

Original Article

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

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Abstract: The aim of the current study was to determine the hepatoprotective effect and possible mechanisms of baicalein on CCl₄-induced acute liver injury. Male C57BL/6 mice were treated with or without baicalein before CCl₄ challenge. We detected the relative liver weight and the histopathology of the liver. Serum aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities were evaluated. Inflammatory mediators and antioxidant parameters were measured in liver homogenate. Moreover, the activity of nuclear factor (NF-κB) was also determined. Our dates showed that baicalein decreased the CCl₄-induced elevation of serum ALT, AST, and LHD activities, and improved hepatic histopathology changes. The levels of tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), and monocyte chemotactic protein 1 (MCP-1) in liver were significantly suppressed. Meanwhile, the activities of superoxide dismutase (SOD) and glutathione peroxidases (GSH-Px) in liver significantly were increased, while the content of malondialdehyde (MDA) was significantly decreased by pretreatment with baicalein. Furthermore, pretreatment with baicalein significantly inhibited NF-κB activation. In conclusion, baicalein has protective effects against CCl₄-induced acute liver injury by inhibition of inflammation and oxidative stress.

Keywords: Acute liver injury, baicalein, inflammation, NF-κB, oxidative stress

Introduction

Liver, one of the main organs for biological metabolism in the body, plays a vital role in regulating various physiological processes including proteins synthesis, glucose homeostasis and detoxification [1, 2]. Liver injury has been recognized as one of the most serious health problems in the world and can be caused by multiple factors including viral infections, excessive alcohol consumption and hepatotoxins [3, 4]. Carbon tetrachloride (CCl₄), a potent inducer of acute liver injury, is widely used in experimental hepatopathy [5]. The pathological lesions caused by CCl₄ affect the liver structure and function leading to liver injury. Increasing evidence supports the role of hepatic inflammation in the pathogenesis of chronic liver disease [6]. Indeed, liver inflammation leads to the secretion of inflammatory mediators including tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), and monocyte chemotactic protein

1 (MCP-1), which in turn contribute to a feed-forward amplification of inflammatory signaling and subsequent development and aggravation of hepatitis [7]. Otherwise, available evidence suggests that oxidative stress, in particular, lipid peroxidation critically participates in the progression of liver damages [8]. Lipid peroxidation may provoke liver damage by compromising the integrity of membranes and changing lipid metabolism balance [9]. Antioxidants are potent free radical scavengers and have been documented to protect hepatocytes from lipid peroxidation. Therefore, blocking or retarding the reactions of oxidative stress and the inflammatory process could be a promising therapeutic intervention for prevention or treatment of liver injuries.

Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one), a main active ingredient purified from the root of *Scutellaria baicalensis* Georgi, has been demonstrated to possess anti-inflam-

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Table 1. Effects of baicalein on relative liver and spleen weight in CCl₄-intoxicated mice

Group	Dose (mg/kg)	Relative liver weight
Normal control	-	3.74 ± 0.18
Model	-	5.66 ± 0.39**
L-baicalein	25	4.72 ± 0.21 [#]
M-baicalein	50	4.69 ± 0.35 ^{##}
H-baicalein	100	4.42 ± 0.25 ^{##}

Values are presented as means ± SD, n=10. **P<0.01 vs. normal control group. [#]P<0.05, ^{##}P<0.01 vs. model group.

matory [6], anti-oxidative [10], anti-allergic [11], and anticarcinogenic activities [12]. A previous study revealed that baicalein protects animals from D-galactosamine (GalN)/LPS induced acute liver failure via inhibition of inflammation in murine models [13]. Liu et al. [14] also demonstrated pretreatment with baicalein evidently diminished liver ischemia/reperfusion injury via inhibition of NF-κB pathway in mice. However, its impact on CCl₄-induced acute liver injury and its molecular mechanisms remains vague. Thus, the present study was conducted to investigate the hepatoprotective effects of baicalein on CCl₄-induced acute liver injury in mice and to explore the underlying mechanisms.

