Original Paper

Baicalin Attenuates Alcoholic Liver Injury through Modulation of Hepatic Oxidative Stress, Inflammation and Sonic Hedgehog Pathway in Rats

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Key Words

Baicalin • Alcoholic liver injury • Oxidative stress • Inflammation • Sonic hedgehog

Abstract

Background/Aims: Lipid accumulation, inflammatory responses and oxidative stress have been implicated in the pathology of alcoholic liver disease (ALD). Targeting inhibition of these features may provide a promising therapeutic strategy for ALD. Baicalin, a flavonoid isolated from *Scutellaria baicalensis Georgi*, has been shown to exert a hepatoprotective effect. However, its effects on ALD remain obscure. This study was aimed to investigate the effects of baicalin on alcohol-induced liver injury and its related mechanisms. **Methods:** For *in vivo* experiments, rats were supplied intragastrical administration of alcohol continuously for 4 or 8 weeks, and then received baicalin treatment in the latter 4 weeks in the presence / absence of alcohol intake. Liver histology and function, inflammatory cytokines, oxidative mediators, and the components of the Sonic hedgehog pathway were evaluated. For *in vitro* experiments, alcohol-stimulated human normal liver cells LO2 were used. **Results:** Baicalin treatment significantly alleviated alcoholic liver injury, improved liver function impaired by alcohol, and inhibited hepatocytes apoptosis. In addition, baicalin decreased the expression levels of proinflammatory cytokines (TNF- α , IL-1 β , IL-6) and malonyldialdehyde (MDA), and increased the activities of antioxidant enzymes SOD and GSH-Px. Furthermore, baicalin modulated the activation of Sonic hedgehog (Shh) pathway. Administration of baicalin upregulated the expression of sonic hedgehog (Shh), patched (Ptc), Smoothened (Smo), and Glioblastoma-1(Gli-1). Blockade of the Shh pathway in cyclopamine abolished the effects of baicalin *in vitro*. **Conclusion:** Both *in vivo* and *in vitro* experimental results indicate that baicalin exerts hepatoprotective roles in alcohol-induced liver injury through inhibiting oxidative stress, inflammatory response, and the regulation of the Shh pathway.

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Introduction

Alcoholic liver disease (ALD) is one of the main causes of liver diseases impairing the health of citizens and is associated with a broad spectrum of liver disorders including steatosis, steatohepatitis, cirrhosis, and hepatocarcinoma [1, 2]. Data from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) show that there were approximately 75,766 of alcohol-related deaths during 2001 in the United States [3]. Thus, it is urgently to develop novel effective therapies to prevent and treat alcohol-related diseases. Despite the pathological mechanisms underlying ALD remain largely unknown, oxidative stress and inflammation have been considered as essential intracellular events contributing to ALD. Hence, targeting these major features may provide a promising therapeutic strategy for ALD.

Baicalin, a flavonoid isolated from the *Scutellaria baicalensis Georgi*, is known to possess multiple biological effects such as anti-oxidant [4], anti-inflammatory [5], and immunoregulatory properties [6]. Recent studies have shown that baicalin can protect liver from various detrimental factors induced damages, including carbon tetrachloride-induced acute liver injury [7], iron or cadmium overload-induced hepatic cytotoxicity [8], and ischemia/reperfusion (I/R)-induced hepatocellular injury [9], suggesting a potent hepatoprotective agent of baicalin. Recently, Kim et al. [10] further showed that baicalin could attenuate I/R-induced inflammatory responses in alcoholic fatty liver condition, indicating that baicalin may exert therapeutic effects in alcoholic liver injury. Thereby, this study was conducted to investigate the potent protective effects of baicalin on ALD, as well as discussed the underlying mechanisms of its therapeutic action.

