Hepatoprotective effects of baicalein against CCl₄-induced acute liver injury in mice

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Abstract

AIM: To investigate the hepatoprotective effect of baicalein against carbon tetrachloride (CCl₄)-induced liver damage in mice.

METHODS: Mice were orally administered with baicalein after CCl₄ injection, and therapeutic baicalein was given twice a day for 4 d. The anti-inflammation effects of baicalein were assessed directly by hepatic histology and serum alanine aminotransferase and aspartate aminotransferase measurement. Proliferating cell nuclear antigen was used to evaluate the effect of baicalein in promoting hepatocyte proliferation. Serum interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) levels were measured by enzyme-linked immunosorbent assay and liver IL-6, TNF-α, transforming growth factor-α (TGF-α), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) genes expression were determined by quantitative real-time polymerase chain reaction.

RESULTS: CCl₄-induced acute liver failure model offers a survival benefit in baicalein-treated mice. The data indicated that the mRNA levels of IL-6 and TNF-α significantly increased within 12 h after CCl₄ treatment in baicalein administration groups, but at 24, 48 and 72 h, the expression of IL-6 and TNF-α was kept at lower levels compared with the control. The expression of TGF-α, HGF and EGF was enhanced dramatically in baicalein administration group at 12, 24, 48 and 72 h. Furthermore, we found that baicalein significantly elevated the serum level of TNF-α and IL-6 at the early phase, which indicated that baicalein could facilitate the initiating events in liver regeneration.

CONCLUSION: Baicalein may be a therapeutic candidate for acute liver injury. Baicalein accelerates liver regeneration by regulating TNF-α and IL-6 mediated pathways.

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Key words: Baicalein; Carbon tetrachloride; Liver injury; Liver regeneration; Hepatocyte proliferation

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INTRODUCTION

Liver is an important organ which plays a central role in metabolic homeostasis[1]. It also has an amazing regenerative capability after liver mass loss, as demonstrated by Higgins et al[2] in 1931. Carbon tetrachloride (CCL₄)-induced hepatic injury is a very classic model widely used for hepatoprotective drug screening[3,4]. The acute hepatotoxicity of CCL₄ lies in its biotransformation to trichloromethyl free radical (CCL₃) or trichloroperoxyl radical (CCL₃O₂⁻) produced by the mixed-function cytochrome P450 oxygenase system of the endoplasmic reticulum, which causes oxidative stress and membrane damage[5]. These free radicals cause lipid peroxidation which results in hepatocellular damage and enhances formation of inflamed tissues. The advantage of this model is that CCL₄ can culminate hepatitis within a few hours, which specifically leads to necrosis and fatty liver, in a similar way as what happens in the cases of acute hepatitis. Meanwhile, following an inflammatory response launched by resident inflammatory cells, CCL₄-induced acute liver injury also involves an intricately regulated process of hepatocyte regeneration when the dosage of CCL₄ is below lethal level which would lead to irreversible liver damage[6,7].

Baicalein (5, 6, 7-trihydroxyflavone, BAE, C₃H₁₀O₇) is a flavonoid extract from the root of Scutellaria baicalensis Georgi, a plant used in traditional Chinese medicine. Previous studies reported that baicalein has multiple functions. It acts as an anti-bacteria and anti-inflammatory agent, inhibits the aggregation of blood platelets, decreases the production of endotoxin, and alleviates the reperfusion injury in ischemic tissues[8,9]. Baicalein was indicated to suppress the growth of human hepatoblastoma cells[10,11], human breast cancer cells[12,13], human lung fibroblasts and peripheral lymphocytes[14] and human leukemia HL-60 cells[15,16]. Baicalein has beneficial effects against the cytotoxicity and genotoxicity to hepatocytes by tert-butylhydroperoxide via quench free radicals. Moreover, baicalein could protect animals from D-galactosamine/lipopolysaccharides induced acute liver failure in murine models, and especially reduce apoptosis (even hepatic necrosis) via cellular FLICE-like inhibitory protein and mitogen-activated protein kinase pathway[16,17]. However, the antifibrotic mechanism of baicalein remains vague so far. The aforementioned investigations for liver diseases on the role of baicalein in selectively inducing apoptosis of cancer cells and inhibiting normal hepatocyte apoptosis, prompted us to study whether baicalein would increase the secretion of various inflammatory cytokines and facilitate regeneration of liver cells. The aim of this study is to assess whether baicalein could prevent acute liver injury induced by CCL₄ in mice and to investigate the possible mechanism of its protective role.

