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The role of baicalin on carbon tetrachloride induced liver fibrosis

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Key words: Liver Fibrosis, Baicalin, Cell Apoptosis

Abstract: The effect of the baicalin, a bio-active flavonoid extracted from *Scutellaria baicalensis* Georgi, on the carbon tetrachloride (CCl_4) induced liver fibrosis was investigated. To compare the effect of baicalin on the liver fibrosis, five different groups of rats treated by 100, 200, and 400 mg/kg baicalin were studied. Upon CCl_4 treatment, the levels of procollagen type III, aspartate aminotransferase, aminotransferase, hyaluronic acid, and hydroxyproline were significantly increased, whereas the superoxide dismutase and glutathione peroxidase content were decreased. These changes in the biochemical parameters, which are associated with liver function, were significantly attenuated by the baicalin treatment, suggesting that baicalin can suppress the liver fibrosis induced by CCl_4. Moreover, the histological staining analysis demonstrated that baicalin could effectively inhibit the degree of liver cell injury. The protein expression of AKT/JAK2/ERK in the serum were markedly increased by CCl_4 but suppressed by the treatment of baicalin in a dose-dependent manner, implying that baicalin can attenuated cell apoptosis induced by CCl_4. Overall, these results suggest that baicalin effectively protects hepatocytes from the CCl_4 oxidative damage, likely due to the inhibition of free radical generation and cell apoptosis during the liver injury.

Introduction

Liver disease is one of the major global challenges. According to the Office for National Statistics in the United Kingdom, liver disease is the fifth most common cause (Toledano *et al.*, 2019). Liver fibrosis is a common chronic liver disease, and it is considered as a critical issue due to its high rate of morbidity and mortality (Huang *et al.*, 2014; Sun *et al.*, 2007). Hepatitis viral infection is the most common cause. Drug abuse, autoimmune disorders, metabolic disorders, and biliary obstruction due to mineral overload are attributed to the liver fibrosis (Friedman, 2003; Kisseleva and Brenner, 2006). Many environmental toxins can also cause liver injury and fibrosis, which is often characterized as the accumulation of extracellular matrix protein and collagen in the perisinusoidal space (Dai *et al.*, 2009; Sun *et al.*, 2012). Moreover, it was found that serious liver fibrosis leads to cirrhosis and liver failure eventually (Are *et al.*, 2020; Zimmermann *et al.*, 2019). However, treatment of liver diseases still remains as a therapeutic challenge. Therefore, there is a need to develop effective therapeutic strategies that will decrease the extent of liver fibrosis or enhance the liver regeneration. Despite technology advances in medicine, only few medical treatment approaches are available for the liver fibrosis (Sun *et al.*, 2012). Meanwhile, modern medicine lacks reliable and effective liver protective activities. Therefore, there has been considerable interest in the development of alternative and complementary medicine for the treatment of liver diseases.

Although the pathogenesis of liver fibrosis is quite complicate, the understanding of biochemical and cellular factors associated with liver fibrosis is gradually increasing. Several reports have suggested that oxidative stress can play a key role in the liver fibrosis (Cederbaum *et al.*, 2009; Poli, 2000). For instance, free radicals, as found during the oxidative process in cells, can attack unsaturated fatty acids of cell membranes, resulting in cell peroxidation, destructing DNA and protein (Sastre *et al.*, 2007). These processes eventually cause various liver injuries. It has been shown that traditional herb medicine, which contains antioxidants, can be an effective approach to prevent liver fibrogenesis (Gebhardt, 2002; Lin *et al.*, 2018).

For example, the radix of *Scutellaria baicalensis* is an eastern traditional medicine, and it has been used as a relaxant for smooth muscle and an anti-inflammatory reagent (Huang *et al.*, 2018; Liu *et al.*, 2019). It has also been used as an ingredient of herb mixture for the hepatoprotective agent (Dong *et al.*, 2020; Gao *et al.*, 1995; Huang *et al.*, 2012; Liu *et al.*, 2015a; Liu *et al.*, 2015b; Wu *et al.*, 2018; Yin *et al.*, 2018; Yu *et al.*, 2020; Zhou *et al.*, 2018). Baicalin is the major active component of the isolated root of *Scutellaria baicalensis*, and it acts as a free radical scavenger for reactive oxygen species and has an

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anti-inflammatory activity (Gao *et al.*, 1995; Zhou *et al.*, 2018). It was found that the baicalin can inhibit the H_2O_2-induced liver injury caused by suppression of oxidation in the cell (Yu *et al.*, 2020). It can also inhibit the activation of redox sensitive nuclear factor-κB (NF-κB) in kidney from the old rats (Liu *et al.*, 2015a; Yin *et al.*, 2018). However, information about the hepatoprotective effect of baicalin is very limited.

