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Reversal of chemical-induced liver fibrosis in Wistar rats by puerarin

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Abstract

Puerarin is a major isoflavonoid compound isolated from *Pueraria lobata*, an edible vine used widely for various medicinal purposes. It has been used for centuries in China to counteract alcohol intoxication. However, the effects of puerarin on chemical-induced liver fibrosis have not been reported. In the present study, we investigated the effects of puerarin on liver fibrosis in Wistar rats induced by alcohol plus carbon tetrachloride administration. Liver fibrosis was produced in rats by treatment with a mixture (50% alcohol, 8 g/kg per day; corn oil, 2 g/kg per day; pyrazole, 24 mg/kg per day; ig) once a day and by intraperitoneal injection of 0.25 ml/kg of a 25% solution of carbon tetrachloride in olive oil twice a week for 8 weeks. After 8 weeks, treatment with puerarin (0.4 and 0.8 g/kg ig, daily for 4 weeks) was conducted to examine its therapeutic effects. At the same time, the model group and treatment group continued to receive the chemical mixture, while the control group received saline instead of the chemical mixture. Upon pathological examination, the puerarin-treated rats significantly reversed the symptoms of liver fibrosis and other hepatic lesions. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as indexes of hepatic cell disruption, were reduced with puerarin treatment, whereas no significant effect was discovered in the levels of alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) activities. A significant increase in apoptosis of activated hepatic stellate cell (HSC) was found by flow cytometric analysis of the hepatic tissues. And the expression of bcl-2 mRNA was down-regulated after puerarin administration. Consequently, all these results showed that puerarin could effectively reverse chemical-induced liver fibrosis in experimental rats, via the recovery of hepatic injury as well as the induction of apoptosis in activated HSC.

Keywords: Liver fibrosis; Puerarin; Alcohol; Carbon tetrachloride; Hepatic stellate cell; Apoptosis

1. Introduction

Chronic injury leading to fibrosis in liver occurs in response to a variety of insults, including viral hepatitis (especially hepatitis B and C), alcohol abuse, drugs, metabolic diseases due to overload of iron or copper, autoimmune attack of hepatocytes or bile duct epithelium, or congenital abnormalities. Typically, injury is present for months to years before significant scar accumulates, although the time course may be accelerated in congenital liver disease. Liver fibrosis is reversible. However, cirrhosis, the end-stage consequence of fibrosis, is generally irreversible [1]. Thus, efforts to understand fibrosis and discover medications for treatment of fibrosis seem to be of great urgency.

Puerarin (4′,7-dihydroxy-8-β-D-glucosylisoflavone) is a C-glycoside compound (Fig. 1). It is present in large amount in the active components of *Pueraria lobata*, a commonly used Chinese herbal medicine. *Pueraria lobata* exerts sedative and antipyretic actions and is often used to treat influenza, wrist stiffness and headache. A number of investigations were carried out internationally to identify the physiological activities of puerarin. Puerarin has numerous biological roles, such as antihyperglycemic [2], antioxidant [3,4], hepatoprotective [5] and estrogenic effects [6]. Puerarin can also treat glutamate-induced apoptosis by suppressing the leakage of lactate dehydrogenase (LDH) in cultured mouse cerebral cortical neurons [7], inhibit alcohol dehydrogenase [8] and xanthine oxidase [9], as well as improve blood circulation and prevent cardiovascular diseases [10,11]. However, the information about whether

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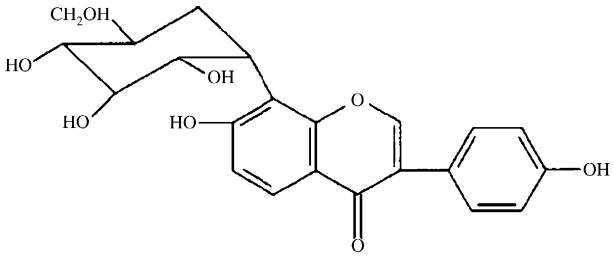

Fig. 1. Chemical structure of puerarin.

puerarin is effective in the establishment of liver fibrosis remains limited.