MATERIALS AND METHODS

Animals and chemicals

Specific pathogen-free male C57 BL/6 mice (8 wk old) were obtained from Shanghai Slac Laboratory Animal Corporation. The mice were maintained in a conventional clean facility in accordance with the National Animal Care and Use Committee. CCL₄ and baicalein were purchased from Sigma-Aldrich Biotechnology (St Louis, MO, United States). Assays kits for the detection of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Jiancheng Biological Technology, Inc. (Nanjing, China). Mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) and the SABC Staining Kit were from Boster Biological Technology (Wuhan, China). Serum levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) were measured by enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, IL-6 and TNF-α from R and D system (Minneapolis, MN, United States). All other chemicals were of the highest grade commercially available.

Induction of liver injury and baicalein administration

Acute liver injury in mice was induced by intraperitoneal injection of CCL₄ at a dose of 1 mL/kg body weight (1:3 diluted in corn oil). A lethal dose was administered by intraperitoneal injection of CCL₄ at 2.6 mL/kg (1:1 diluted in corn oil). At the indicated time points, serum and liver specimens were collected. Mice were orally administered with baicalein (80 mg/kg) dissolved in CMC-Na to 200 mg/L 1 h after CCL₄ injection, and the same dose of baicalein was given twice a day for 4 d, and control mice were treated with same dosage CMC-Na.

Serum AST and ALT

Serum AST and ALT levels were determined with a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc., China). Enzyme activities were presented in international unit per liter (IU/L).

Histology-injury grading

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin-eosin for the histological studies. To evaluate the degree of necrosis after acute liver injury, we created an injury grading score (Grades Ⅰ-Ⅳ) based on severity of necrotic lesions in the liver parenchyma (Table 1).

Proliferating cell nuclear antigen staining

For PCNA immunohistochemical staining, de-paraffinized sections of liver blocks were used. Liver tissues were fixed for 24 h in neutral buffered formalin, processed routinely and embedded in wax. Immunohistochemical staining was performed as previously described[18]. The sectioned liver tissues were stained using a mouse monoclonal antibody against PCNA and the SABC Staining Kit (Wuhan Boster Biological Technology, Wuhan, China) according to the
manufacturer's protocol, then subjected to photomicroscopic observation (NIS-Elements Basic Research, Nikon Eclipse 50i, Kanagawa, Japan).

ELISA
Serum IL-1β, IL-6 and TNF-α levels were measured by ELISA kit (RD system, Minneapolis, MN, United States) according to the manufacturer’s instructions. Cell lysates were generated by adding 1 mL fresh medium to 100 mg liver specimen or 1 × 10^6 cells followed by three freeze-thaw cycles. Transforming growth factor-α (TGF-α), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) and ELISA kits were used to determine protein concentrations. ELISA was performed in triplicate for each sample of lysate.

Real-time quantitative polymerase chain reaction
Total RNA was obtained from the liver of mice and was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The quantification and qualification of RNA were performed using ultraviolet absorbance assay and electrophoresis in 1.2% agarose. RNA quality was satisfactory for the 28s rRNA band on gel and had twice the intensity of the 18s rRNA band without significant smearing of rRNA. Real-time quantitative polymerase chain reactions (PCRs) were performed with the MJ chromo 4 reverse transcription-PCR detection system (Bio-Rad Laboratories, Hercules, CA, United States). Specific primers were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, United States) and their sequences are listed in Table 2. As an internal control, the expression of the housekeeping gene β-actin was measured and remained constant at all the experimental conditions studied.

Table 1  Liver injury grading system

| Group   | Day 2 | Day 3 | Day 5 | Day 7 |
|---------|-------|-------|-------|-------|
| Baicalein | III   | I     | I     | 0     |
| 1       |       |       |       |       |
| 2       | III-IV| II    | 0     | 0     |
| 3       | III   | I     | 0     | 0     |
| 4       | III   | II    | 0     | 0     |
| 5       | III-IV| I     | I     | 1     |
| 6       | III   | I     | 0     | 0     |
| Control | IV    | II-III| I-II  | 0     |
| 1       |       |       |       |       |
| 2       | III-IV| III   | II    | 1     |
| 3       | III-IV| III   | II    | II    |
| 4       | IV    | III   | 0     | 0     |
| 5       | IV    | II    | I     | 1     |
| 6       | IV    | III   | I     | 1     |