Carbon tetrachloride (CCl_4) is commonly employed as an inducer of the liver injury in small animals (Huang *et al.*, 2014; Yu *et al.*, 2020). It is known that CCl_4 is hepatotoxic as well as nephrotoxic to humans (Kodavanti *et al.*, 1989; Ritesh *et al.*, 2015). It has been reported that hepatic necrosis induced by CCl_4 involves the activation by a microsomal cytochrome P450-depentent (Sun *et al.*, 2007) monooxygenase system, resulting in the generation of ROS and trichloromethyl free radicals. It eventually leads to damage of cell membrane (Mccay *et al.*, 1984), following by the release of inflammatory mediators from the activated macrophages. They are believed to be the cause of the CCl_4-induced hepatic injury. In this work, we investigated the role of baicalin in CCl_4 induced liver injury and how it can protect the liver injury. The possible molecular mechanism and the effect of inhibition of the oxidative stress and inflammation will be discussed.

Materials and Methods

Materials

Carbon tetrachloride (CCl_4) was purchased from Aladdin (part no.: C112043, China). Commercial kits for SOD, GSH-Px, hydroxyproli, MDA, AST/GOT, and ALT/GPT were purchased from Jiancheng Co. (Nanjing, China). 85% Baicalin was purchased from Fusion Biology Co. (Part No. 21967-41-9, China).

Experiments were carried out on 17 ICR male rats (Jestier, China) of 18–22 g body weight. They were housed at 22–24°C and exposed to alternate cycles of 12 h light and dark. They were given free access to a standard pellet diet and tap water. The animal study was approved by the Laboratory Animal's Ethic Committee of the Hospital. It was carried out throughout the experiment following the international laboratory animal use and care guideline.

Experimental model and treatment

We first prepared a 2% CCl_4 solution by mixing CCl_4 and corn oil thoroughly mixed for 2 min. Then the CCl_4-corn oil solution was administered subcutaneously at a dose of 0.1 mL/kg of body weight, twice per week and continued for 6 weeks, in order to induce the liver fibrosis.

The animals were randomly divided into 5 groups: (1) Control group (normal rats), in which 3 rats were injected subcutaneously with corn oil with no CCl_4. (2) CCl_4 control group, in which 5 rats were administered 2% CCl_4 without adding baicalin. (3) CCl_4 + low dose baicalin group, in which 3 rats were administered with 2% CCl_4 and baicalin (100 mg/kg). (4) CCl_4 + intermediate dose baicalin group, in which 3 rats were administered with 2% CCl_4 and baicalin mixture (200 mg/kg) (5) CCl_4 + high dose baicalin group, in which 3 rats were administered with 2% CCl_4 and baicalin (400 mg/kg).

At the end of six weeks, animals were scarified. The blood samples were obtained from orbital veins. The serum samples were collected by centrifugation in a Sorvall RC centrifuge (Thermo Scientific, Germany) of the whole blood at 4000 rpm for 10 min. The samples were stored frozen at −80°C until further use. The livers were immediately removed and were fixed in 10% formalin for histological analysis.

Liver index, kidney index, and spleen index

Relative weights of liver, kidney and spleen were represented as the percentage of the total body weight in gram.

Biochemical metabolic parameters

The activities of ALT and AST were measured by spectrophotometry using commercial kits, according to the manufacturer's instructions. The levels of LN, HA, PC III were obtained by radio-immunoassay kits, according to the manufacturer's instructions. TGF-beta1 was determined by the rat ELISA kit.

Evaluation of oxidative stress and antioxidant status

The tissue homogenates of livers were used to determine SOD, MDS, and GDH-PX levels, according to the manufacturer's instructions.

Evaluation of collage

Liver collage concentration was determined by measuring the level of hydroxyproline, according to the method published previously (Sun *et al.*, 2007).

Histological observation

Livers were isolated from rats and the tissues were fixed using 10% formalin for 24 h. The fixed tissues were then dehydrated and immersed in paraffin solution, followed by cutting into small sections for staining. The hematoxylin-eosin staining was used to observe liver injury. The tissues were stained with Masson staining reagents to detect the deposition of collagen and stained with Sirius red staining reagents to measure the type and content of collagen. The pathological observation was evaluated by a Nikon microscope with a digital camera and polarization microscope.

