Effect of compound rhodiola sachalinensis A Bor on CCl₄-induced liver fibrosis in rats and its probable molecular mechanisms

Xiao-Ling Wu, Wei-Zheng Zeng, Pi-Long Wang, Chun-Tao Lei, Ming-De Jiang, Xiao-Bin Chen, Yong Zhang, Hui Xu, Zhao Wang

INTRODUCTION
Transforming growth factor beta 1 (TGF-β 1) is the most potent profibrogenic mediator in liver fibrosis and cirrhosis as shown in animal models and human chronic hepatic injury[14-16]. It plays critical roles in the activation of hepatic stellate cell (HSC) and inhibiting the TGF-β signal transduction pathway has become a new effective target for the prevention or treatment of liver fibrosis[8-11]. Several traditional Chinese herbs have been shown to have the ability of intervention in liver fibrosis[12-20], however, most of them were limited in morphological and serum studies, lacking of deep research in their molecular biological mechanisms. Our previous study has shown another Chinese medicine, compound rhodiola sachalinensis A Bor, can effectively prevent CCl₄-induced liver fibrosis in rats[21-23]. In this study, the probable biological mechanism of it, especially in the expression of TGF-beta 1 mRNA, α₁(Ⅰ) mRNA and Na⁺/Ca²⁺ exchanger mRNA was explored.

MATERIALS AND METHODS
Animals
Male SD rats (weighing 140-160 g) were obtained from the Experimental Animal Center of Sichuan University (Chengdu, Sichuan Province, China). The rats were housed in a room with controlled temperature (15-20 °C) and lighting (10 h light, 14 h dark). Free access to water and food was allowed during the experimental period. All the rats were randomly divided into three groups: normal group (n=10), treatment group (n=40) and CCl₄-induced model group (n=40). The liver fibrosis was induced by CCl₄ subcutaneous injection. Treatment group was administered with compound rhodiola sachalinensis A Bor (0.5 g/kg) once per day. The model group was given normal food and water, received injection of liquid paraffin with the same dosage and duration as CCl₄.

At the end of the 15-week experimental period, all the rats were anesthetized with intramuscular injection of sodium pentobarbital (30 mg/kg) before sacrificed. Blood was collected from the heart and the serum obtained through centrifugation. The liver was removed rapidly, part of it was conserved in refrigerator at -20 °C and the rest was frozen in a freezer at -20 °C.

Serum parameters of hepatic fibrosis
Parameters of hepatic fibrosis were determined by levels of type III procollagen (PCHI), type IV collagen (CIV) and hyaluronic acid (HA), using radioimmunoassay (commercial kit obtained from Shanghai Navy Medical Institute, China). Serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed by a automatic analyzer. Another serum enzyme N-acetyl-beta-D-glucosaminidase (β-NAG) was assayed with spectrophotometric method.

METHODS
Ninety healthy male SD rats were randomly divided into three groups: normal group (n=10), treatment group of compound rhodiola sachalinensis A Bor (n=40) and CCl₄-induced model group (n=40). The liver fibrosis was induced by CCl₄ subcutaneous injection. Treatment group was administered with compound rhodiola sachalinensis A Bor (0.5 g/kg) once a day at the same time. Then the activities of several serum fibrosis-associated enzymes: alanine aminotransferase (ALT), aspartate aminotransferase (AST), N-acetyl-beta-D-glucosaminidase (β-NAG) and the levels of serum procollagen III (PCIII), collagen IV (CIV), hyaluronic acid (HA) were assayed. The histopathological changes were observed with HE, VG and Masson stain. The expression of TGF-β1 mRNA, α₁(Ⅰ) mRNA and Na⁺/Ca²⁺ exchanger (NCX) mRNA was detected by reverse transcription polymerase chain reaction (RT-PCR) in situ.

RESULTS
Compound rhodiola sachalinensis A Bor significantly reduced serum activities of ALT, AST, β-NAG and decreased the levels of PCIII, CIV, HA, improved the liver histopathological changes, inhibited the expression of TGF-β1 mRNA, α₁(Ⅰ) mRNA and Na⁺/Ca²⁺ exchanger mRNA in rats.

CONCLUSION
Compound rhodiola sachalinensis A Bor can intervene in CCl₄-induced liver fibrosis in rats, in which potential mechanisms may be decreasing the production of TGF-β1, reducing the production of collagen, preventing the activation of hepatic stellate cell (HSC) and inhibiting the expression of TGF-β1 mRNA, α₁(Ⅰ) mRNA and Na⁺/Ca²⁺ exchanger mRNA.