Therefore, in the present study, we designed to evaluate the therapeutic effects of puerarin on chemical-induced liver fibrosis in rats. Further, the underlying mechanism of its reverse effect was evaluated by the estimation of histopathology, measurement of serum enzymes over hepatic injury and analysis of apoptosis in level of mRNA expression.

2. Materials and methods

2.1. Materials

The puerarin preparation was obtained from Shanghai University of Traditional Chinese Medicine (Shanghai, China). Reagent grade of CCl_4 was purchased from Shanghai Chemical. The sources for the other materials are given below.

2.2. Animals and treatments

Male Wistar rats (150–170 g, SPF, Shanghai Center of Experimental Animals, Chinese Academy of Sciences) were housed in conventional cages with free access to water and rodent chow at 20–22°C with a 12-h light–dark cycle. All procedures involving the use of laboratory animals were in accordance with National Institutes of Health guidelines. After 1 week of acclimatization, the rats were randomly divided into three groups: normal group, model group and treatment group at high or low dose. On the basis of a precedent [12], liver fibrosis was produced in rats by treatment with a mixture (50% alcohol, 8 g/kg per day; corn oil, 2 g/kg per day; pyrazole, 24 mg/kg per day; ig) once a day and by intraperitoneal injection of 0.25 ml/kg of a 25% solution of carbon tetrachloride in olive oil twice a week for 8 weeks. After 8 weeks of treatment, liver fibrosis was established. Then, puerarin (0.4 and 0.8 g/kg ig, daily for 4 weeks) was administered to rats to examine its therapeutic effects on chemical-induced liver fibrosis. At the same time, the model group and treatment group continued to receive the chemical mixture, while the control group received saline instead of the chemical mixture.

2.3. Sample collection

At the end of the experiment, all rats were anaesthetized with diethyl ether; samples of blood were drawn from the eye socket and collected in polyethylene tubes. The serum samples obtained by centrifugation for 10 min at 3000×*g* at 4°C were kept frozen at −80°C until assayed. The livers were rapidly removed and rinsed in cold saline. A portion of the liver was fixed for histopathology, another portion was dissected for flow cytometry assay and the remaining tissue was stored at −80°C until assayed.

2.4. Serum ALT, AST, ALP and GGT determinations

ALT (EC 2.6.1.2), AST (EC 2.6.1.1), ALP (EC 3.1.3.1) and GGT (EC2.3.2.2) were assayed in samples of serum obtained at the end of the experiment. The activity was determined by using a commercial clinical test kit (Shanghai Rongsheng Biotech).

2.5. Histopathology

Immediately after sacrifice, the liver tissue was removed and a portion of the tissue was instantly fixed in 10% phosphate buffered formalin (prepared by dissolving 3.5 g anhydrous sodium phosphate monobasic and 6.5 g anhydrous sodium phosphate dibasic in 100 ml of 40%