Western immunoblotting

Western immunoblotting analysis of liver tissue was undertaken using the following monoclonal antibodies: β-actin was purchased from Zsbio (Beijing, China). α-SMA was purchased from Abcam Inc. (Shanghai, China). JAK2, phosphor-JAK2 (or p-JAK2), phosphor-ErK (or p-ErK), Erk, Akt, phosphor-Akt (or p-Akt) and cleaved Caspase 3 were purchased from CST (Beverly, MA). Rock1, P-53, Bcl-2, and Bax were purchased from Santa Cruz biotechnology (Santa Cruz, CA). These antibodies were used to identify proteins expressed. The extracted proteins were subjected to electrophoresis on a 10% SDS-PAGE after normalization for protein content. After resolution, the protein samples were electrotransferred onto PVDF.

The membrane was blocked for 1 h in 0.1% nonfat dry milk in PBS. Membranes were incubated overnight at room temperature with the primary antibody in TBS. Membranes were washed twice for 5 min in PBS before the addition of the secondary antibody in TBS containing 0.1% nonfat dry

milk for 1 h. The membranes were then washed in TBS for 5 min followed by water for 5 min. Reactive bands were identified using enhanced chemiluminescence and autoradiography according to the manufacturer's instructions.

Statistical analysis

Quantitative data was demonstrated as the mean ± standard deviation (SD). The significance of the difference *vs.* the CCl_4 group was analyzed by a Student's *t*-test. A Mann–Whitney rank sum test was used to measure the degree of histopathological liver fibrosis. The *P*-value less than 5% ($P < 0.05$) was considered to be statistically significant.

Results

The effect of baicalin on body weight, liver, spleen, and kidney index

The roles of baicalin on the rats' body weight, and the index of liver, spleen and kidney were evaluated, as shown in Fig. 1. The liver, kidney and spleen index were measured as the percentage of the total body weight. The results in Figs. 1B and 1C show that the body weight, the liver, kidney, and spleen index do not exhibit any statistically difference between normal and liver-fibrosis rats. The baicalin treatment does not have a significant impact on these factors.

The effects of baicalin on liver function

The effect of baicalin on the liver function was evaluated by measuring the therapeutic serum regimens, hyaluronic acid (HA), Procollagen type III (HPCIII), Aspartate aminotransferase (ALT), and Alanine aminotransferase (AST), on the hepatic fibrosis in CCl_4 rat model (Hu *et al.*, 2010; Ozer *et al.*, 2010). The results are summarized in Fig. 2. The significantly higher levels of HA, HPCIII, AST and ALT in the rats' serum in the CCl_4 group were observed than that of the control group. However, HA and HPCIII levels (Figs. 2A and 2B) were effectively reduced at a higher dose of baicalin (above 100 mg/kg). For example, by treating with 400 mg/kg baicalin, the HA and HPCII levels were reduced significantly by approximately 80% and 40%, respectively. Additionally, baicalin also significantly suppressed CCl_4-induced increase in AST level at relatively lower baicalin dose (Fig. 2D), while ALT level does not show a notably change upon treating baicalin (Fig. 2C). This result indicated that baicalin has a protective effect for the CCl_4-induced liver injuries.

The baicalin effect of hepatic activities

Next, we investigated the effect of baicalin on the hepatic anti-fibrotic and anti-oxygenation capability by evaluating the hydroxyproline (HyP), malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) in liver. HyP is an amino acid, and it exists almost exclusively in collagens (Alcock *et al.*, 2019; Katayama *et al.*, 1997; Kim *et al.*, 2009). Measurement of HyP in fibrotic tissues has been used as a reliable method to study fibrosis and to investigate the potentially anti-fibrotic agent (Kim *et al.*, 2009). As shown in Fig. 3A, the