Materials and methods

Animal group

Male C57BL/6 mice, 8 to 10 weeks old, 20 to 22 g body weight, were purchased from Experimental Animal Center of Suzhou Aiermaite technology Co. Ltd. (SPF grade, Certificate No. SCXK20160002). All experiments were performed in accordance with China National Institutes of Healthy Guidelines for the Care and Use. The mice were maintained in a specific pathogen free facility under controlled conditions of 23 ± 3°C, relative humidity of 50-60% and a 12 h light/dark cycle. Basal diet and water was provided and allowed at least 1 week to adapt to the environment. Mice were randomized into five dietary groups (n=8/group): normal control group, model group, low-dose baicalein (L-baicalein) group, medium-dose baicalein (M-baicalein) group and high-dose (H-baicalein) group. Mice in L-baicalein, M-baicalein and high-baicalein group were intraperitoneally administered with baicalein (25, 50, 100 mg/

kg body weight respectively, dissolved in 0.01% DMSO phosphate-buffered saline) for 7 days consecutively, while the mice in normal control group and model were administrated 0.01% DMSO phosphate-buffered saline (1 ml/kg body weight). The doses were used based on our preliminary experiments. Two hours after the final administration, the mice in model group and baicalein treatment group were intraperitoneally injected with 0.3% (v/v) CCl₄ (10 ml·kg⁻¹, dissolved in olive oil). Simultaneously, the animals in normal control group intraperitoneally received equal volume of olive oil alone. Twenty-four hours after the CCl₄ challenge, the mice were weighed and then euthanized. Blood samples for biochemical analyzes were obtained from the inferior vena cava. Serum was separated by centrifugation at 4°C, 4000× g for 15 min. Livers were washed in ice-cold saline, blotted on a filter and then weighed to calculate relative liver weight (liver weight/body weight × 100). The left lobe of the liver was excised for histological examination and the remaining parts were stored at -80°C for other assays.

Histopathology examination

Liver tissues were fixed in 10% buffered neutral formalin for 10 h, and then embedded in molten paraffin. Specimens were sectioned at 4 μm and stained with haematoxylin and eosin stain (H & E). The histopathological changes were observed by light microscopy.

Measurement of liver enzymes

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities were measured with enzymatic kinetic method by using commercially available reagent kits (Nanjing Jiancheng Co., Nanjing, China) according to the manufacturer's instructions.

Measurement of inflammatory markers

The liver tissues were homogenized with ice-cold 0.9% NaCl solution (pH 8.6), and the homogenates were centrifuged at 3000× g for 15 min at 4°C to obtain the supernate. Then, the levels of tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), and monocyte chemoattractant protein 1 (MCP-1) were measured with enzyme-linked immunosorbent assay (ELISA)

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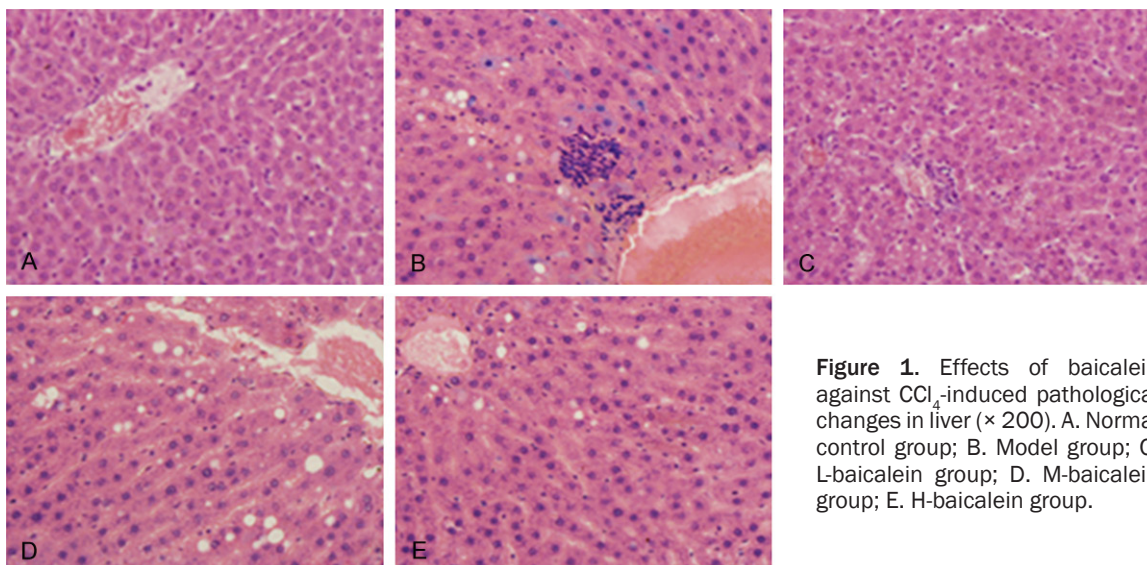


Figure 1. Effects of baicalein against CCl₄-induced pathological changes in liver ($\times 200$). A. Normal control group; B. Model group; C. L-baicalein group; D. M-baicalein group; E. H-baicalein group.

kits (Nanjing Jiancheng Co., Nanjing, China) according to the manufacturer's instructions.