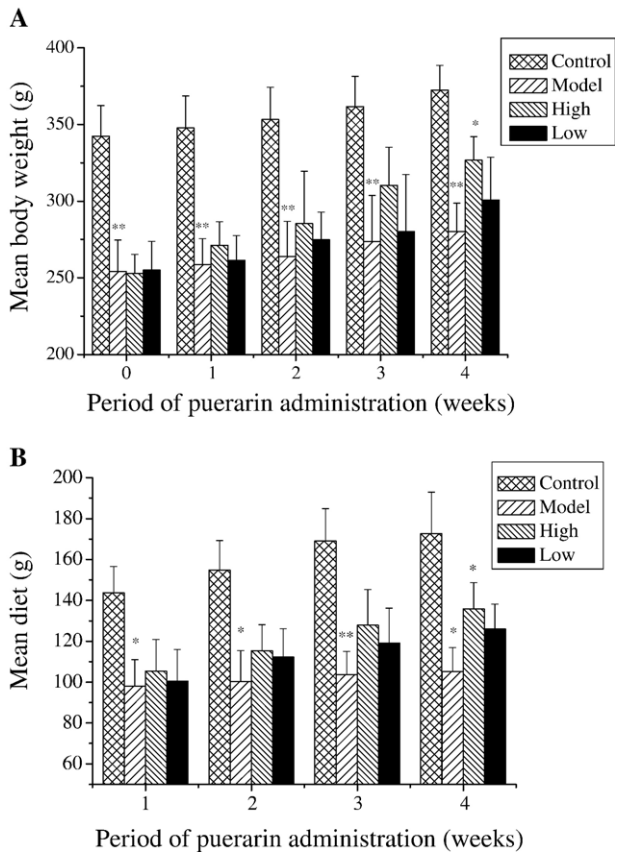

Fig. 2. Changes in body weight (A) and diet consumption (B) in rats treated with ethanol plus CCl_4 during puerarin administration. 'Control' represents normal group, 'model' represents puerarin-untreated group, 'high' represents puerarin-treated group with 0.8 g/kg and 'low' represents puerarin-treated group with 0.4 g/kg. *P<.05, **P<.01 (model vs. control, high or low vs. model), ANOVA and post hoc test.

formaldehyde and made up to 1 L with water), processed by routine histology procedures, embedded in paraffin, cut in 5-μm pieces and mounted on the slide. The samples were stained with hematoxylin and eosin (H&E) for histopathological examination.

Specimens were evaluated in terms of 'grading' (necroinflammatory activity) and 'staging' (fibrosis score) using Ishak's classification [13]. Necroinflammatory was graded according to the modified histological activity index. Steatosis was graded according to the percentage of hepatocytes with fatty accumulation. In detail: Grade 0 (absent), Grade 1 (<10%), Grade 2 (10–30%), Grade 3 (30–60%) and Grade 4 (>60%). Fibrosis was graded as 0, no fibrosis; Grade 1, enlarged fibrous portal tracts; Grade 2, periportal or portal–portal septa but intact architecture; Grade 3, fibrosis with architectural distortion; and Grade 4, probable or definite cirrhosis [14].

2.6. Flow cytometry assay

The liver tissue was rapidly removed, weighed and placed into 10 ml of ice-cold PBS containing 0.2% bovine serum albumin (BSA) (Sigma), 0.01 mol/l EDTA and 10 mg/ml of deoxyribonuclease (Sigma), and then the tissue was disrupted in a glass homogenizer and passed through a 40-μm nylon cell stainer (Becton Dickinson). The suspension was centrifuged at 500×*g* for 10 min at room temperature. The pellet was resuspended in 500 μl of PBS with BSA and transferred into a fresh tube. The cells obtained by the above method were fixed with ice-cold 70% ethanol in PBS at 4°C for 8 h, then incubated with RNase (20 μg/ml) for 30 min at 37°C and labeled with propidium iodide (50 μg/ml). DNA contents were measured by a FACSCalibur cytometer (Becton Dickinson). Multicycle software (CELLQUEST software, Becton Dickinson) was used to produce histograms of DNA content frequency.