Materials and Methods

Animals and treatment

Male Wistar rats (weighing 180-220g) were housed in plastic cages with free access to food and water. Animals were kept at a temperature $22\pm 2^{\circ}\text{C}$ and a relative humidity of 50% environments. Animal experimental procedures were approved by the Experimental Animal Ethics Committee of China-Japan Friendship Institute and in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. Rats were randomly assigned into five groups ($n=6$ in each group): Control group (no alcohol intake, no baicalin treatment), ALD model I group (8 weeks of alcohol intake, no baicalin treatment), ALD model II group (4 weeks of alcohol intake, no baicalin treatment), Baicalin treatment I group (8 weeks of alcohol intake, 4 weeks of baicalin treatment), Baicalin treatment II group (4 weeks of alcohol intake, 4 weeks of baicalin treatment). Except the control group, the remaining four groups were provided with alcohol by gavage. The dose of alcohol was 65 % (vol/vol), 5 ml/kg/day in the first three days, and then 65 % (vol/vol), 10 ml/kg/day in the following days. After 4 weeks, the ALD I group was continuously supplied with alcohol for another 4 weeks; while the ALD II group stopped alcohol consumption. The baicalin I and baicalin II groups were intragastrically received baicalin at a concentration of 120mg/kg/day (baicalin purity $\geq 98\%$, Meilun Biotech, Dalian, China) for four weeks. The dosage of baicalin was based on the previous reports [10, 11] and our preliminary experiments. Animal body conditions were monitored through the experimental process. Rats were euthanized at 8 weeks, and the liver tissues and blood samples were collected for the following experiments.

Biochemical assays

Several biochemical indicators including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and triglyceride (TG) are used in the diagnosis of liver diseases [12-14]. The activities of ALT and AST, and the content of TG were detected using commercial kits (Nanjing Jiancheng Bioengineering Research Institute) according to the instructions.

Liver histology

Liver tissues were fixed in 10% formalin and embedded in paraffin. Then the paraffin-embedded tissues were cut into 5 μm thicknesses of sections. After being stained with hematoxylin and eosin (H&E),

the liver tissue sections were examined microscopically to evaluate tissue damages using an OLYMPUS DP71 microscope.

Measurement of TNF- α , IL-1 β , and IL-6

The concentration of TNF- α , IL-1 β , and IL-6 in serum and liver tissue homogenate was measured using commercial enzyme-linked immunosorbent assay kits specific for rat according to the manufacturer's instructions (Boster, Wuhan, China).

Measurement of MDA, SOD, and GSH content

Liver tissue samples were homogenized. The supernatants of homogenate were used to determinate the levels of malonyldialdehyde (MDA), glutathione (GSH-Px) and superoxide dismutase (SOD) accordingly (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western blot analysis

Liver samples were homogenized and total proteins from the homogenate were extracted using NP-40 Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China), the protein concentration of each sample was determined using bicinchoninic acid method (Beyotime). Equal amounts of protein were separated by SDS-PAGE (10% and 13% gels) and the separated proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies against Shh, Smo, Ptc, Gli1, Bcl-2, Bax, and cleaved-caspase 3 at 4 °C overnight, respectively (anti-Shh and anti-Smo, Bioss, Beijing, China, 1:500 diluted; anti-Ptc, Sangon, Shanghai, China, 1:400 diluted; anti-Gli1, USCN life science, Wuhan, China, 1:200 diluted; anti-Bcl-2 and anti-Bax, Boster, Wuhan, China, 1:400 diluted; anti-cleaved-caspase 3, Abcam, Cambridge, MA, 1:1000 diluted). After washed three times, the membranes were again incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:5000 diluted, Beyotime) at 37°C for 45 min. Protein blots on the membranes was visualized using electrochemiluminescence (ECL) reagents (7Sea Biotech, Shanghai, China), and the scanned images were analyzed with Gel-Pro-Analyzer software 4.0 (Media Cybernetics, Silver Spring, MD, USA).

TUNEL staining

Apoptotic cells in sections of liver tissues were detected using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL, Roche, Basel, Switzerland) according to the manufacturer's instructions.

