Correlation between anti-fibrotic effect of baicalin and serum cytokines in rat hepatic fibrosis

Xiao-Dong Peng, Li-Li Dai, Chang-Quan Huang, Chun-Mei He, Li-Juan Chen

AIM: To investigate the correlation between the antifibrotic effect of baicalin and serum cytokines production in rat hepatic fibrosis.

METHODS: Forty male Sprague-Dawley rats were divided randomly into four groups: normal control group, model group, baicalin-treated group, and colchicine-treated group. Except for the normal control group, all rats in the other groups were administered with carbon tetrachloride to induce hepatic fibrosis. At the same time, the last two groups were also treated with baicalin or colchicine. At the end of the 8 wk, all animals were sacrificed. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), transforming growth factor (TGF)-β1, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 were measured. Liver index, hepatic hydroxyproline content and the degree of liver fibrosis were also evaluated.

RESULTS: The levels of ALT, AST and liver index in the baicalin-treated group were markedly lower than those in the model group (ALT: 143.88 ± 14.55 U/L vs 193.58 ± 24.35 U/L; AST: 263.66 ± 44.23 U/L vs 404.37 ± 68.29 U/L; liver index: 0.033 ± 0.005 vs 0.049 ± 0.009, P < 0.01). Baicalin therapy also significantly attenuated the degree of hepatic fibrosis, collagen area and collagen area percentage in liver tissue (P < 0.01). Furthermore, the levels of serum TGF-β1, TNF-α and IL-6 were strikingly reduced in the baicalin-treated group compared with the model group, while the production of IL-10 was up-regulated: (TGF-β1: 260.21 ± 31.01 pg/mL vs 375.49 ± 57.47 pg/mL; TNF-α: 193.40 ± 15.18 pg/mL vs 260.04 ± 37.70 pg/mL; IL-6: 339.87 ± 72.95 pg/mL vs 606.47 ± 130.73 pg/mL; IL-10: 506.22 ± 112.07 pg/mL vs 316.95 ± 62.74 pg/mL, P < 0.01).

CONCLUSION: Baicalin shows certain therapeutic effects on hepatic fibrosis, probably by immunoregulating the imbalance between profibrotic and antifibrotic cytokines.

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Key words: Baicalin; Hepatic fibrosis; Hepatic stellate cell; Cytokines

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INTRODUCTION

Hepatic fibrosis is a common pathological process of chronic liver injury, regardless of etiology, and its progression leads to cirrhosis and liver cancer[1]. Despite extensive efforts, its etiology and pathogenesis remain unclear, and effective therapy with limited side effects is still lacking[2]. Baicalin is a major bioactive flavonoid contained in dried roots of Scutellaria baicalensis...
Liver index calculation
Liver index was measured according to the formula: (rat liver weight/rat weight) × 100%[18].

Histopathology
Samples were obtained from the same liver lobe in all animals and fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) or van gieson (VG) stain.

The degree of liver fibrosis was evaluated on HE-stained sections as described previously[16,17]. The collagen content of the sections was also determined on VG-stained sections by a computer image analysis system (CM2000B, Beijing University of Aeronautics & Astronautics, China). Five random fields were chosen in each section and the amount of total collagen was detected in the area stained by VG, and expressed as percentage relative to the total area[18].

ELISA for serum TGF-β1, TNF-α, IL-6 and IL-10
Cytokine levels in the serum samples were measured by a commercially available ELISA kit (Biosources, San Jose, CA, USA) according to the manufacturer’s instructions.

Statistical analysis
Statistical analysis was performed with SPSS for Windows, version 13.0 (Chicago, IL, USA). Parametric data were analyzed statistically by one-way ANOVA followed by post-hoc tests when appropriate. Degree of hepatic fibrosis was analyzed by Kruskal-Wallis nonparametric test. Data were expressed as the means ± SD. A significant difference was defined as $P < 0.05$.

### Table 1 Level of liver index and serum AST, ALT in different treatment groups (mean ± SD)

| Group        | Liver index | ALT (U/L) | AST (U/L) |
|--------------|-------------|-----------|-----------|
| Normal       | 9           | 0.026 ± 0.004 | 114.50 ± 8.16 | 183.09 ± 26.70 |
| Model        | 7           | 0.049 ± 0.009 | 193.58 ± 24.35 | 404.37 ± 68.29 |
| Baicalin     | 9           | 0.033 ± 0.005 | 143.88 ± 14.55 | 263.66 ± 44.23 |
| Colchicine   | 9           | 0.031 ± 0.004 | 167.60 ± 21.66 | 325.61 ± 52.83 |

* $P < 0.05$, $P < 0.01$ vs normal control group; $P < 0.05$, $P < 0.01$ vs model group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

**MATERIALS AND METHODS**

### Reagents and rats

Baicalin (7-gluconic acid, 5,6-dihydroxyflavone, CAS No: 21967-41-9) was provided by Sichuan Guanghan Bencao Plant Chemical Co., Ltd (Sichuan, China), and purity was assessed by HPLC (> 98%). Colchicine was purchased from Xiamen Sanland Chemical Co., Ltd (Fujian, China). CCl₄ was obtained from Chongqing Chemical Reagent Co., Ltd (Chongqing, China). Male Sprague-Dawley (SD) rats weighing 150-180 g were purchased from the Experimental Animal Center of Third Military Medical University. All studies involving rats were approved by the Institutional Animal Care and Use Committee.