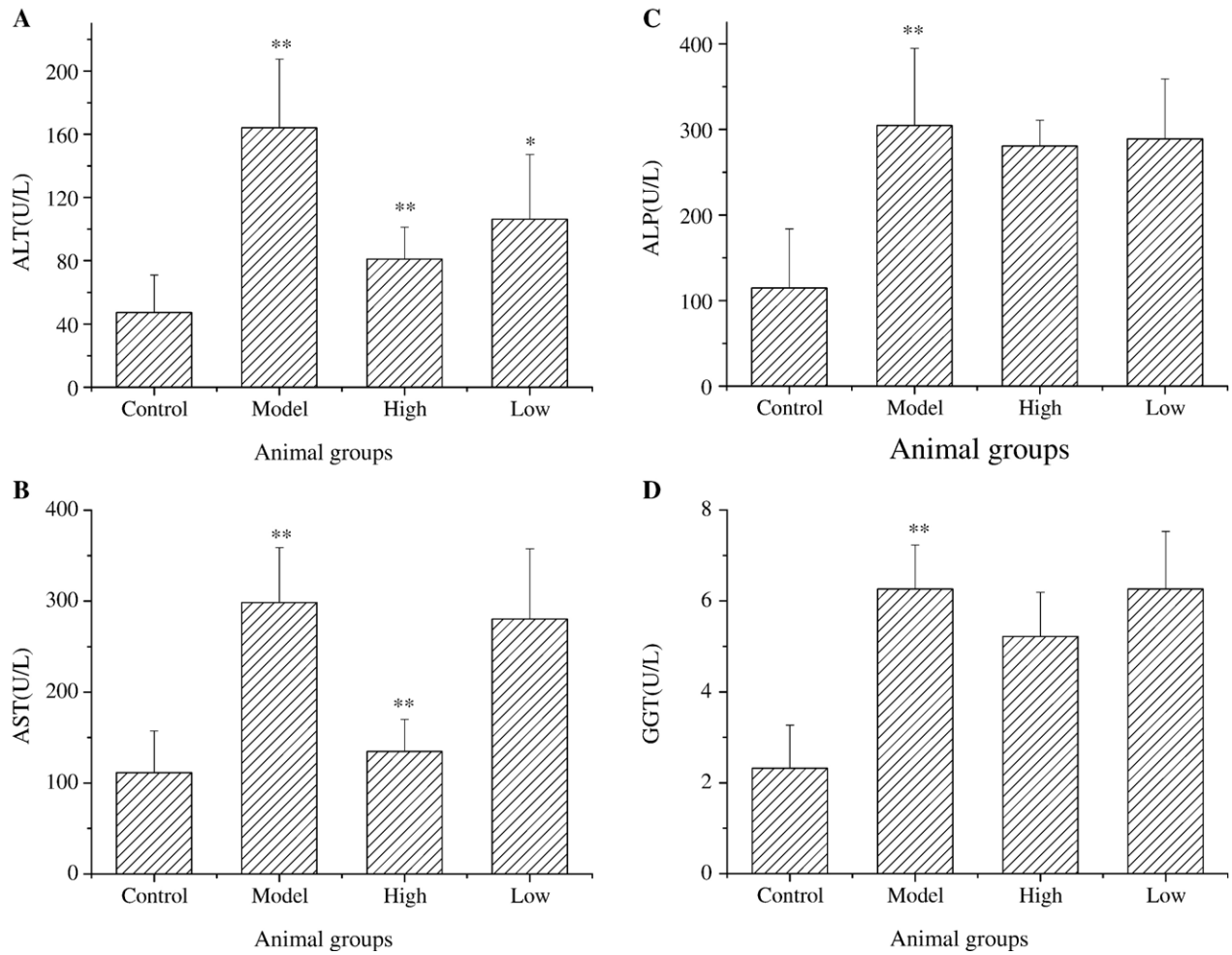

Fig. 3. Serum ALT (A), AST (B), ALP (C) and GGT (D) activities in toxicant-treated rats with and without puerarin administration. One enzyme unit is the quantity of enzyme needed to cause a reaction to process 1 μmol of substance per minute under specified conditions. 'Control' represents normal group, 'model' represents puerarin-untreated group, 'high' represents puerarin-treated group with 0.8 g/kg and 'low' represents puerarin-treated group with 0.4 g/kg. Values are the mean±S.D. of animals for each group. *P<.05, **P<.01 (model vs. control, high or low vs. model), ANOVA and post hoc test.