Immunohistochemistry

The expressions of Shh, Smo, Ptc, and Gli1 in liver were examined by immunohistochemical staining. Briefly, slices of 5 μ m thicknesses were deparaffinized, and rehydrated, then the slices were performed for antigen retrieval using citrate buffer (pH 6.0) at 100 °C for 10 min and placed in 3% H₂O₂ for 10 min to exhaust endogenous peroxidase activity. After being blocked with goat serum for 30 min, the slices were incubated with primary antibodies (anti-Shh, anti-Smo, and anti-Ptc were from Sangon biotech, Shanghai, China, 1:50 diluted; anti-Gli1, USCN life science, Wuhan, China, 1:200 diluted) at 4°C overnight. After washed three times in PBS, the slices were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:200 diluted) at 37° C for another 30 min. At last, the slides were developed with diaminobenzidine and hematoxylin counterstaining and the immunohistochemistry changes were observed under a light microscope.

Cell culture and treatments

Human normal liver cell LO2 was obtained from Zhongqiaoxin Zhou Biotech (Shanghai, China). The cells were cultured in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Cells were maintained in a humidified atmosphere of 5 % CO₂ at 37 °C. The cells were exposed to 100mM alcohol in the absence / presence of baicalin (25, 50 μ M) or cyclopamine (20 μ M) for 24 h and then harvested. The dosage of these drugs and alcohol were chosen by our preliminary experiments.

Hoechst 33258 fluorescence staining

L02 cells were stimulated with alcohol in the absence / presence of baicalin (25, 50 μ M) or cyclopamine (20 μ M) for 24 h. The cells were fixed, then stained with Hoechst staining kits (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions. Apoptotic cells were detected under a fluorescence microscope.

Statistical analysis

All values are expressed as the mean $\pm$ standard deviation (SD), and data are processed in GraphPad Prism 5.0 software (GraphPad Software Inc, San Diego, CA, USA). Differences among groups were performed using analysis of variance (ANOVA), followed by the Bonferroni test for post hoc comparisons. A P value <0.05 was considered statistically significant.

Results

Baicalin alleviates alcohol-induced liver injury in rats

To evaluate the protective effects of baicalin on alcohol-induced liver injury in rats, we first examined the histological changes of liver tissues from five different groups. As shown in Fig. 1A, liver tissues in the control group exhibited normal lobular architecture that contained central veins and radiating hepatic cords. In contrast, live tissues of ALD groups

Fig. 1. Baicalin treatment alleviates alcohol-induced liver injury in rats. Rats were grouped: normal control (no alcohol, no treatment); ALD I model group (with alcohol 8 weeks, no treatment); Baicalin I treatment group (baicalin 120 mg/kg+alcohol 8 weeks); ALDII model group (with alcohol 4 weeks, no treatment); Baicalin II treatment group (baicalin 120 mg/kg+alcohol 4 weeks). (A) Liver histologic changes determined by hematoxylin and eosin staining. Magnification $\times 400$, scale bars = 50 μ m. The serum levels of ALT (B), AST (C), TG (D), and hepatic TG (E) were determined. Data are presented as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$ vs. ALD model group.

Fig. 2. Baicalin attenuates alcohol-induced oxidative stress and inflammatory cytokines production in liver. Effects of baicalin on hepatic MDA (A), SOD (B) and GSH-Px (C) expression levels in liver were measured. Effects of baicalin on TNF-α (D), IL-1β (E), and IL-6 (F) production were determined. Data are presented as mean ± SD. * $p < 0.05$ and ** $p < 0.01$ vs. ALD model group.

showed abnormal lobular architecture, evidenced by loss of hepatic lobule structure, partial loss of membrane, karyopyknosis, and fatty degeneration. In baicalin treatment groups, the hepatic structure was partially repaired. Furthermore, we found that baicalin promoted liver functional repair. The levels of serum AST, ALT, TG, and hepatic TG were significantly increased in the ALD model groups compared to those of the control group. Baicalin treatment notably reversed the elevation of these parameters (Fig. 1B-E). Biochemical results were in accord with the histology changes.