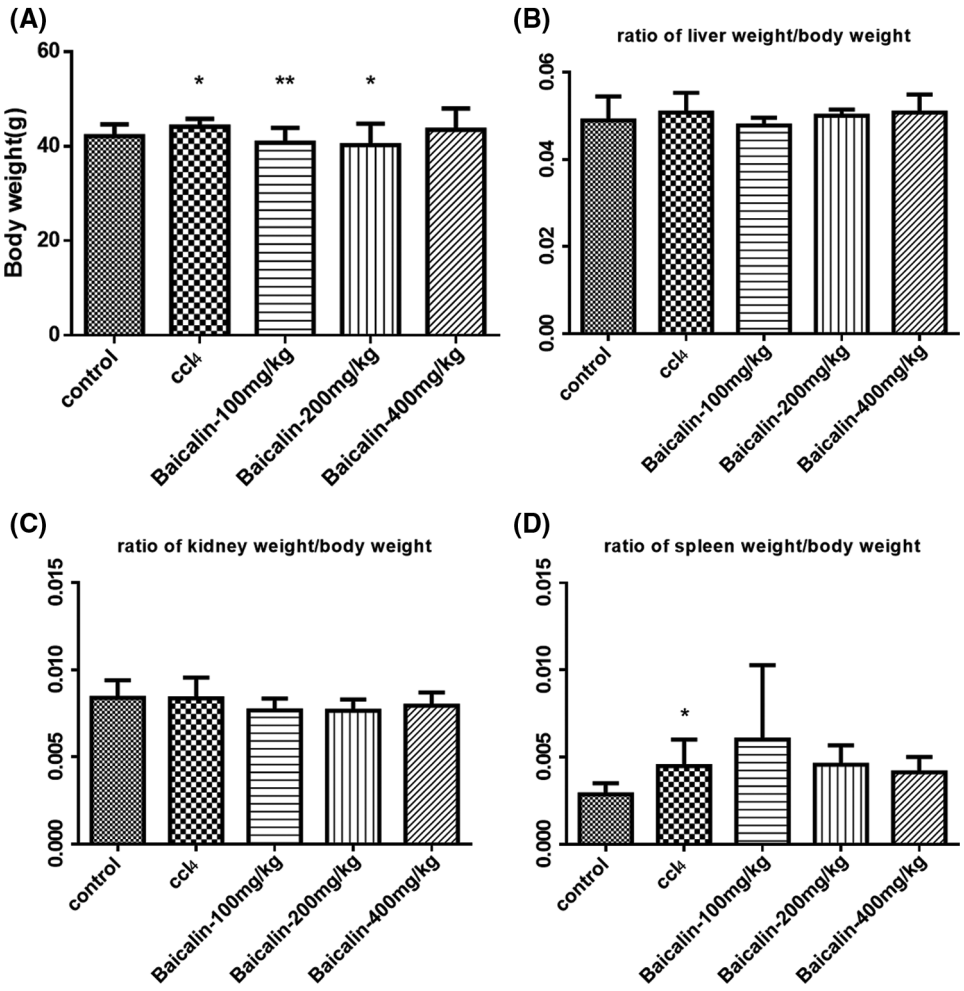

FIGURE 1. Body weight (A), liver (B), kidney (C) and spleen (D) index in the different groups.
Data were presented as mean ± SD. * and **represent the significant difference from the control group with $P < 0.5$, and $P < 0.01$, respectively.