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Table 1 Primer sequences of α1(I), TGF-β1 and Na+/Ca2+ exchanger mRNA

| mRNA            | Upstream (5'→3') | Downstream (5'→3') |
|-----------------|-----------------|--------------------|
| α1(I)           | CAC CCT CAA GAG CAG TC | GTT CGG GCT GAT GTA CCA GT |
| TGF-β1          | CTT TGT ACA ACA GCA CCC GC | GTC AAA AGA CAG CCA CTC AGG |
| NCX             | TAT TGC CGA ACC GGT TTA TGT | CTC GTC TCT CCA TCT GGG AC |

Table 2 Liver fibrosis-associated enzymes and fibrosis markers in serum (x±s)

| Group          | ALT (IU/L) | AST (IU/L) | β-NAG (μmol/L) | PCIII (μg/L) | CIV (μg/L) | HA (μg/L) |
|----------------|------------|------------|----------------|--------------|------------|-----------|
| Normal         | 87.93±18.61 | 104.3±32.40 | 189.00±26.70  | 89.99±10.85  | 35.69±9.68 | 12.41±45.62 |
| Model          | 198.64±71.02 | 514.59±160.22 | 415.77±133.37 | 265.5±98.21  | 159.67±29.64 | 455.7±113.55 |
| Treatment      | 114.17±47.89 | 291.62±141.75 | 244.67±46.80  | 164.25±45.68 | 96.73±16.48 | 289.35±75.68 |

*p <0.01 vs model group.

Histopathological grading
Liber samples from each rat were embedded in paraffin, stained with hematoxylin-eosin (HE), Van Gieson (VG) and Masson trichrome collagen stain, and then examined under an optical microscope. Fibrosis-degree of liver sections was graded numerically based on the criteria described below: 0, no fibrosis; +, slight fibrosis, fibrosis located in the central liver lobule; +2, moderate fibrosis, widen central fibrosis; +3, severe fibrosis, fibrosis extended to the edge of liver lobule; +4, liver cirrhosis.

Molecular biological detection: RT-PCR in situ
Each liver sample embedded in paraffin was sectioned and fixed onto a poly-L-lysine covered glass. The expression of α1(I) mRNA, TGF-β1 mRNA and Na+/Ca2+ exchanger (NCX) mRNA was detected with RT-PCR in situ. (primers obtained from Shanghai Sangon Biotechnology Co. Ltd.) (Table 1).

Statistical analysis
Data were analyzed using t-test and Microsoft Excel 2000.

RESULTS

Changes of serum fibrosis-associated markers
In model group, the serum activities of ALT, AST and β-NAG were significantly increased (P<0.01), the serum levels of PCIII, CIV and HA were also elevated (P<0.01). With administration of compound Rhodiola Sachalensis A Bor (RSC), serum activities of ALT, AST, β-NAG and levels of PCIII, CIV, HA were decreased obviously (P<0.01), although they were still higher than those in normal group (P<0.05) (Table 2).

Histopathological changes of the liver
The control livers showed a normal lobular architecture with central veins and radiating hepatic cords. The staging score was 0. Subcutaneous injection of CCl4 caused severe liver pathological damages such as: inflammation, necrosis and excessive collagen deposition. The semiquantitative staging score of hepatic fibrosis was raised to 3.53±0.68 in model group. The livers in treatment group showed less inflammation, necrosis, collagen deposition and a significantly decreased staging score of 2.43±0.47 (P<0.05) (Table 3, Figures 1, 2).

Molecular biological changes
Scoring method: according to the number of positive cells within one visual field on average. (-), no positive cells, scoring 0; (+), positive cells <1/3, scoring 1; (++), positive cells <2/3, scoring 2; (+++), positive cells >2/3, scoring 3. There were less positive signals of α1(I) mRNA, TGF-β1 mRNA and Na+/Ca2+ exchanger mRNA detected with RT-PCR in situ in normal group, in which scores were 1.11, 0.75, 1.00 and the ratio of positive samples was 77.8 %, 62.5 %, 10.0 % respectively (Tables 4-6). In model group, the positive signals of RT-PCR in situ for α1(I) mRNA, TGF-β1 mRNA and Na+/Ca2+ exchanger mRNA were significantly enhanced. The semiquantitative scores of them were increased to 2.80, 2.40, 2.30 and the ratio of positive samples raised to 100.0 %, 90.0 %, 100.0 % respectively (P<0.01). Treatment with RSC made the scores reduced to 1.63, 1.20, 1.50, and positive ratio lowered to 87.5 %, 80.0 %, 80.0 % respectively in comparison with model group (P<0.05) (Tables 4-6, Figures 3-6).

Table 3 Histopathological semiquantitative scores in the liver

| Group          | n  | 0 | + | ++ | +++ | Staging scores |
|----------------|----|---|---|----|-----|----------------|
| Normal         | 10 | 0 | 0 | 0  | 0   | 0              |
| Model          | 30 | 0 | 0 | 3  | 8   | 3.53±0.68      |
| Treatment      | 35 | 0 | 2 | 18 | 2   | 2.43±0.47      |

*p <0.01, p <0.05 vs model group.

Table 4 Expression of α1(I) mRNA (semiquantitative scores and positive ratio)

| Group          | n  | - | + | ++ | +++ | Scores | Positive ratio (%) |
|----------------|----|---|---|----|-----|--------|-------------------|
| Normal         | 9  | 2 | 4 | 3  | 0   | 1.11±0.7 | 77.8%             |
| Model          | 10 | 0 | 2 | 2  | 2   | 2.80±2  | 100.0%            |
| Treatment      | 8  | 1 | 3 | 4  | 1   | 1.63±0.7 | 87.5%             |

*p <0.01, p <0.05 vs model group.