Baicalin attenuates alcohol-induced oxidative stress in liver

Long-term alcohol abuse leads to metabolism dysbolism [15]. Oxidative stress plays a critical role in the pathology of ALD [16]. To assess the effects of baicalin on alcohol-induced oxidative stress, we examined the content of MDA (a marker of lipid peroxidation) and the activities of GSH-Px and SOD in liver tissues. As shown in Fig. 2A, hepatic level of MDA was markedly increased in the ALD model groups. Synchronously, the activities of antioxidant enzymes SOD and GSH-Px were decreased compared with the control group (Fig. 2B-C). Administration of baicalin increased the levels of SOD and GSH-Px and reduced MDA production in liver tissues. Together, these results indicate that baicalin alleviates alcohol-induced oxidative stress in the liver.

Baicalin inhibits pro-inflammatory cytokines production and hepatocyte apoptosis in induced by alcohol

Alcohol abuse has been reported to induce hepatic inflammation [17]. We further determined the expression of inflammatory cytokines TNF-α, IL-1β, and IL-6 in liver tissues. As shown in Fig. 2D-F, significant induction of pro-inflammatory TNF-α, IL-1β, and IL-6 was observed in ALD model rats. Baicalin markedly reduced TNF-α, IL-1β, and IL-6 production. Furthermore, baicalin diminished the hepatotoxicity induced by alcohol. TUNEL staining showed that more TUNEL-positive cells were found in the ALD model groups compared to

Fig. 3. Administration of baicalin inhibits hepatocyte apoptosis in alcohol-induced rats. (A-B) The apoptotic cells in the liver were assayed by TUNEL staining. (C-F) Expression levels of cleaved caspase-3, Bax, and Bcl-2 were determined by Western blot analysis. β -actin was used as an internal control for grayscale analysis. Data are presented as mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$ vs. ALD model group.

the control group. However, number of apoptotic cells was reduced in the baicalin treatment groups (Fig. 3A-B). Western blot results also showed that baicalin treatment downregulated the expression of pro-apoptotic Bax and cleaved caspase-3 but increased the level of anti-apoptotic Bcl-2 (Fig. 3D-F). These results indicate that baicalin inhibits inflammatory response and hepatocellular apoptosis in alcoholic liver injury.

Baicalin regulates activation of the Sonic hedgehog (Shh) pathway in liver

Sonic hedgehog (Shh) pathway plays vital roles in tissue morphogenesis and liver repair [18-20]. To assess whether baicalin affected Shh pathway in alcohol-induced liver injury, we further determined the expression of Shh pathway components including Shh ligand, patched (Ptc) and Smoothened (Smo) receptors, and Glioblastoma-(Gli)-1 transcription factor. RT-PCR and Western blot results showed that the expression of Shh, Ptc, Smo, and Gli-1 were markedly increased at both mRNA and protein levels after alcoholic injury. Baicalin further promoted upregulation of these molecules (Fig. 4). Immunofluorescence assay also confirmed the increase in Shh, Ptc, Smo, and Gli-1 in the baicalin treatment groups compared to the ALD model groups (Fig. 5). Together, these results indicate that Shh signaling pathway was activated in alcohol-induced liver injury, baicalin treatment further promotes activation of the Shh pathway.

Fig. 4. Baicalin regulates the Shh signaling pathway in liver. mRNA expression of Shh (A), Ptc (B), Smo (C), and Gli1 (D) in the liver were determined by RT-PCR analysis. Protein levels of Shh, Ptc, Smo, and Gli1 (E-F) were determined by Western blot analysis. β -actin was used as an internal control for grayscale analysis. Data are presented as mean $\pm$ SD. * p < 0.05 and ** p < 0.01 vs. ALD model group.

