（3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide，MTT）比色法检测细胞活力，流式细胞术检测细胞周期，实时定量聚合酶链反应法分析细胞 I 型纤溶酶原激活剂抑制物（plasminogen activator inhibitor-1，PAI-1）、转化生长因子 β1（transforming growth factor-β1，TGF-β1）、尿激酶型纤溶酶原激活因子（urokinase-type plasminogen activator，uPA）和基质金属蛋白酶 2(matrix metalloproteinase-2，MMP-2) 的基因表达。

结果：MTT 比色法检测表明丹参素可抑制由 200 μmol/L 乙醛诱发的 HSC 增殖，流式细胞术分析发现丹参素可使处于 S 期的细胞数量明显增加（P<0.05）。此外，丹参素还能下调 TGF-β1 和 PAI-1 的基因表达，并增加 uPA 的转录水平（P<0.01），但对 MMP-2 的表达没有明显影响。

结论：丹参素可抑制乙醛诱发的 HSC-T6 的活化与增殖，可调节参与细胞外基质沉积的细胞因子的表达。

关键词：丹参素；肝细胞；细胞增殖；细胞外基质；体外研究