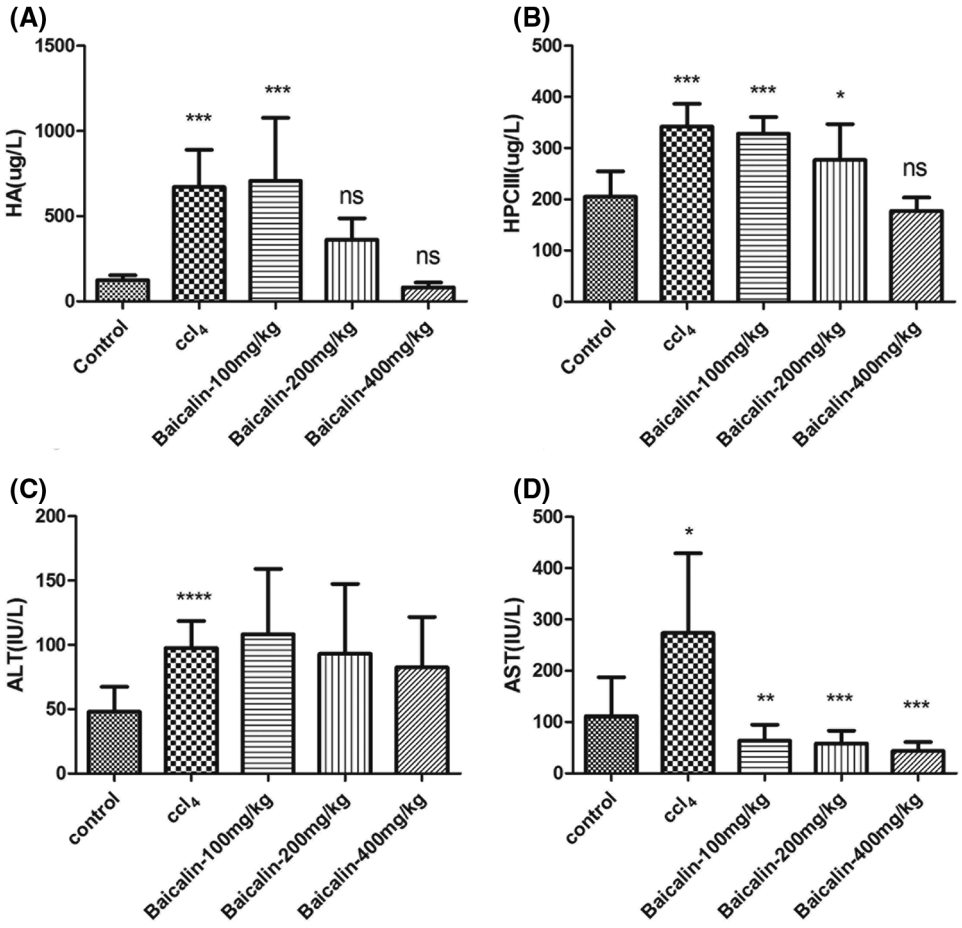

FIGURE 2. Hyaluronic acid (HA, A), Procollagen type III (HPCIII, B), Aspartate aminotransferase (ALT, C) and Alanine aminotransferase (AST, D) in different experimental groups. Data were expressed as mean ± SD. *, **, ***, and **** represent the significant difference from the control group with $P < 0.5$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

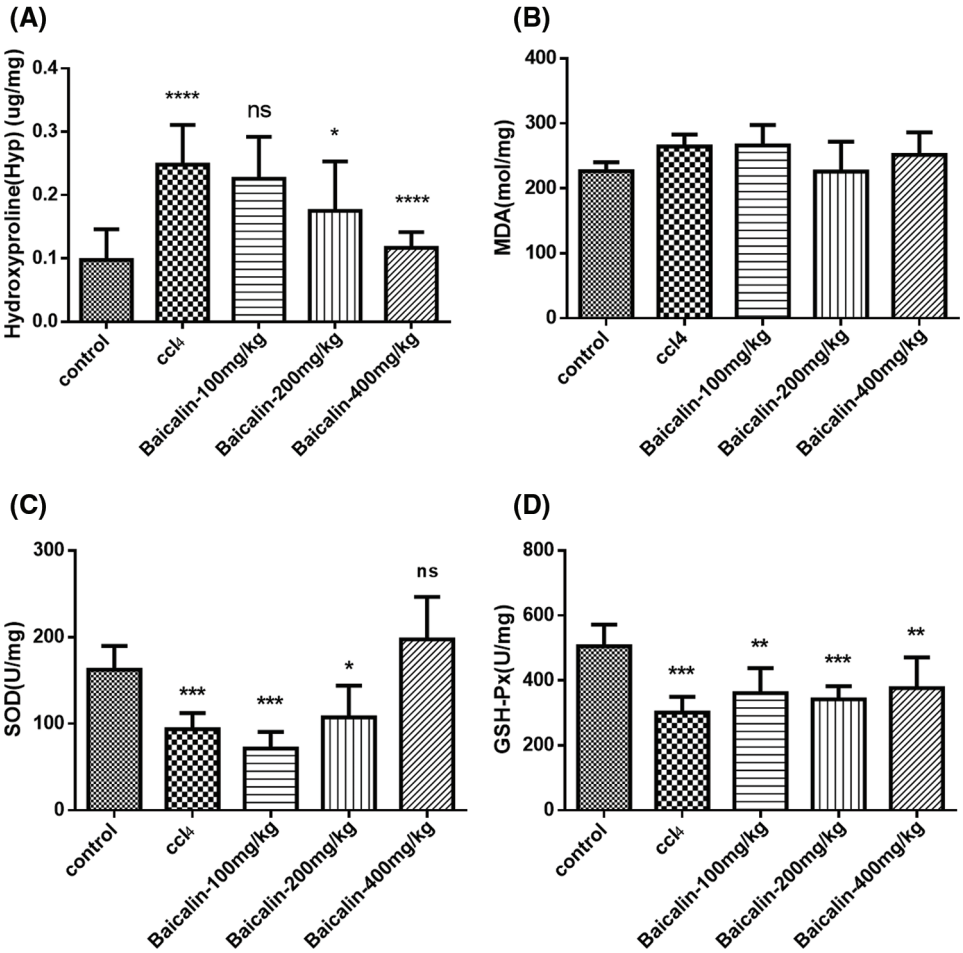

FIGURE 3. Hydroxyproline (HyP, A), malondialdehyde (MDA, B), superoxide dismutase (SOD, C), and glutathione peroxidase (GSH-Px, D) in experimental groups.
*, **, ***, and **** represent the significant difference from the control group with $P < 0.5$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively.

content of HyP in the livers after treating CCl_4 were significantly higher than those in the normal livers, suggesting the liver fibrosis has been formed in the CCl_4 treated rats. After treating different doses of baicalin, the hepatic HyP levels were markedly decreased. This finding suggests that baicalin is an effective anti-fibrotic agent in CCl_4 injury rats.