Table 2  Primer sequences used for real-time quantitative polymerase chain reaction

| Gene | Sense | Anti-sense |
|------|-------|------------|
| IL-6 | CCACCTCCCAACAGCCCT-GTCTTATAC | CACCAACTCTTITCTCAATTC-CAAGA |
| TNF-α | AAGGCTGTAGCCCAGTCT-GT | CAGGATAAGGCGGAGCCCTT-GTC |
| HGF | GTGCTGGCATTACTAT-GATGG | CTCGACTTCCTTCTCACAGG |
| TGF-α | GCCGGGCTGAGTGGT-GTCTC | AGCCACACAGCCAGGAG-GTHGF |
| EGF | CGGACAGCTACACGGAATG | CAGGCGACAGAACAGAATA-ACCC |
| β-actin | AGCCTTCTTCTTTGTAGTTG | GTTGTGGCATAAGGTTCT-TAC |

Table 3  Survival functions

| Group   | Cum survival |
|---------|--------------|
| Baicalein | 1.0          |
| Control  | 0.8          |
| 1-censored | 0.6          |
| 2-censored | 0.4          |
| 3-censored | 0.2          |
| 4-censored | 0.0          |

Figure 1  Baicalein increased probability of survival after a lethal dose of carbon tetrachloride (2.6 mL/kg). Mice (n = 15) were administered with or without baicalein twice a day for 5 d. Survivals were scored twice a day, and the results were analyzed using the log-rank test and expressed as the Kaplan-Meier survival curves. P = 0.009 between control and baicalein groups.

Statistical analysis
Student’s t test (unpaired, two-tailed) was used for comparisons between data from specified different conditions. Results from survival experiments were analyzed using the log-rank test and presented as Kaplan-Meier survival curves.

RESULTS
Baicalein reduces mortality after a lethal dose performance
In a previous experiment to observe the dosage-dependent effect of CCl₄, we found that 2.6 mL/kg CCl₄ was a median lethal dose (a mortality of 50%, data not shown) within 24 h. Oral baicalein administration offers a survival
benefit for mice, increasing the probability of survival significantly one day after CCl4 injection (P = 0.009, Figure 1).

**Baicalein protects mice from acute hepatocellular damage**

To confirm the effect of baicalein in protecting mice from hepatic damage, we used serum ALT and AST levels as indicators for liver injury. In the control group, the serum level of ALT and AST rapidly reached the peak level at day 1, and decreased thereafter, while baicalein significantly inhibited the elevated ALT and AST from day 1 to day 5 (n = 6) (Figure 2A and B). The attenuated increase of serum AST and ALT indicated that baicalein plays a direct protective role in hepatocytes. To evaluate the effect of baicalein on hepatocellular necrosis and inflammation, histological changes in the liver after CCl4 administration with or without baicalein treatment were examined by histology-injury grading (Table 1). Liver sections from the baicalein-treated mice demonstrated only moderate necrosis involving the centrilobular areas, maintaining a rather normal architecture. The necrotic areas were significantly diminished around the central vein and centrilobular regions in baicalein-treated mice at day 3 (Figure 2C-E). These findings indicated that baicalein has potential anti-hepatotoxic activity.

**Baicalein promotes hepatocyte proliferation from an early phase**

To confirm whether baicalein has the potent advantage of accelerating hepatocyte proliferation from an early phase, we investigated the proliferation of hepatocytes using immunostaining of PCNA in sections of liver tissue at days 2 and 3. The PCNA staining confirmed that baicalein administration increased the number of positive staining cells more significantly at day 2 compared with the control group (Figure 3A and B). A great number of PCNA+ hepatocytes could be detected in the liver sections of baicalein-treated mice at day 3 (Figure 3C and D), which demonstrated that baicalein significantly increased the number of PCNA+ cells. Numbers of PCNA+ cells in at least 12 mm² tissue sections were counted for each mouse, and data showed that baicalein could accelerate hepatocyte proliferation (Figure 3E).

**Serum levels of IL-1β, IL-6 and TNF-α**

To evaluate the hepatoprotective mechanism of baicalein, serum IL-1β, TNF-α and IL-6 levels were determined by ELISA kit. Serum IL-1β was found to be elevated after CCl4 treatment,[21] whereas baicalein administration resulted in significant attenuation of the elevation (Figure 4A). CCl4-induced acute liver injury could activate hepatic non-parenchymal cells (including Kupffer cells and stellate cells) and increase the production of TNF-α and IL-6.[22] In our study, we found that serum TNF-α and IL-6 were rapidly increased and reached the peak level within 12 h in baicalein administration group as compared with the control group, and then decreased within 24 h (Figure 4B and C).
Expression of TNF-α and IL-6 in liver
Real-time quantitative PCR was used to quantify the expression of TNF-α and IL-6 in mouse liver. Data showed that in baicalein administration group, the production of TNF-α and IL-6 mRNA reached a peak level, which was even higher than in the control group, and then decreased rapidly in 24 h (Figure 5A and B).