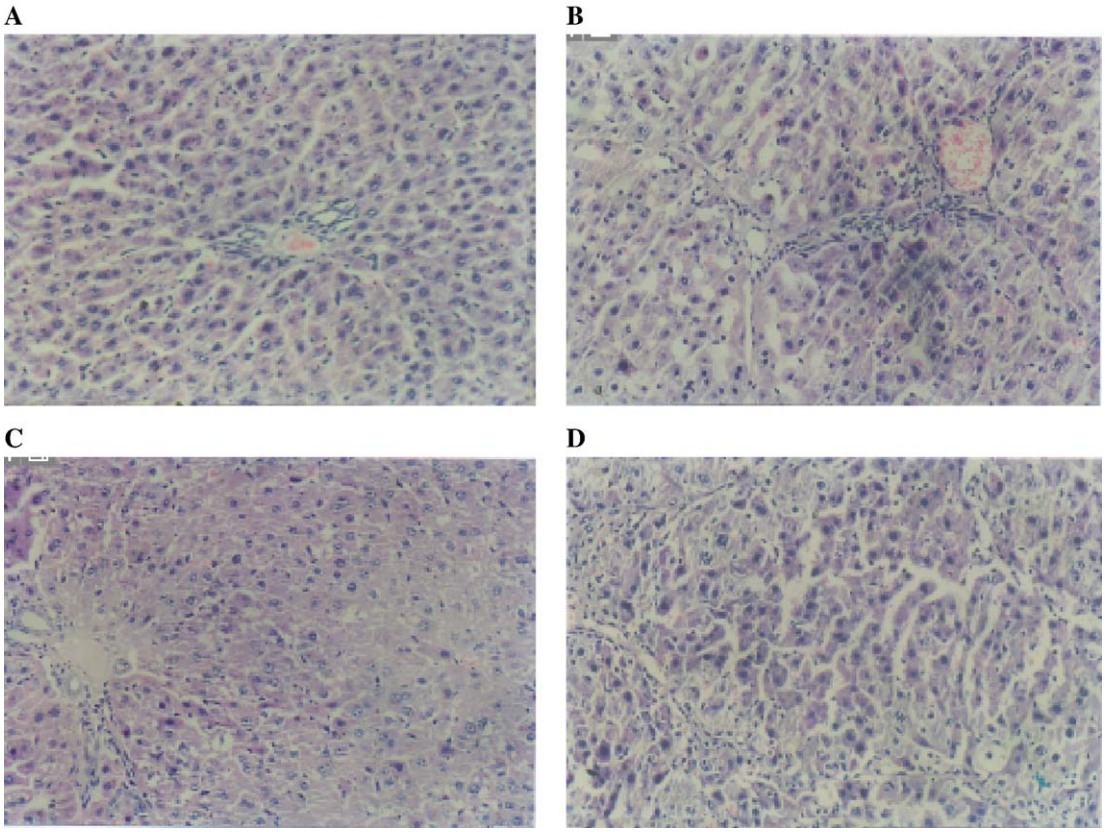

Fig. 4. Liver histology in toxicant-treated rats with and without puerarin administration. H&E-stained sections of rat liver in the different groups: (A) control, (B) model, (C) puerarin (0.8 g/kg) and (D) puerarin (0.4 g/kg). Magnitude 200×.

Subdiploid DNA peaks were quantified from the DNA content data.

2.7. Total RNA isolation and semiquantitative RT-PCR analysis

Total RNA of the hepatic tissues was extracted using TRIZOL reagent according to the supplier's instruction. RNA was quantitated by optical density measurement at 260 and 280 nm using a spectrophotometer, and integrity was confirmed by running 4 μl RNA on a 1.2% agarose gel.

Reverse transcription was performed in 20 μl of reaction mixture containing nuclease-free water (5.75 μl), 10× buffer (2 μl), dNTP (1 mM), $MgCl_2$ (5 mM), 4 μl of RNA of total RNA, ribonuclease inhibitor (1 U/μl), oligo primer (25 μg/ml) and avian myeloblastosis virus reverse transcriptase (0.75 U/μl) at 42°C for 60 min. Then, heating at 95°C inactivated reverse transcriptase for 5 min. Resulting reverse transcription products were stored at −80°C until used.

The PCR primers of bcl-2 and β-actin were synthesized by Shanghai Shenergy Biocolor BioScience and Technology. The sequences of the primers used in this study were bcl-2 sense primer: 5′CAC CCC TGG CAT CTT CTC CT 3′; bcl-2 antisense primer: 5′GTT GAC GCT CCC CAC ACA CA 3′(349 bp product); β-actin sense primer: 5′TAA AGA CCT CTA TGC CAA CAC AGT 3′; β-actin antisense primer: 5′CAC GAT GGA GGG GCC GGA CTC ATC 3′ (260 bp product).