Moreover, MDA, SOD, and GSH-Px are commonly used as biomarkers to evaluate the oxidative stress and the antioxidant status in liver injuries (Katayama *et al.*, 1997; Romani *et al.*, 1988; Sampey *et al.*, 2003). As can be seen in Figs. 3C and 3D, GSH-Px and SOD levels are reduced in CCl_4-groups, suggesting that the end-product of lipid peroxidation due to the oxygenation process is higher in CCl_4 group than in the control group. As increasing the dose of baicalin treatment (Figs. 3C and 3D), the SOD and GSH-Px levels effectively increase. In contract, MDA level does not display a statistical difference among the group studied (Fig. 3B).

The effect of protein expression level in the liver tissue

Liver fibrosis is often investigated by the accumulation of proteins in extracellular matrix, which occurs in the most types of chronic liver diseases (Chen *et al.*, 2015; Li *et al.*, 2019). To determine whether the protective effect of baicalin on CCl_4-induced liver injury is mediated by cell apoptosis, the protein levels of AKT, phosphorylate (p-) AKT, ERK, and phosphorylate (p-) ERK in cell were detected. The results indicated that CCl_4 injection enhanced the levels of AKT, p-AKT, p-JAK2, ERK, p-ERK and ROCK1, compared with the levels in control group (Figs. 4 and 5). However, as shown in Fig. 5, treating with a small dose of baicalin does not significantly affect the p-JAK2 and ERK level in CCl_4-injuried rats. These levels were only suppressed significantly

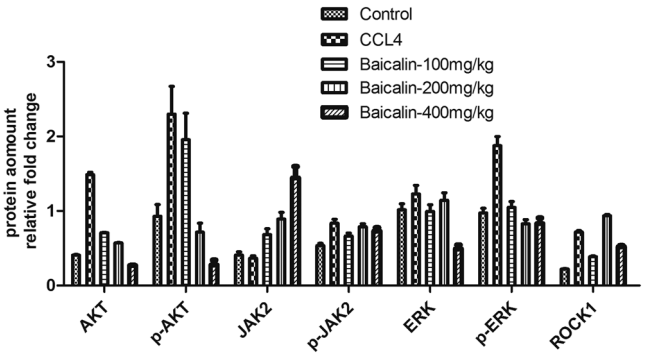

FIGURE 5. The effect of baicalin on AKT/ JAK2/ ERK and ROCK protein expression in the rats' livers.

at higher doses of baicalin. On the other hand, a small dose of baicalin (100 mg/kg) can significantly suppress AKT, P-AKT, and ROCK1 level in the CCl_4-injuried rats. It has been shown that high responsiveness of cell apoptosis is related to the increased expression of AKT and ROCK1 levels in liver cells (Liu *et al.*, 2015b). Our findings indicate that baicalin treatment may suppress the AKT and ROCK1 signaling pathway in CCl_4 induced liver injury, suggesting the potential capability of baicalin to suppress cell apoptosis.

B-cell lymphoma-2 (Bcl-2) is considered to be an anti-apoptotic protein, and Bcl-2 associated X protein (Bax), mammalian target of rapamycin (mTOR), p53 and caspase-3 are known to be the biomarker of apoptosis (Cao *et al.*, 2013; Guo *et al.*, 2002). To better understand the role of baicalin on the cell apoptosis in liver injury, the western blot analysis was performed to detect the expression of apoptosis markers at mRNA and protein levels. As indicated in Figs. 6 and 7, the relative expression of Bax, Bcl-2, mTOR, p53, and

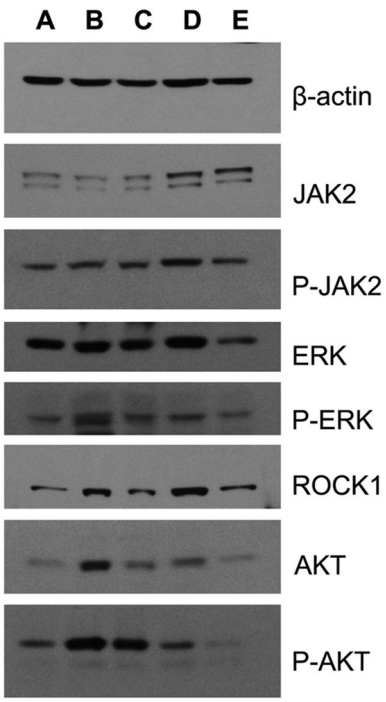

FIGURE 4. The effect of baicalin on β-actin, JAK2, p-ERK, ROCK1, AKT, and P-AKT in the rats' livers.
(A) Control group, (B) CCl_4 control group, (C) baicalin (100 mg/kg), (D) baicalin (200 mg/kg), (E) baicalin (400 mg/kg).