Determination of MDA, GSH-Px, and SOD activity

The malondialdehyde (MDA) content, superoxide dismutase (SOD) and glutathione peroxidases (GSH-Px) activities in the liver homogenates were determined by ELISA kits (Nanjing Jiancheng Co., Nanjing, China) according to the producer's instructions.

NF- κ B activity assay

Nuclear factor-kappa B (NF- κ B) activity was detected by using nuclear extracts from the liver tissues. Activation of NF- κ B was quantified using the TransAM NF- κ B assay kit (Active Motif, Carlsbad, USA) according to the manufacturer's instructions.

Western blotting analysis

Liver tissues were homogenized in RIPA lysis buffer containing protease inhibitor, and then were centrifuged at 15000 r for 15 min. The protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Chengdu Must Biotechnology Co., Ltd, Chengdu, China). The protein samples (20 μ g) were subsequently subjected to 12% SDS-PAGE gels, and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked by 5% non-fat dry milk at room temper-

ature for 2 h and then were incubated overnight at 4°C with anti-NF- κ B (p65) antibody (1:1000, Cell Signaling Technology, MA, USA), anti-I κ B α antibody (1:1000, Cell Signaling Technology, MA, USA) and β -actin (1:1000, Cell Signaling Technology, MA, USA) antibody (1:2000, Cell Signaling Technology, MA, USA) and corresponding secondary antibodies subsequently. The resulting band was analyzed by enhanced chemiluminescence (ECL system, Amersham, Sydney, Australia) and western blots were visualized on the Kodak Image Station (Carestream Health Inc, New York, NY).

Statistical analysis

Statistical analysis was implemented using SPSS18.0 for windows. All data were reported as the mean \pm SD, and differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. The level of statistical significance was set at $P < 0.05$.

Results

Effects of baicalein on liver weight

As shown in **Table 1**, the relative liver weight was significantly increased in model compared to normal control group ($P < 0.01$), and baicalein treatment markedly suppressed the increase in relative liver weight in a dose-dependent manner.

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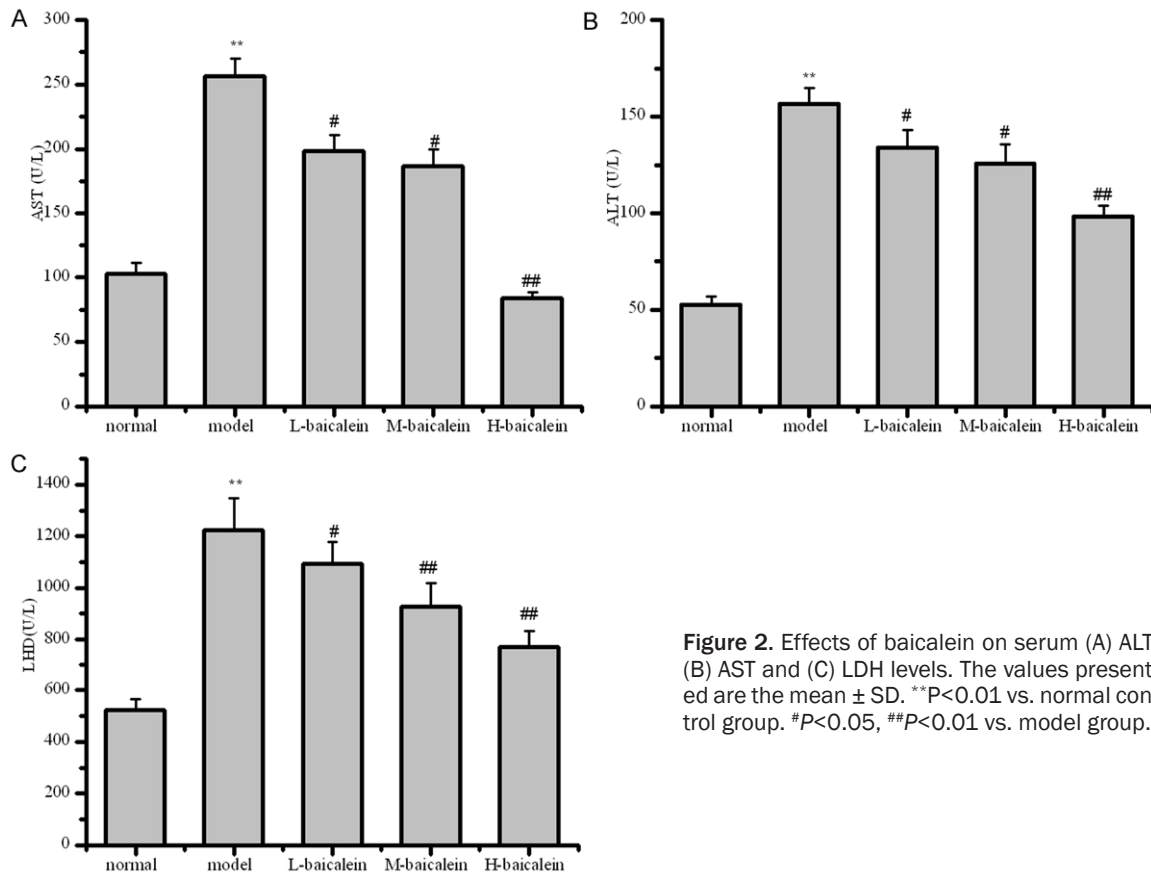