Polymerase chain reaction was carried out in 20 μl of reaction mixture, which contained 2 μl of 10× Taq buffer with KCl, DNTP (2 mM), 0.6 μl (10 pmol/μl) of each specific PCR primer, Taq DNA polymerase (0.1 U/μl), $MgCl_2$ (2.5 mM), cDNA 2 μl, nuclease-free water (10.8 μl). The cycles were 94°C for 45 s, 55°C for 45 s and 72°C for 50 s. Final extension was for 10 min at 72°C. The number of cycles performed was 30. Polymerase chain reaction of β-actin chosen as an internal control was carried out in the same tubes as for the genes. PCR products were run on a

Table 1
Effect of puerarin administration on histopathological changes in rats with chemically induced liver fibrosis

Group	*n*	Necroinflammatory					Steatosis					Fibrosis				
		0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Control	7	7					7					7				
Model	6	2	4						1	2	3			2	3	1
High	7	4	3					3	4				5	2		
Low	7	2	5						4	2	1		1	6		

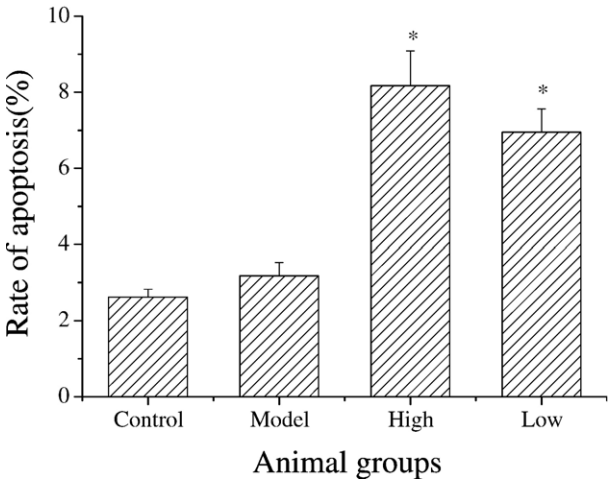

Fig. 5. Quantitative analysis of apoptosis in liver tissues of toxicant-treated rats with and without puerarin administration by flow cytometry. A portion of liver tissues was processed to isolate cells, and then the cells were stained with propidium iodide and analyzed in a FACScan flow cytometer. Data are presented as the mean±S.D. for three rats per group. Significant difference from the treatment group is indicated by $*P<.05$ vs. model group, ANOVA and post hoc test.

1.5% agarose gel containing ethidium bromide and viewed under UV light. The PCR signal intensities were semi-quantified by scanning the gels using the Smartview software, and bcl-2 densitometric measurement was normalized with the internal control, β-actin.

2.8. Statistical analysis

Data were expressed as mean±S.D. and analyzed by the ANOVA and post hoc test. P values below .05 were regarded as statistically significant.

3. Results

3.1. Body weight and diet consumption

To determine the potential toxicity and safety of the Chinese herbal medicine puerarin in Wistar rats, the changes in body weight and diet consumption were assayed (Fig. 2). The model group exerted a retarded gain in body weight and diet consumption when compared with the control group. Changes in body weight and diet consumption could be observed during the whole period of treatment, and a significant increase was displayed in the treated group relative to the model group at week 4. Also, puerarin treatment at high dose showed a greater effect than the low-dose group. These results suggest that treatment of puerarin can markedly reverse the decrease in body weight and diet consumption at the doses studied, which is safe in Wistar rats.