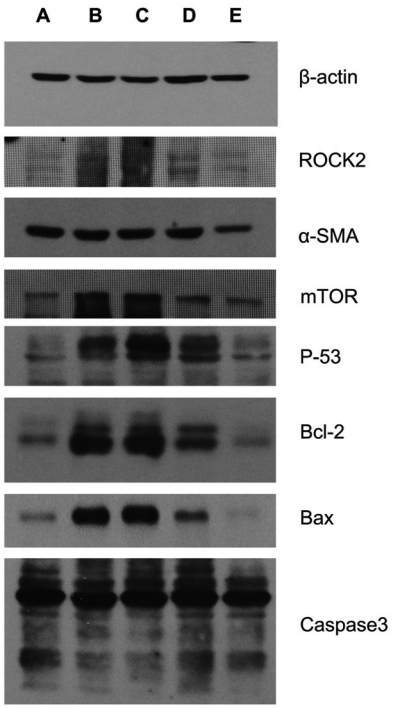

FIGURE 6. The effect of baicalin on β-actin, ROCK2, α-SMA, mTOR, p-53, Bcl-2, Bax in the rats' livers mediated by cell apoptosis.
(A) Control group, (B) CCl_4 control group, (C) baicalin (100 mg/kg), (D) baicalin (200 mg/kg), (E) baicalin (400 mg/kg).

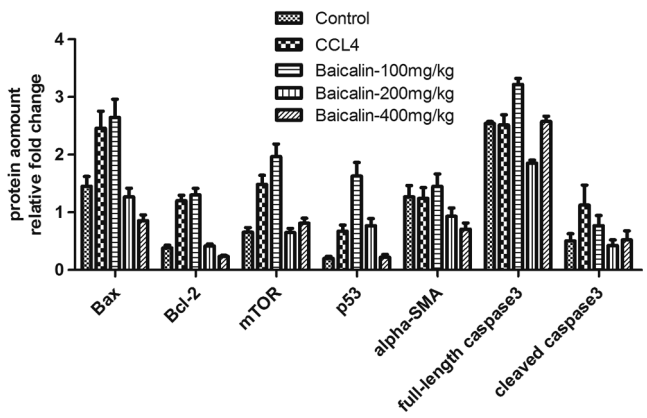

FIGURE 7. The effect of baicalin on the cell apoptosis in the rats' livers.

cleaved caspase-3 was significantly enhanced after CCl_4-treatment, compared with the control. However, this enhancement was markedly attenuated by treatment of baicalin. Several studies have shown that high level of Bax, Bcl-2, mTOR, and p53 associates with cell apoptosis (Gao *et al.*, 2002, 2003; Liu *et al.*, 2019). Thus, these results suggest that baicalin may attenuate hepatocyte apoptosis in liver injury in the dose dependence matter.

Histological staining analysis

Histological characteristics of the liver in the control group showed a normal lobular architecture and structure (Fig. 8A). However, after treating with CCl_4, the extensive hepatocellular damages were observed, as evidenced by the presence of hepatocellular degeneration and infiltration inflammatory cells (Fig. 8B). The treatment of baicalin in various dosages can attenuate the degree of injury changes (Figs. 8C–8E). These results confirmed that baicalin has the anti-fibrotic effects in CCl_4–liver injury model, and the effect is dose dependent.

The effect of baicalin on the changes of liver cells was presented in Fig. 9. The VG staining results indicated that CCl_4 treated liver showed extensive changes in microstructures, including marked enlarged domains of portal inflammation, hepatic necrosis, and fibrotic septa (Fig. 9B). No abnormalities were observed in the control group (Fig. 9A). Baicalin treatment could significantly alleviate the degree of liver injury in the cellular level, and their cell morphologies are close to that of normal cells.

Discussion

Herein, we evaluate the role of baicalin on liver fibrosis induced by CCl_4 in rats. The treatment of CCl_4 in rats has been commonly used as an ideal model system to study liver fibrosis (Kodavanti *et al.*, 1989; Ritesh *et al.*, 2015). In this work, liver fibrosis was induced successfully by subcutaneous 2% CCl_4 injection for 6 weeks. The histological data has showed that the microstructure of liver lobules was destroyed and pseudolobules generated. In addition, the high hydroxyproline level in liver, as well as high HA, HPCIII, and AST in serum consistently confirmed the formation of liver fibrosis.

We showed that baicalin treatment appeared to be beneficial in this model of CCl_4 injury by means of improving liver function, reducing liver index, and inhibiting the process of developed hepatic fibrosis. This protective function could associate with the increase of SOD and GSH-Px activities and the reduction of MDS level, thus suggesting antioxidant activity may be the possible mechanism of baicalin in liver fibrosis. Additionally, baicalin can suppress several biomarkers associated with cell apoptosis in CCl_4 induced liver cells. It indicates that baicalin can alleviate liver cell injury by suppressing the cell apoptosis process.