Expression of TGF-α, HGF and EGF in liver
Real-time quantitative PCR was used to quantify the levels of TGF-α, HGF and EGF mRNA in liver. Data showed that the production of TGF-α, HGF and EGF mRNA was upregulated more rapidly in the baicalein administration group during the early phase and kept at a generally higher level within the process of liver regeneration (Figure 5C-F).

DISCUSSION
The model of acute intoxication with CCl₄ has been used for decades to investigate the response of acute liver injury, because the elementary lesions caused by this hepatotoxin replicate those seen in most cases of human liver diseases, which makes it a good model to study both signal transduction and cell cycle events in vivo [23,24]. Using this delicate model, we have identified the protective effect of baicalein against the typical acute liver injury.

Oral administration of baicalein to mice which have received a LD₅₀ dosage of CCl₄ resulted in a significantly reduced mortality rate. Since the pathological effect of CCl₄ in the animals has been proved to be mainly restricted to the liver and lethality of high-dose CCl₄ is mostly related with organ failure following acute liver failure instead of direct injury to other organs, it is reasonable to hypothesize that administration of baicalein can reduce animal mortality mainly through attenuating acute liver damage by CCl₄, and facilitating the preservation and restoration of liver functions.

It has been proved that baicalein administration indeed attenuated acute liver damage. The indicators for
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![Graphs A, B, C showing levels of interleukin-1β, interleukin-6, and tumor necrosis factor-α in serum in control and baicalein groups after CCl4 (1 mL/kg) treatment.](image)

The liver damage we have utilized are serum aminotransferase activities, including AST and ALT. They are commonly referred to as “liver enzymes”, because the levels of these enzymes are released from damaged hepatocytes into the blood, and their levels in the serum have been widely recognized as a very important indicator to judge the severity of acute hepatic injury. In our experiment, administration of baicalein attenuated the elevated serum ALT and AST induced by CCl4 in mice, which indicated that the proportion of damaged hepatocytes was reduced as a direct result of baicalein administration. Elevated ALT level was found to be significantly attenuated 12 h after CCl4 treatment, while similar phenomenon appeared at 24 h after CCl4 treatment for AST. Both time points are defined as the early-stage liver damage in which cell apoptosis and necrosis dominate the process. When the liver damage progresses over time, the speed of cell damage as a result of either cell apoptosis or necrosis is reduced, as indicated by the relative decrease of AST/ALT levels at later time points of days 3 to 5. On the other hand, regeneration of liver gradually took place from the middle to late stages of liver damage, during which cell proliferation rate would naturally increase till the original weight and shape of the liver and its functions, is restored. We used another statistical index to measure the possibility of baicalein in the regeneration of liver tissue. It is the density of positive cells in a certain area of tissue section immunostained with PCNA antibody. The index strongly indicated that baicalein treatment contributes to a faster liver recovery after CCl4-induced liver injury by promoting the endogenous regeneration process from the middle stage of the entire liver damage process. We also used histological methods as supportive means to reveal the degree of cell necrosis and inflammation. Data also showed that oral baicalein administration inhibited inflammation, necrosis, and destruction of liver architecture.

To investigate the underlying mechanism, we evaluated the effects of baicalein treatment on the serum level of certain key cytokines tightly related to inflammation and cell proliferation. IL-1β, IL-6 and TNF-α, as acute-phase proteins, are considered to be the special biomarkers that reflect inflammatory status. IL-1β plays a key role in inflammation, usually leading to tissue destruction. Furthermore, IL-1β has been previously shown to antagonize hepatocyte proliferation. Serum IL-1β can increase dramatically during different inflammatory and no-inflammator processes. In the present study, we observed that baicalein administrated mice demonstrated a significantly lower serum level of IL-1β at days 1, 2, 3 and 5, compared with the control group. The decreased level of inflammatory cytokines may explain the accelerated liver regeneration observed in baicalein administrated mice. IL-6 and TNF-α expression has been identified as attractive targets for liver regeneration. The release of TNF-α, as a pro-inflammatory mediator in liver apoptosis, is also linked to cytotoxicity induced by CCl4. Kupffer cells (macrophages in liver) produce TNF-α in rapid response to tissue injury, which then up-regulates the expression of IL-6. TNF-α and IL-6 together activate the neighboring hepatocytes, leading to signal transducer and activator of transcription STAT3 activation and...
the production of several other proteins that are shared within the growth-factor-mediated pathway network. In previous studies, pretreatment with IL-6 before CCl\textsubscript{4} reduces acute CCl\textsubscript{4}-mediated cell apoptosis, and accelerates regeneration in both wild-type and IL-6-/- livers\cite{30}. The mechanism of IL-6 and TNF-\textalpha in protecting the liver against injury has not been fully clarified\cite{31-33}. Previous studies showed that liver regeneration and hepatoprotection require the cytokine IL-6 immediately after liver injury\cite{34,35}. But overexpression of IL-6 inhibits hepatocyte growth and causes liver injury\cite{36,37}. In the present study, the expression of TNF-\textalpha and IL-6 in baicalein administrated mice reached a high level at day 0.5 and then was kept at a relatively lower level at days 1, 2, 3 and 5 compared with the control. We consider that the lower levels of TNF-\textalpha and IL-6 which are cell death mediators from days 1 to 5 may facilitate liver regeneration. In term of the mechanisms, we found that gene expression of IL-6 and TNF-\textalpha in treated liver was enhanced in a similar pattern as the level of corresponding proteins, leading to the conclusion that baicalein could indeed alter the expression of certain cytokines to affect the liver damage process.