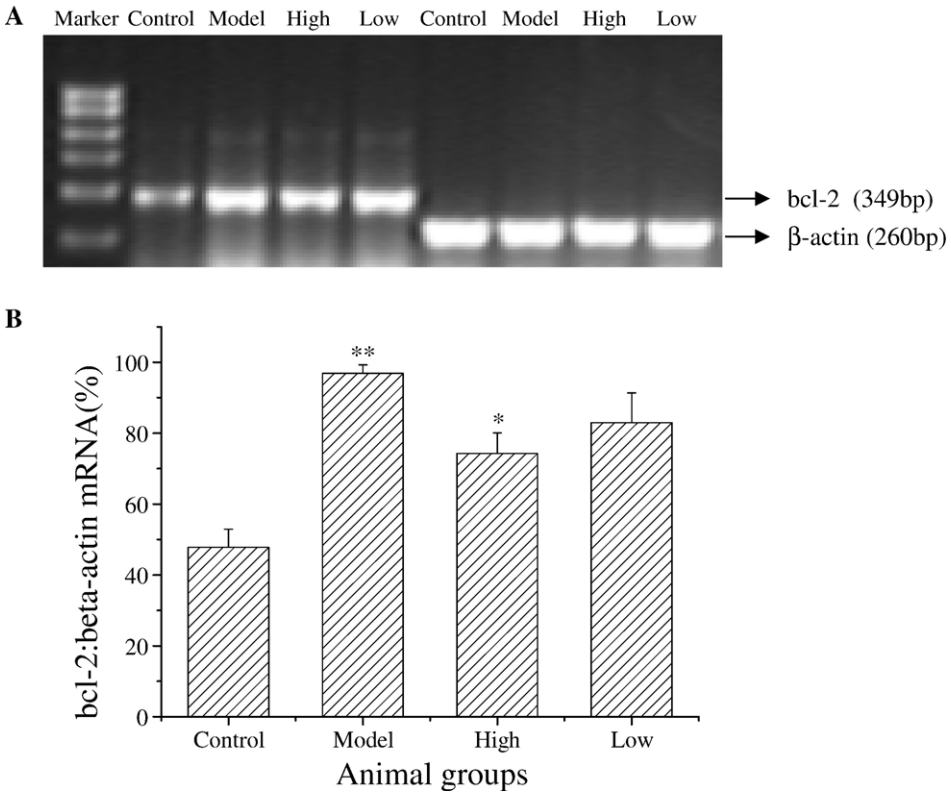

Fig. 6. Expression of bcl-2 mRNA in liver tissues of toxicant-treated rats with and without puerarin administration. (A) Agarose gel eletrophoresis of RT-PCR product of bcl-2 mRNA isolated from hepatic rats. (B) Semiquantitative analysis of the bcl-2 mRNA level, using densitometric scanning of the eletrophoresis. $*P<.05$, $**P<.01$ (model vs. control, high or low vs. model), ANOVA and post hoc test.

3.2. Effects of puerarin on serum ALT, AST, ALP and GGT

To evaluate the extent of liver injury in liver fibrosis, we carried out an analysis of serum ALT, AST, ALP and GGT activities. ALT, AST, ALP and GGT activities at the end of the experiment are shown in Fig. 3. A significant increase in the activities of the four enzymes was observed in the model group compared with those of the control group. Puerarin treatment was able to reduce the ALT and AST activities. However, no effect was observed in the ALP and GGT activities.

3.3. Effects of puerarin on liver pathology

We also conducted an immunohistochemical staining of the liver tissues. The control group showed normal lobular architecture with central veins and radiating hepatic cords. The establishment of the model group was quite successful: marked fatty degeneration, slight confluence, portal inflammation and necrosis, obvious collagen deposition, perihepatocyte fibrosis and hepatocyte loosening. As shown in Fig. 4, the treatment group could markedly decrease those pathological characters at the two studied doses. Histopathological changes are summarized in Table 1, indicating the significant inverse relationship between the puerarin treatment and chemically induced liver fibrosis.

3.4. Apoptosis of HSC

It has been demonstrated that apoptosis is the major mechanism by which activated HSCs are removed during recovery from fibrosis [15–17]. In order to further determine whether puerarin could induce apoptosis of activated HSC during recovery from fibrosis, we measured apoptosis in the liver tissue by FCAS analysis. As shown in Fig. 5, apoptosis in the treatment group was more obvious than that in the model group. These observations suggest that puerarin treatment may be attributed to the recovery from fibrosis in activated HSC.

3.5. Expression of bcl-2 mRNA in liver

The result of semiquantitative RT-PCR analysis of bcl-2 mRNA in the liver tissue of each group is shown in Fig. 6. It was obvious that the level of bcl-2 mRNA was higher in the model group than that in the control group, while its expression decreased greatly in rats treated with puerarin at both high and low doses. The results suggest that puerarin possessed the ability of down-regulating the expression of bcl-2 mRNA in liver tissues.