The increased content of aminotransferase (AST) could be due to the leakage from the damaged hepatic cells and has been used as a marker for liver injury (Hu *et al.*, 2010; Ozer *et al.*, 2010). It was shown in Fig. 2D that the abnormally high AST level was noted in rats treated with CCl_4. AST level is reduced by administration of baicalin. This finding provided strong evidence that the baicalin can improve the hepatic damages in rats without significant hepatotoxicity. The role of baicalin was further evaluated by the improvement in the histopathological analysis.

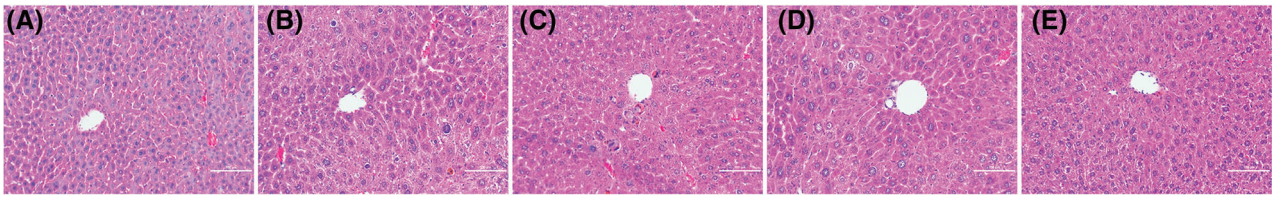

FIGURE 8. The representative hematoxylin-eosin staining (HE) histological photographs of (A) control group, (B) CCl_4 control group, (C) CCl_4 and low-dose baicalin group (100 mg/kg), (D) CCl_4 and intermediate-dose baicalin group (200 mg/kg), (E) CCl_4 and high-dose baicalin group (400 mg/kg).

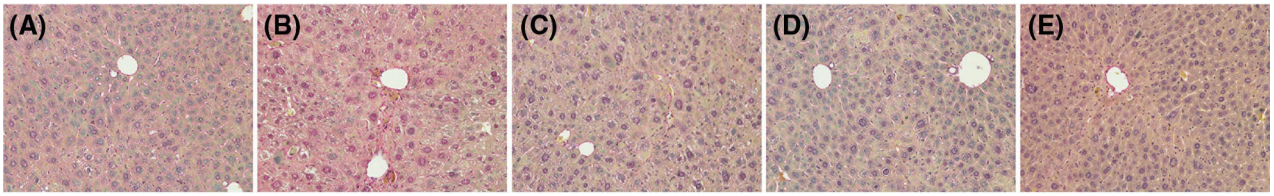

FIGURE 9. Representative Van Gieson's (VG) Stain photographs *x*200 of (A) control group, (B) CCl_4 control group, (C) CCl_4 and low-dose baicalin group (100 mg/kg), (D) CCl_4 and intermediate-dose baicalin group (200 mg/kg), (E) CCl_4 and high-dose baicalin group (400 mg/kg).

HPCIII and HA levels in serum are key markers of hepatic fibrogenesis. Hepatic HA level reflects the total amount of collagen, and it has been used to evaluate the extent of fibrosis (Alcock *et al.*, 2019; Kim *et al.*, 2009). Therefore, HPCIII and HA are important indexes to evaluate liver fibrosis. The result shown in Fig. 2 demonstrated that baicalin could significantly reduce HA and PCIII in serum, which suggested that baicalin could suppress deposition and accumulation of collagen in the liver.

Conclusion

The present study showed that baicalin from traditional medicine herb, *Scutellaria baicalensis*, has a rich bioactive profile and has preventive functions to liver fibrosis. The possible mechanism of the effects might be related to its antioxidant activities and suppression of cell apoptosis, decreasing the level of Hyp, SOD, GSH-Px, AKT/JAK2/ERK protein and inhibition of collagen synthesis. Baicalin could be a potential agent for liver fibrosis treatment, which targeting the apoptosis of hepatocytes and preventing cell oxidation. This research can provide alternative options for the development of pharmaceutical therapeutic compounds in medical practices.

Availability of Data and Materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contribution: The authors confirm contribution to the paper as follows: study conception and design: Meng-Ting Li; data collection: Kaifeng Zheng; analysis and interpretation of results: Yi-er Qiu; draft manuscript preparation: Mengting Li. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval: The animal study was approved by the Institutional Animal Care and Use Committee of Shanghai Rat & Mouse Biotech Co., Ltd. (SHRM) (Approval No. SHRM-IACUC-045). It was carried out throughout the experiment following the international laboratory animal use and care guideline.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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