Figure 2. Effects of baicalein on serum (A) ALT, (B) AST and (C) LDH levels. The values presented are the mean \pm SD. ** $P < 0.01$ vs. normal control group. # $P < 0.05$, ## $P < 0.01$ vs. model group.

Baicalein treatment alleviated CCl₄-induced histopathology changes in liver

Histopathology evaluation of liver sections stained with hematoxylin and eosin (H & E) was performed under a light microscope (**Figure 1**). The histologic features of the normal control group showed a well-preserved cytoplasm, prominent nucleus and nucleolus, and normal lobular architecture. In the model group, pathologic changes including hepatocytes necrosis, destruction of hepatic architecture, and inflammatory cell infiltration were observed. However, baicalein pretreatment significantly attenuated these pathologic changes induced by CCl₄.

Baicalein inhibits CCl₄-induced serum ALT, AST and LDH levels

The liver function was assessed by detecting plasma ALT, AST and LDH activities. As shown in **Figure 2**, the model group exhibited a significant increase in AST, ALT and LDH levels compared with the normal control group ($P < 0.01$). However, pre-treatment with baicalein at three different doses for 7 days consecutively signifi-

cantly suppressed the increase of serum ALT and AST activities induced by CCl₄.

Baicalein inhibited the release of inflammatory mediators in liver

To identify the anti-inflammatory property of baicalein, the level of inflammatory mediators including TNF- α , IL-6 and MCP-1 in liver were determined by ELISA method. The results showed that liver TNF- α , IL-6 and MCP-1 levels increased significantly in the model group compared with the normal control group ($P < 0.01$). Pre-treatment with baicalein dose-dependently inhibited the increase of TNF- α , IL-6 and MCP-1 levels (**Figure 3A-C**).

Effects of baicalein against CCl₄-induced oxidative stress

In order to evaluate the effect of baicalein treatment on oxidative stress induced by CCl₄, levels of MDA, activities of SOD and GSH-Px were measured in liver tissue. The liver MDA level in the model group markedly increased compared with the normal control group ($P < 0.01$), and