Baicalin mitigates alcohol-induced cytotoxicity in hepatocytes through Shh signaling pathway

To further elucidate the modulatory effect of baicalin on Shh pathway, we carried out *in vitro* experiments by using the Shh antagonist cyclopamine and alcohol-stimulated LO2 cells. Fig. 6A-C showed that alcohol induced high production of AST, ALT, and TG in hepatocytes, which were dose-dependently reversed by the administration of baicalin (Fig. 6A-C). Furthermore, hoechst assay revealed that DNA fragmentation with brilliant blue staining were markedly increased in alcohol-induced LO2, baicalin reduced the number of apoptotic cells (Fig. 6D). We further detected the levels of Bcl-2 and Bax. Baicalin dose-dependently increased the expression of Bcl-2, and decreased the expression of Bax and cleaved caspase 3 (Fig. 6E-F). However, the effects of baicalin were abrogated when the combination of baicalin and cyclopamine. The alcohol-induced lesion and apoptosis were aggravated after blockade of the Shh pathway when compared with the baicalin treatment group. These results suggest that the protective role of baicalin in alcohol-induced injury might be related to the Shh signaling pathway.

Fig. 5. Immunohistochemistry used to detect the expression level of Shh, Ptc, Smo, and Gli1 in liver from five groups. Baicalin enhances the immunoreactivity of Shh, Ptc, Smo, and Gli-1 in liver. Magnification $\times 400$, scale bars = $50\mu\text{m}$.

Discussion

Alcohol intake is one of the most common causes of liver disease, leading to steatosis, cirrhosis, and even liver cancer. Although the mechanisms remain inconclusive, several molecular events like oxidative stress and inflammatory response have been implicated in the pathology of ALD. Thus, inhibition of these features might offer a promising therapeutic strategy for ALD. Here we demonstrate that baicalin, a polyphenolic compound isolated from *Scutellaria baicalensis* Georgi, ameliorates alcoholic liver injury in rats. Furthermore, we also show the direct evidence that the hepatoprotection of baicalin is probably due to the inhibition of oxidative stress and inflammatory mediators, and these effects of baicalin may attribute to the Shh signaling pathway.

Baicalin possesses a number of pharmacological activities such as anti-inflammatory, anti-oxidant, and anti-tumor properties, the therapeutic potential of baicalin has been extensively studied [21-23]. Previous studies also demonstrated that baicalin exerted hepatoprotective effect against several types of liver diseases, such as carbon tetrachloride-induced acute hepatic injury [7], cadmium induced injury [8], and ischemia/reperfusion induced liver injury [9]. To investigate whether baicalin has therapeutic efficacy in alcoholic liver injury, we first established the liver injured animal model via alcohol feeding. After continuous 4 weeks of ingestion, rats received intragastric administration of baicalin for an additional 4 weeks in the presence or absence of alcohol intake. Liver function assay showed that baicalin significantly improved the impaired liver function, as indicated by the decrease in serum ALT, AST, TG, and hepatic TG after baicalin treatment. Furthermore, baicalin alleviated pathological changes of liver caused by alcohol, suggesting a hepatoprotective action of baicalin in alcohol-induced rats.

A growing body of literature has shown that oxidative stress plays a critical role in the pathogenesis of ALD [24]. Alcohol consumption has been reported to induce free radical intermediates production like 1-hydroxyethyl radicals and ROS, and decrease the content of antioxidants. The imbalance leads to oxidative stress and tissue damage [24, 25]. Lipid peroxidation is a pathological event initiated by toxic oxygen radicals. MDA, the

Fig. 6. Baicalin alleviated alcohol-induced injury in hepatocytes. LO2 cells were exposed to alcohol (100mM) in the absence or presence of baicalin (25μM, 50 μM, or 50μM plus cyclopamine) for 24 h. The levels of (A) ALT, (B) AST in culture medium, and (C) TG in hepatocytes were determined by commercial kits. (D) Apoptotic cells were detected by Hoechst 33258 fluorescence staining. A bright blue fluorescence was shown in the nucleus of apoptotic LO2. Magnification ×400, scale bars = 50μm. * $p < 0.05$ and ** $p < 0.01$ vs. alcohol group; & represents $p < 0.01$ vs. baicalin 50μM group; # represents $p < 0.01$ vs. control group.