### Induction of liver fibrosis and baicalin treatment

Forty male SD rats were divided randomly into four groups: normal ($n = 9$); model ($n = 11$); baicalin-treated ($n = 10$); and colchicine-treated ($n = 10$). Except for the normal control group, all rats in the other groups were treated with subcutaneous injection of 40% CCl₄ (initial dose of 0.5 mL/kg, followed by 0.3 mL/kg), mixed with vegetable oil, twice weekly for 8 wk. The latter two groups were also treated with baicalin (70 mg/kg, dissolved in sterile saline water, intraperitoneal injection, once daily), or colchicine (50 μg/kg, dissolved in sterile saline water, intraperitoneal injection, once daily) on the same day as CCl₄ administration and continued for the 8-wk experimental period. The two drug doses were selected based on a previous study[13]. Simultaneously, normal control and model groups were intraperitoneally administered with the same volume of vehicle (sterile saline water) once daily. At the end of the 8-wk experimental period, all animals were anesthetized with 3% chloral hydrate and dissected. Blood and liver were obtained for further analysis.

### Measurement of serum aspartate aminotransferase (AST)

Serum AST and alanine aminotransferase (ALT) levels were measured using on an automated analyzer of biochemistry (Hitachi 7170, Tokyo, Japan) according to the manufacturer’s instructions.

### Liver index calculation

Liver index was measured according to the formula: (rat liver weight/rat weight) × 100%[18].

**8** **References**

[1] Huangqin (common name: *Huangqin* in China, a traditional Chinese herbal medicine) and it possesses a multitude of pharmacological activities. For instance, Baicalin exerts the inhibitory effects against several virus including influenza virus, human T cell leukemia virus and acquired human immunodeficiency virus type I[3-5]. It can act as potent anti-inflammatory, anti-allergic and anti-bacterial agent in a variety of inflammatory diseases[6-8]. It may also be potentially useful in the treatment of prostate and bladder cancers as well as hepatoma via multiple cellular mechanisms[9,10]. Most importantly, previous studies show that baicalin has significant scavenging effects on oxygen free radicals and protective effects on liver injury induced by iron overload and CCl₄, suggesting that it is a potent free radical scavenger and hepatoprotective drug[11,12]. However, the exact mechanisms of anti-fibrotic effect of baicalin remain unclear. Therefore, it is necessary to be further elucidated.

CCl₄-induced hepatic fibrosis is a well-established animal model to study the pathogenesis and therapy of chronic liver injury. Zhang et al[14] have reported that several profibrotic cytokines, including transforming growth factor (TGF)-β₁, tumor necrosis factor (TNF)-α and interleukin (IL)-6, play an important role in the initiation and perpetuation of CCl₄-induced liver fibrosis, whereas IL-10 plays an antifibrogenic role by counterbalancing the former effects. This study aimed to further investigate the effect of baicalin on hepatic fibrosis induced by CCl₄ and its relationship with the expression of TGF-β₁, TNF-α, IL-6 and IL-10.

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**Table 1 Level of liver index and serum AST, ALT in different treatment groups (mean ± SD)**

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* $P < 0.05$, $P < 0.01$ vs normal control group; $P < 0.05$, $P < 0.01$ vs model group. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.
RESULTS

Animals
Irritability, aggression and weight loss were present predominantly in the model group. At the end of the 8-wk experimental period, no death was found in the normal control group. There were four deaths in the model group, one in the colchicine-treated group, and one in the baicalin-treated group.

Liver index and serum aminotransferases
Liver index in the normal control group was 0.026 ± 0.004. However, 8 wk after CCl4 injection, the liver index increased markedly. The increase was significantly attenuated by baicalin or colchicine treatment (P < 0.01, Table 1).

We then measured serum aminotransferase activity in different experimental groups. The levels of serum AST and ALT were significantly increased in the model group compared with those in the normal control group. In contrast, baicalin or colchicine treatment significantly suppressed upregulation of these parameters induced by CCl4 (P < 0.05 or P < 0.01, Table 1).