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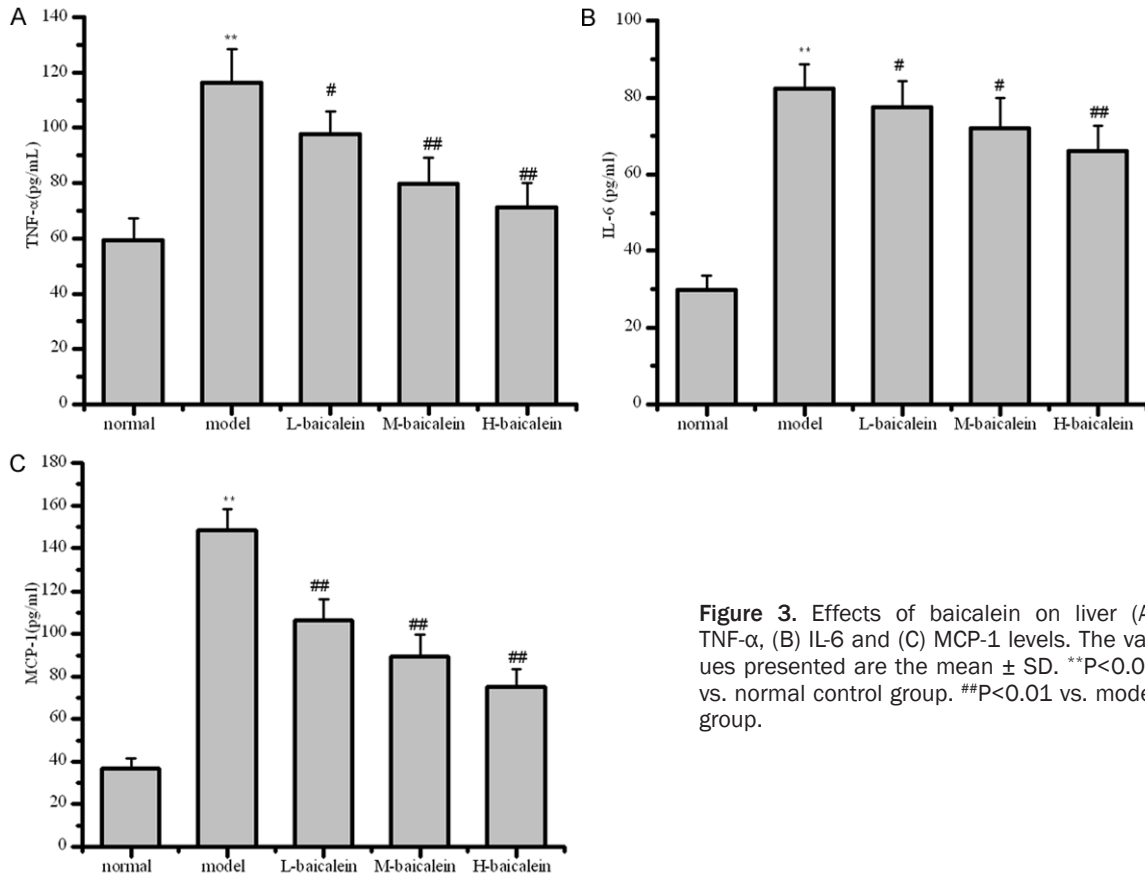


Figure 3. Effects of baicalein on liver (A) TNF- α , (B) IL-6 and (C) MCP-1 levels. The values presented are the mean \pm SD. **P<0.01 vs. normal control group. ##P<0.01 vs. model group.

baicalein treatment reversed the increase in a dose-dependent manner (**Figure 4A**). In contrast, liver SOD and GSH-Px levels were conspicuously decreased in the model group compared with those in the normal control group (P<0.01), whereas intervention with baicalein significantly increased the level of SOD and GSH-Px in a dose-dependent manner (**Figure 4B, 4C**).

Baicalein modulates NF- κ B activation in the liver

As shown in **Figure 5A**, compared to the normal control group, liver in model group exhibited an increase of NF- κ B and a decrease of I κ B α . However, baicalein pretreatment significantly prevented the degradation of I κ B α and the increase of NF- κ B. As demonstrated in **Figure 5B**, baicalein pretreatment blocked the NF- κ B activity induced by CCl₄.

Discussion

CCl₄ intoxication has been extensively used to establish experimental model of liver injury for

screening of the hepatoprotective activities of drugs [8]. It is well established that the cleavage of the carbon-chloride bond of CCl₄ leads to the formation of a trichloromethylperoxy radical that is involved in the pathogenesis of liver injury [1]. In this study, the activities of AST, ALT and LHD were dramatically increased in model group, which suggested the hepatic structural was damaged. However, baicalein pretreatment markedly reversed the alternations and protected hepatocytes from CCl₄-induced liver damage. Histopathological observations showing hepatocytes necrosis, destruction of hepatic architecture, and inflammatory cell infiltration further confirmed the severity of hepatic injury induced by CCl₄. The injury was reduced by pretreatment with baicalein. These results revealed that baicalein have protective effects on CCl₄-induced acute liver injury.