main metabolite of lipid peroxidation, has been used as an indicator of ROS-induced lipid peroxidation [26]. The antioxidant effect of baicalin has been largely studied, an earlier study also reported that baicalin significantly inhibited oxidative stress in iron overload-induced liver injury [27]. Wen et al. also demonstrated that baicalin exerted antioxidant property in cadmium induced hepatic cytotoxicity [8]. Here we detected the effect of baicalin on alcohol-induced oxidative stress by measuring MDA, GSH-Px, and SOD. Our results showed that alcohol induced significant increase in MDA expression and a decrease in antioxidant SOD and GSH-Px. Baicalin treatment effectively reversed these changes. These results indicate that the hepatoprotective function of baicalin is likely attribute to its antioxidant activity.

Inflammation is another crucial event associated with ALD progression. Alcohol abuse increases gut permeability and leading to endotoxin translocation into the circulation, which induces the production of proinflammatory cytokines such as TNF- α [28, 29]. These inflammatory mediators further result in hepatocellular apoptosis or necrosis[30]. In the present study, we found that alcohol intake results in hepatic inflammation, as implicated by inflammatory cells infiltration and increase in pro-inflammatory cytokine TNF- α , IL-1 β , and IL-6 expression in liver tissues. Administration of baicalin attenuated the alcoholic inflammation. Moreover, baicalin inhibited hepatocyte apoptosis and enhanced the expression of anti-apoptotic Bcl-2. Our results here were in agreement with the previous findings [22, 31].

The Sonic Hedgehog (Shh) signaling is an important morphogenic signaling regulating embryonic development and tissue differentiation [19, 32]. Components of Shh pathway consist of Shh ligand, Ptc and Smo receptors, and Gli transcription factors [33]. When Hh ligand interacts with Ptc receptor, inhibition of Ptch to Smo protein is removed, which can transmit downstream signal and trigger the activation of Gli family transcription factors [34]. Recently, increasing evidence demonstrates the importance of the Shh pathway in liver injury and tissue repair [20, 35]. Activation of Hh signaling promotes the proliferation and differentiation of hepatic progenitor population [36, 37]. Furthermore, the Shh signal has been shown to affect inflammatory cytokines and oxidative stress related indexes [38, 39]. Zhou et al. reported that the Shh signal attenuated inflammation in acute pancreatitis via upregulation of IL-10 [40]. Huang et al. showed the anti-oxidative and anti-apoptotic role of Shh signal in focal cerebral ischemia model [41]. Consistent with these findings, here we found that alcohol intake activated the Shh pathway, as observed by increased expression of Shh, Smo and Ptc receptor, and transcription factor Gli1 in the liver. Baicalin treatment further enhanced the elevation of Shh components. To illuminate the modulatory action of baicalin on Shh pathway in alcoholic liver injury, we performed *in vitro* studies, alcohol-stimulated LO2 cells were used as an *in vitro* model. As expected, baicalin mitigated ethanol-induced lesion and apoptosis of hepatocytes. However, these effects of baicalin were abolished by cyclopamine, a specific inhibitor of Shh signaling through direct interaction with SMO. Together, both *in vivo* and *in vitro* experimental results indicate that baicalin may help regeneration of hepatocytes and liver remodeling after alcoholic liver injury via Shh pathway. However, how baicalin regulates Shh signaling and is the effect of baicalin on Shh signaling involved in other molecules or signals remains to be illustrated.

In summary, our results indicate that baicalin can alleviate alcohol-induced hepatic injury in rats, the hepatoprotective action of baicalin may be due to its anti-oxidant, anti-inflammatory properties, and shh signaling activation. Therefore, baicalin may be a novel therapeutic strategy ALD protection.

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Disclosure Statement

The author declares no conflict of interest.

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