Another group of molecules we have investigated are growth factors such as HGF, TGF-\textalpha and EGF. They promote hepatic survival by stimulating liver regeneration and providing hepatoprotection in various models of liver injury, such as toxic damage caused by CCl\textsubscript{4}\cite{38}. It has been proven that HGF, TGF-\textalpha and EGF are the main growth factors secreted after hepatic injury\cite{39}. HGF is the most...
potent mitogen for mature hepatocytes and acts as a hepa-totropic factor. HGF level is increased markedly in mouse liver after various liver injuries such as hepatitis, ischemia, physical crush and partial hepatectomy. HGF acts as a trigger for liver regeneration and strongly enhances EGF expression. Previous studies indicated that the liver regen-erative response is blocked if antibodies to HGF are ad-ministered at the same time as CCl4 treatment[60]. HGF ad-ministration to rodents was confirmed to reduce the level of CCl4-induced injury. HGF has been shown to regulate DNA synthesis partially through upregulation of other growth factors in hepatocytes in vivo and in vitro, which indicates that all of them are crucial for liver regeneration[42,43]. In our study, a significant increase of HGF, EGF and TNF-α expression occurred in livers from baicalein-treated groups during the proliferation phase (from days 1 to 3). Such expression reached a lower level in baicalein-treated mice at day 5 compared with the control, which indicated that the liver regeneration was terminated at an earlier phase.

In conclusion, we found baicalein from the Chinese herbal medicine possesses strong beneficial effects in a mouse model against acute liver injury caused by CCl4. The expression of inflammatory cytokines IL-6 and TNF-α are markedly increased at the very early stage, which activate crucial signal transducers, including signal transducer and activator of transcription 3 and trigger certain signal cascades related to liver regeneration. During the middle stage, the expression level of such cytokines was significantly lowered to reduce inflammation cell apoptosis. The subsequent elevation of HGF, TGF-α and EGF may promote hepatic survival by stimulating hepatocyte regeneration. The protective effect of baicalein represents a clinical potential in the development of novel therapeutic agents for acute liver injury.

**COMMENTS**

**Background**

Baicalein is one of the bioactive compounds of *Scutellaria baicalensis* Georgi which has been shown to have anti-inflammatory, anti-bacteria and anti-hepatotoxic effects. However, the underlying mechanisms by which baicalein protects the liver from drug-induced injury still remain speculative.

**Research frontiers**

Previous investigations of liver diseases on the role of baicalein in selectively inducing apoptosis of cancer cells and inhibiting normal hepatocyte apoptosis, have prompted studies whether baicalein would increase the secretion of various inflammatory cytokines and facilitate regeneration of liver cells. This study assessed whether baicalein administration could prevent acute liver injury induced by carbon tetrachloride in mice and investigated the possible mechanism of its protective role.

**Innovations and breakthroughs**

The authors found that baicalein significantly elevated the serum level of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 at the early phase, which indicated that baicalein could facilitate the initiating events in liver regeneration. This study supports the possibility that baicalein may be a therapeutic candidate for acute liver injury, and indicates that baicalein could accelerate liver regeneration by regulating the TNF-α and IL-6 mediated pathways.

**Applications**

All these results support the possibility of baicalein being a therapeutic candidate for acute liver injury, and indicate that baicalein accelerates liver regeneration by regulating the TNF-α and IL-6 mediated pathways.

**Peer review**

The authors concluded that baicalein could facilitate the initiating events in liver regeneration. The experiments were well done and the results were clearly shown. This study is well designed and performed, and is of great interest for its novelty and impact in the field.

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