The inflammatory response has been recognized as a main pathological cause implicated in CCl₄-induced acute liver injury [15]. Pro-inflammatory cytokines such as TNF- α , IL-6 and MCP-1 are known to be crucial in inflammatory

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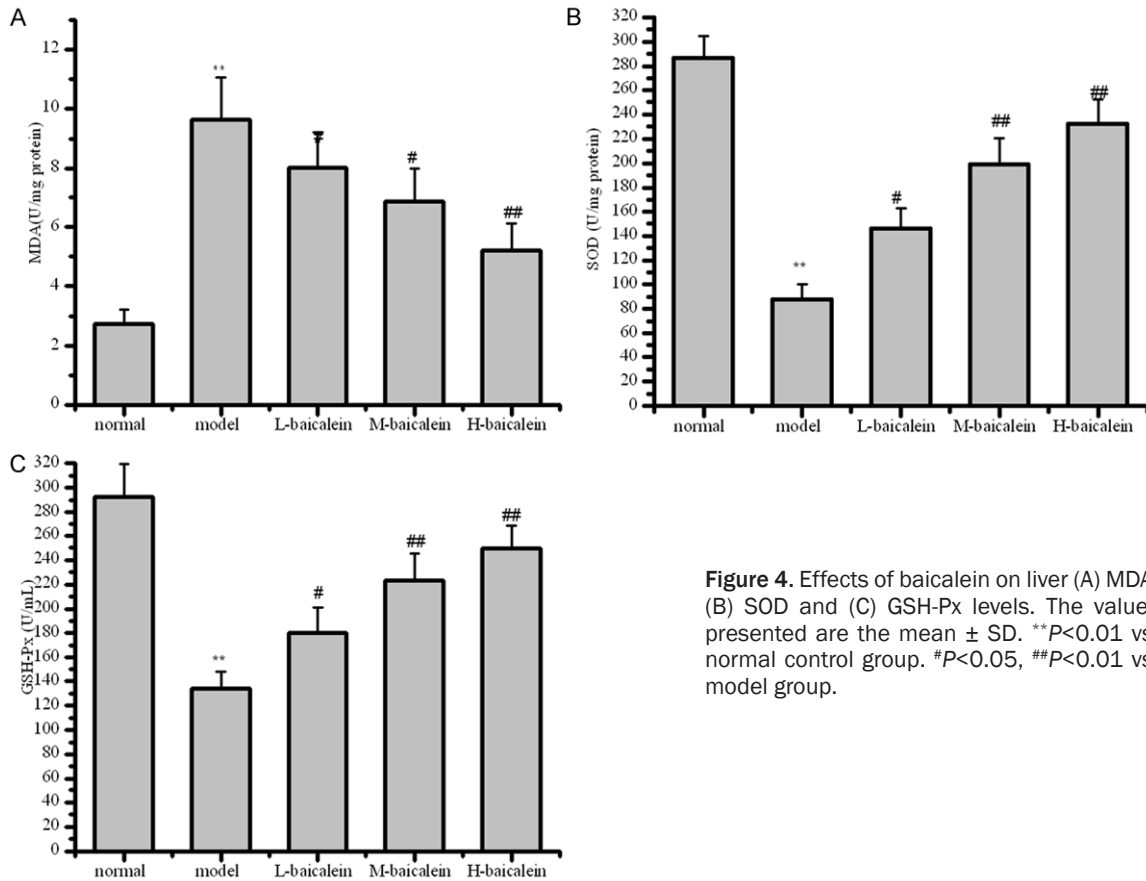


Figure 4. Effects of baicalein on liver (A) MDA, (B) SOD and (C) GSH-Px levels. The values presented are the mean \pm SD. ** P <0.01 vs. normal control group. # P <0.05, ## P <0.01 vs. model group.