Table 5 Expression of TGF-β1 mRNA (semiquantitative scores and positive ratio)

| Group          | n  | - | + | ++ | +++ | Scores | Positive ratio (%) |
|----------------|----|---|---|----|-----|--------|-------------------|
| Normal         | 8  | 3 | 1 | 0  | 0   | 0.75±0.7 | 62.5%             |
| Model          | 10 | 1 | 1 | 7  | 0   | 2.40±0.7 | 90.0%             |
| Treatment      | 10 | 2 | 5 | 2  | 1   | 1.20±0.7 | 80.0%             |

*p <0.01, p <0.05 vs model group.

Table 6 Expression of Na+/Ca2+ exchanger mRNA (semiquantitative scores and positive ratio)

| Group          | n  | - | + | ++ | +++ | Scores | Positive ratio (%) |
|----------------|----|---|---|----|-----|--------|-------------------|
| Normal         | 10 | 0 | 0 | 0  | 0   | 0.10±0.7 | 10.0%             |
| Model          | 10 | 0 | 2 | 3  | 5   | 2.3±0.7  | 100.0%            |
| Treatment      | 10 | 2 | 3 | 3  | 2   | 1.50±0.7 | 80.0%             |

*p <0.01, p <0.05 vs model group.
DISCUSSION

Liver fibrosis is generally preceded by chronic liver injury despite of its primary causes, including alcohol, hepatic virus, oxidant stress and other persistent damages. The activation of hepatic stellate cells (HSC) is considered to be of great importance during the long period of liver fibrosis, which is induced by some critical cytokines and then becomes the main source of most collagen proteins\(^{[24]}\). Among the cytokine mediating factors, transforming growth factor beta 1 (TGF-\(\beta\)1) has been considered as the essential profibrogenesis factor and the main target of treatment\(^{[25-31]}\). Additionally, \(\text{Na}^+\text{Ca}^{2+}\) exchanger was a newly noticed factor whose expression increased along with the activation of HSC, and its real role in liver fibrosis has not been interpreted\(^{[32]}\). Thus, detection of the expression of TGF-\(\beta\)1 and \(\text{Na}^+\text{Ca}^{2+}\) exchanger mRNA is useful in exploring the probable mechanisms of anti-fibrotic drugs.

Several drugs, including cytokines, antioxidant, chemical drugs, soluble type II receptor of TGF-\(\beta\)1\(^{[9, 33]}\), antibody of TGF-\(\beta\)1 have been used to block liver fibrosis. But their effects are not as prosperous as we expected\(^{[1]}\). Besides, some traditional Chinese drugs have been found to be effective on preventing fibrogenesis and other chronic liver injury, which develop a more hopeful future in controlling liver fibrosis and cirrhosis. The present study aimed at exploring the effects of a traditional Chinese herb, compound rhodiola sachalinensis A Bor, which consists of rhodiola sachalinensis A Bor, sophora flavescens Ait and other herbs, on the prevention of CCL\(_4\)-induced liver fibrosis in rats. The potential mechanisms of RSC was explained at the same time.

In this study, chronic administration of CCL\(_4\) caused liver fibrosis and cirrhosis as indicated by the changes of serum markers, histopathological changes, and molecular biological changes. The activities of serum fibrosis-associated enzymes, namely ALT, AST, \(\beta\)-NAG and contents of extracellular matrix (ECM) components (PCIII, CIV, HA) were significantly
increased along with increased expression of α(1)I mRNA, TGF-β1 mRNA and Na+/Ca2+ exchanger mRNA. Under the light microscope, the liver fibrosis/cirrhosis was verified by the typical liver structure: inflammation, necrosis and excessive collagen deposition, some of the samples even had pseudolobules. With the therapy of compound rhodiola sachalinesis A Bor, serum parameters of liver fibrosis (ALT, AST, β-NAG, PCIII, CIV, HA) were significantly decreased (P<0.01). HE, VG and Masson stained histopathological sections showed mild inflammation, necrosis and fewer collagen deposition. The semiquantitative fibrosis staging scores were also decreased obviously (P<0.01). The expression of α(1)I mRNA, TGF-β1 mRNA and Na+/Ca2+ exchanger mRNA was significantly inhibited using RT-PCR in situ (P<0.01). These results suggest that compound rhodiola sachalinesis A Bor may prevent experimental liver fibrosis by modulating the synthesis and releasing of critical cytokines, such as TGF-β1, thus inhibiting the activation of HSC and its production of collagen proteins. The inhibition of Na+/Ca2+ exchanger mRNA may partly relate to its anti-fibrotic effects. In conclusion, traditional Chinese medicine compound rhodiola sachalinesis A Bor has significant anti-fibrogenesis effects on CCl4-induced liver fibrosis in rats. The probable molecular mechanisms may include blocking the synthesis of TGF-β1, interfering with the activation of HSC, preventing production and deposition of collagen, and inhibiting the expression of Na+/Ca2+ exchanger mRNA. The exact molecular mechanisms remain to be explored.

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