4. Discussion

Now a major challenge to curing liver fibrosis is to find novel chemical entities with less toxicity and greater effectiveness than those used in current chemotherapy. Since natural products often have such features, recently, research for new drugs has refocused on natural products. This rediscovery of natural products has yielded promising compounds such as taxanes and camptothecins. Moreover, recent reports have indicated the possible use of natural products as a source of potential chemopreventive agents [18,19]. In this study, we demonstrated that puerarin, a nature product, has certain effects in antifibrosis.

Liver fibrosis is common in various chronic liver diseases. There is clear evidence that persistent fibrosis can lead to the development of hepatocellular carcinoma [20,21]. Interrupting and/or reversing liver fibrosis is important in preventing its progression to hepatocellular carcinoma. However, there is as yet no clearly established therapy for reversing liver fibrosis. Therefore, at first, animal models of liver fibrosis are important for research into the underlying mechanisms or treatments associated with this disease.

Currently, cirrhosis is mainly induced either by the ligation of common bile duct or by application of hepatotoxins such as CCl_4 injection or thioacetamide [22]. In this study, experimental liver fibrosis was induced by low dose of CCl_4 (ip) and ethanol solution (ig) within 12 weeks in Wistar rats. It has been reported that hepatic histological changes in ethanol-CCl_4-induced fibrosis model were similar to those found in human alcoholic cirrhosis [23]. Analysis of pathology highly demonstrated our successfully established rat model of chemical-induced liver fibrosis. And the puerarin treatment group could markedly decrease pathologic characters of the liver tissues.

Analysis of serum enzymes is one of the most sensitive tests employed in the diagnosis of hepatic diseases. In the present study, we have observed increased activities of serum ALT, AST, ALP and GGT in chemical-induced liver fibrosis in rats. This can be attributed to the damaged structural integrity of the hepatic cells. The enzyme ALP is located in the cytoplasm and will be released into circulation after cellular damage [24]. In addition, the soluble enzyme AST is released when injury involves organelles such as mitochondria [25]. As a result, the establishment of liver fibrosis in this study caused both plasma membrane and organelle membrane damage. On administering puerarin, we have observed decreased levels of serum ALT and AST. Together with the results of histopathology mentioned above, we hypothesize that puerarin may possess the ability of preserving the structural integrity of the liver from the adverse effects of ethanol-CCl_4 and therefore reverse hepatic injury.

Besides improvement of the hepatocyte against hepatic dysfunction, recovery from liver fibrosis is associated with apoptosis of activated HSC. Recently, it is widely believed that the most essential characteristic of hepatic fibrosis is HSC activation, inducing apoptosis, which turns out to be a potential hopeful target for treatment in fibrosis [1]. So we performed flow cytometry assay to investigate the effect of puerarin on apoptosis. It was discovered in this work that puerarin could significantly induce the apoptosis of activated HSC, so that the compound might attenuate liver fibrosis.

Next, we designed to examine the mechanism of apoptosis induced by puerarin. Measurement of the expression of bcl-2 mRNA in liver tissues by RT-PCR was then conducted. The bcl-2 family of proteins may be either pro- or anti-apoptotic. Bcl-2 itself inhibits apoptosis in response to a wide variety of signals [26], and over-expression of bcl-2 can protect cardiac myocytes from apoptosis [27]. We found that puerarin treatment could down-regulate bcl-2 mRNA expression. These results suggest that puerarin reversed chemical-induced hepatic fibrosis, at least in part, by inducing apoptosis of activated HSC through down-regulation of bcl-2 mRNA expression.

In conclusion, results from the present study demonstrated that puerarin was effective in the treatment of chemical-induced liver fibrosis in rats. The primary mechanisms of this therapeutic effect could be due to its protection against hepatic injury by reducing serum levels of ALT and AST, as well as its induction of apoptosis in activated HSC by down-regulating bcl-2 mRNA expression. Therefore, on the basis of our work, puerarin should be regarded as a new drug and should be useful for the prevention and treatment of liver fibrosis and even cirrhosis.

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