Histopathology
Using HE staining, we observed that the liver tissue in normal control rats showed normal lobular architecture with central veins and radiating hepatic cords. However, liver sections taken from rats in the model group exhibited more inflammatory infiltration, steatosis, hepatocyte coagulative necrosis and fibrous septa compared with the normal control rats after 8 wk of CCl4 treatment. In contrast, baicalin or colchicine treatment markedly ameliorated these histopathological changes (Figure 1A-D). The results were further supported by a significantly decreased staging score of hepatic fibrosis after baicalin or colchicine therapy (P < 0.01, Table 2).

Table 2 Degree of liver fibrosis in different treatment groups (mean ± SD) (pg/mL)

| Group       | n | Degree of hepatic fibrosis | Average |
|-------------|---|----------------------------|---------|
|             | 0 | I | II | III | IV |
| Normal      | 9 | 9 | 0 | 0 | 0 | 0 |
| Model       | 7 | 0 | 0 | 4 | 3 | 3.43<sup>b</sup> |
| Baicalin    | 9 | 0 | 3 | 2 | 0 | 1.89<sup>a,b</sup> |
| Colchicine  | 9 | 0 | 3 | 5 | 1 | 1.78<sup>a,b</sup> |

<sup>a</sup>P < 0.01 vs normal control group; <sup>b</sup>P < 0.01 vs model group.

Table 3 Comparison of collagen area and collagen area percentage in liver tissue from rats of different treatment groups (mean ± SD)

| Group       | n | Collagen area (μm²) | Collagen area percentage |
|-------------|---|---------------------|--------------------------|
| Normal      | 9 | 993.54 ± 145.31    | 1.97 ± 0.30              |
| Model       | 7 | 2599.99 ± 488.32   | 10.15 ± 2.87<sup>b</sup> |
| Baicalin    | 9 | 1407.74 ± 284.49<sup>a,b</sup> | 4.54 ± 2.08<sup>a,b</sup> |
| Colchicine  | 9 | 1396.00 ± 276.07<sup>a</sup> | 4.65 ± 2.11<sup>a</sup> |

<sup>a</sup>P < 0.01 vs normal control group; <sup>b</sup>P < 0.01 vs model group.

We evaluated by VG staining the collagen level in the liver tissue from different treatment groups. Compared with the normal control group, both collagen area and collagen area percentage were significantly increased in the model group. The increases were reduced by baicalin or colchicine treatment, similar to the changes in hepatic histological examination (P < 0.01; Figure 1E-H, Table 3). Therefore, the above findings show that baicalin can prevent CCl4-induced hepatic fibrosis in rats.

Effect of baicalin on serum TGF-β1, TNF-α, IL-6 and IL-10 production
As shown in Table 4, the levels of serum TGF-β1, TNF-α and IL-6 in the model group were significantly higher than those in the normal control group (P < 0.01). Upregulation was markedly inhibited by treatment with 70 mg/kg baicalin (P < 0.01). On the other hand, IL-10 production in the model group was sharply decreased compared with that in the normal control group (55% reduction, P < 0.01). However, baicalin therapy significantly recovered the decrease induced by CCl4 (P < 0.01).

DISCUSSION

In the present study, baicalin significantly lowered the levels of serum ALT, AST and liver index, reduced histological changes of liver fibrosis, suppressed the expression of cytokines, including TGF-β1, TNF-α and IL-6, and improved significantly the serum level of IL-10. Furthermore, our previous study showed that the increases of several fibrosis indices including serum hyaluronic acid, type IV collagen and hepatic hydroxyproline content after the CCl4 injection can be notably inhibited by baicalin treatment<sup>13</sup>. The above findings demonstrated that baicalin can effectively prevent CCl4-induced hepatic fibrosis in rats and regulate the production of cytokines correlated with fibrosis.

Table 4 Serum levels of TGF-β1, TNF-α, IL-6 and IL-10 in rats in the different treatment groups (mean ± SD) (pg/mL)

| Group       | n | TGF-β1            | TNF-α           | IL-6          | IL-10           |
|-------------|---|-------------------|-----------------|---------------|-----------------|
| Normal      | 7 | 199.78 ± 18.92    | 157.62 ± 11.77  | 187.98 ± 51.97 | 700.52 ± 138.63 |
| Model       | 7 | 375.49 ± 57.47<sup>b</sup> | 260.04 ± 37.70<sup>b</sup> | 606.47 ± 130.73<sup>b</sup> | 316.95 ± 62.74<sup>b</sup> |
| Baicalin    | 7 | 260.21 ± 31.01<sup>b</sup> | 193.40 ± 15.18<sup>b</sup> | 339.87 ± 72.95<sup>a,d</sup> | 506.22 ± 112.07<sup>b</sup> |

<sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01 vs normal control group; <sup>d</sup>P < 0.01 vs model group.
Carbon tetrachloride (CCl₄), is a known hepatotoxin that can cause liver necrosis, fibrosis and cirrhosis when administered repeatedly. Hepatotoxicity is thought to involve two phases. The initial phase involves bioactivation by a microsomal cytochrome-P450-dependent monooxygenase system, which results in the formation of free radicals and oxidative stress/lipid peroxidation which exhibits the increase of malondialdehyde (MDA) amounts and decrease of superoxide dismutase (SOD) levels. The second step involves the activation of Kupffer cells, which is accompanied by the production of profibrotic mediators such as TGF-β, TNF-α and IL-6. Hepatic stellate cells (HSC), activated by pro-fibrotic factors, lose

Figure 1 Representative pathological changes in liver sections taken from four experimental groups (A-D: HE, × 100; E-H: VG, × 200). A, E: Normal; B, F: Model; C, G: Baicalin; D, H: Colchicine.
vitamin A and transform into myofibroblasts (MFN), expressing α-smooth muscle actin (α-SMA) and thus gaining the function of contractility, proliferation and fibrogenesis[32,33]. In this study, we observed that baicalin significantly reduced the increase in profibrotic cytokines such as TGF-β1, TNF-α and IL-6 induced by CCl4. The reduction in profibrotic cytokines may be correlated closely with previous results that baicalin has good radical scavenging action (lessening the MDA level and activating the SOD activity) and can thus reduce the production of activated Kupffer cells[12,13]. The down-regulation of pro-fibrotic cytokines induced by baicalin treatment then significantly inhibits the activation and proliferation of HSC and enhances HSC apoptosis in vitro or in vivo studies[13], which results in the extenuation of hepatic fibrosis. Thus, the reduction of profibrotic cytokines such as TGF-β1, TNF-α and IL-6 levels is one important mechanism associated with anti-fibrotic effect of baicalin.

IL-10 is a pluripotent cytokine produced by many activated immune cell types, including T-helper cells, B cells, macrophages, monocytes and keratinocytes[27]. Recent studies have indicated that IL-10 might play an important role in antifibrogenesis during CCl4-induced hepatic fibrogenesis[29-31]. Our study showed that the level of circulating IL-10 in the model group was lower than that in the normal control group, which was consistent with a previous study[13]. In contrast, baicalin significantly restored the decrease in IL-10 content induced by CCl4, probably contributing to the anti-fibrotic effect of baicalin.

In conclusion, baicalin has significant antifibrogenic effects on CCl4-induced liver fibrosis in rats. In addition to the inhibition of HSC activation and lipid peroxidation, as previously reported, immunoregulation of the imbalance between profibrotic and antifibrotic cytokines is one of the most important factors involved in the preventive effect of baicalin on CCl4-induced liver fibrosis. The exact molecular mechanisms remain to be explored.

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COMMENTS

Background
Hepatic fibrosis is a common pathological process of chronic liver injuries, regardless of etiology, and its progression leads to cirrhosis and liver cancer. Despite extensive efforts, its etiology and pathogenesis remain unclear and regardless of etiology, and its progression leads to cirrhosis and liver cancer.

Research frontiers
Baicalin is a flavonoid purified from the medicinal plant Scutellaria baicalensis Georgi, a well known Traditional Chinese Medicine. The previous studies show that baicalin has significant scavenging effects on oxygen free radicals and protective effects on liver injuries induced by CCl4. In this study, the effect of baicalin on hepatic fibrosis induced by CCL and its relationship with the expression of pro-fibrotic and anti-fibrotic cytokines were first investigated. The levels of liver index and serum aminotransferases in baicalin-treated group were markedly lower than those in model group. Baicalin therapy also significantly attenuated the degree of hepatic fibrosis, collagen area and collagen area percent in liver tissue. Furthermore, the levels of serum transforming growth factor-β1, tumor necrosis factor-α and interleukin (IL)-6 were strikingly reduced in baicalin-treated group compared with model group while the production of IL-10 was up-regulated. The above results show that baicalin has certain therapeutic effects on hepatic fibrosis probably by immunoregulating the imbalance between pro-fibrotic and anti-fibrotic cytokines.

Applications
The study demonstrates that baicalin is a good hepatoprotective drug for preventing and treating human liver fibrosis probably by immunoregulating the imbalance between pro-fibrotic and anti-fibrotic cytokines.

Terminology
Hepatic fibrosis is characterized by elevated deposition and altered composition of extracellular matrix, which is a common stage in most chronic liver injuries. Baicalin is a bioactive anti-inflammatory flavone purified from the medicinal plant Scutellaria baicalensis Georgi, a well known Traditional Chinese Medicine.

Peer review
The authors analyzed the preventative effects and mechanism of baicalin in the treatment of liver fibrosis in rats. The results are interesting and suggest that baicalin may be a clinical useful agent for preventing and treating human liver fibrosis.
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