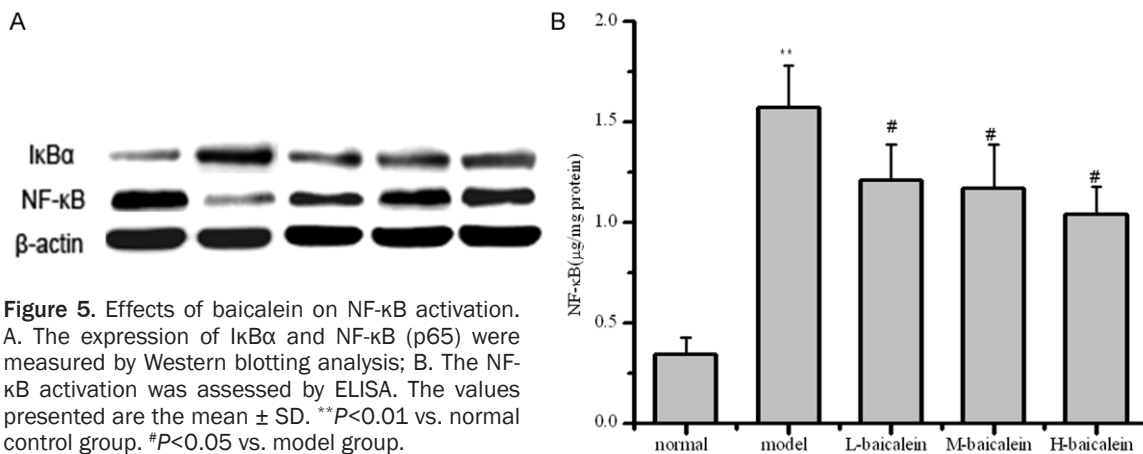


Figure 5. Effects of baicalein on NF-κB activation. A. The expression of IκBα and NF-κB (p65) were measured by Western blotting analysis; B. The NF-κB activation was assessed by ELISA. The values presented are the mean \pm SD. ** P <0.01 vs. normal control group. # P <0.05 vs. model group.

process and hepatic damage [16, 17]. TNF- α and IL-6 have been shown to correlate with many human liver diseases [18]. MCP-1, a chemokine mainly expressed by macrophages and vascular endothelial cells in the liver, was quickly up-regulated by inflammation stimuli, and could lead to the recruitment of more inflammatory cells, particularly neutrophils and

macrophages, infiltrating into the damaged tissues [19]. In the present study, baicalein pretreatment significantly inhibited production of several pro-inflammatory cytokines including IL-6, TNF- α , and MCP-1 in liver tissues. The dates indicate that the protective effects conferred by baicalein may be partially via the inhibition of inflammation.

NF-κB is a critical transcription factor for inflammatory gene expression and plays a vital effect in the pathogenesis of acute liver injury [8, 14]. NF-κB is activated upon CCl₄-induced liver injury and regulates the production of proinflammatory cytokines/chemokines, such as TNF-α and IL-6. The inhibition of NF-κB activation leads to a decrease of pro-inflammatory cytokine production and an amelioration of tissue injuries. Under normal physiological conditions, NF-κB is maintained in the cytoplasm in an inactive form bound by inhibitory protein IκBα. Activation of the NF-κB pathway leads to phosphorylation of p50 and p65, resulting in the transcription of genes [17, 20]. The present study demonstrated that CCl₄ stimulation dramatically increased the expression of NF-κB p65 and the degradation of IκBα in liver. However, baicalein pretreatment inhibited IκBα degradation and NF-κB p65 activation. Therefore, we believe that the modulation of the IκBα/NF-κB signaling pathway in liver accounts, at least in part, for the anti-inflammatory and the protective effects of baicalein.

Oxidative stress has been accepted as a major molecular mechanism in CCl₄-induced acute liver injury, which is responsible for cell membrane damage and the consequent release of marker enzymes of hepatotoxicity [21, 22]. Oxidative injury induced by CCl₄ can be monitored in experimental animals by detecting oxidative stress parameters, such as MDA, SOD, and GSH-Px. MDA is the final product generated in the metabolism of lipid peroxides, which can further damage the cells, which can reflect the degree of sensitivity of lipid peroxidation [23]. SOD is conceived of as the first line of cell defense against oxidative stress, which functions by eliminating reactive oxygen radicals, including superoxide and hydrogen peroxide, and preventing the generation of more hydroxyl radicals [24]. GSH-Px acts as an important enzyme catalyzing the reduction of H₂O₂ and lipid hydroperoxides into water and corresponding alcohols and then terminating the chain reaction of lipid peroxidation [25]. It is illustrated in this study that the pretreatment with baicalein can increase SOD and GSH-Px activities, and reduce MDA content in the liver tissues, suggesting that the anti-acute liver injury function of baicalein may be related to its function of anti-oxidative stress.

In conclusion, the present study demonstrated that baicalein ameliorated CCl₄-induced acute liver injury through attenuating oxidative stress, reducing the production of inflammatory cytokines and inhibiting NF-κB signaling pathway. Baicalein shows a promising reagent for the prevention or treatment of acute liver injury.

Disclosure of conflict of interest

None.

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