PROTECTION BY AND ANTI-OXIDANT MECHANISM OF BERBERINE AGAINST RAT LIVER FIBROSIS INDUCED BY MULTIPLE HEPATOTOXIC FACTORS

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SUMMARY

1. The aim of the present study was to investigate the effect and mechanism of berberine, an alkaloid extracted from the traditional Chinese medicine coptis, on rat liver fibrosis induced by multiple hepatotoxic factors.

2. Male Wistar rats were separated into five groups, a normal control group, a fibrotic control group and fibrotic groups treated with three different doses of berberine. The fibrotic models were established by introduction of multiple hepatotoxic factors, including CCl₄, ethanol and high cholesterol. Rats in the treatment groups were administered 50, 100 or 200 mg/kg berberine, intragastrically, daily for 4 weeks. Serum levels of alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST), hepatic activity of superoxide dismutase (SOD) and hepatic malondialdehyde (MDA) and hepatic hydroxyproline (Hyp) content were determined. Liver biopsies were obtained for histological and immunohistochemical studies to detect the expressions of α-smooth muscle actin (SMA) and transforming growth factor (TGF)-β1.

3. The results showed that, compared with the fibrotic control group, serum levels of ALT and AST and hepatic content of MDA and Hyp were markedly decreased, but the activity of hepatic SOD was significantly increased in berberine-treated groups in a dose-dependent manner. In addition, histopathological changes, such as steatosis, necrosis and myofibroblast proliferation, were reduced and the expression of α-SMA and TGF-β1 was significantly downregulated in the berberine-treated groups (P < 0.01).

4. These results suggest that berberine could be used to prevent experimental liver fibrosis through regulation of the anti-oxidant system and lipid peroxidation.

Key words: anti-oxidant, berberine, hepatic stellate cells, liver fibrosis, α-smooth muscle actin, transforming-growth factor-β1.

INTRODUCTION

Liver fibrosis is a common response to chronic liver injury, manifested as hepatocyte necrosis, regeneration and collagen deposition, which is irreversible at best and, at worst, may result in cirrhosis that is asymptomatic and may remain so for more than a decade. Therefore, there is practical significance in finding an agent that can prevent the progression of liver fibrosis.

It is well known that hepatic stellate cells (HSC) play an important role in liver fibrogenesis. Activation of HSC refers to the conversion of quiescent cells into proliferative, fibrogenic and contractile myofibroblasts, which is a complex but tightly programmed procedure. This procedure could be divided into two stages: initiation and perpetuation. Initiation is the stage in which phenotypes change quickly in response to a paracrine stimulus. The paracrine secretion stimulus could be from inflammatory cells (e.g. Kupffer cells, leucocytes, lymphocytes), injured hepatocytes and other cells and is a major factor in the activation of HSC. Previous studies have demonstrated that the appearance of α-smooth muscle actin (SMA) is a classical marker of activated HSC, coinciding with the infiltration of Kupffer cells to further synthesise extracellular matrix (ECM) by releasing cytokines, such as transforming growth factor (TGF)-β1. The initiation stage is followed by the perpetuation stage, in which activated phenotypes are amplified by enhanced growth factor expression (i.e. TGF-β1).

Transforming growth factor-β1 is one of the major fibroblast growth factors. The HSC are a major source of TGF-β1. Kupffer cells and hepatocytes can also secrete TGF-β1. In the process of liver fibrogenesis, TGF-β1 continues to increase to stimulate the change of static HSC to activated HSC. As described above, perpetuation is a continuously dynamic process, as demonstrated by sequential changes in TGF-β1 signalling with progressive activation of HSC. Therefore, a strategy of inhibiting the secretion of TGF-β1 may be an effective approach to prevent fibrogenesis. During HSC activation, the synthesis of the ECM is increased and its degradation is reduced. Excess deposition of ECM proteins disrupts the normal architecture of the liver, ultimately leading to liver fibrosis. Taking these events together, it is believed that an effective treatment that reduces the harmful effects of activated HSC would prevent the development and progression of liver fibrosis.

Excess production of reactive oxygen species (ROS) has been implicated in many pathological processes, including chronic liver injury and fibrogenesis. Growing evidence demonstrates that ROS can be mainly derived from activated Kupffer cells and damaged...
hepatocytes and that ROS are associated with the activation of HSC through paracrine stimulation.12-16 Factors increasing ROS production may also contribute to the stimulation of the production of excessive ECM.17 Increased oxidation leads to activation of TGF-β1.18 When the production of oxidation is more than the anti-oxidant capacity, damage to lipids may occur.19 Thus, it was proposed that plant-derived anti-oxidants act as potential antifibrotic agents by either protecting hepatocytes against ROS or inhibiting the activation of HSC.20

Berberine is an alkaloid extracted from coptis, cork and other traditional Chinese medicines that is used for the treatment of bacterial infectious diseases. Recently, it has been reported that berberine exhibits multiple pharmacological activity, such as correcting dyslipidaemias, anti-inflammatory effects, antidiabetic effects and anticancer effects.21-25 Many experiments have shown that berberine acts to reduce the formation of ROS.20-22 Therefore, we hypothesized that berberine may inhibit the activation of HSC and delay fibrogenesis of the liver by, at least in part, deceasing oxidative stress. However, whether berberine has an antifibrotic effect in the early stages of liver fibrogenesis is not known. Therefore, it was necessary to investigate the effects and mechanism of action berberine in liver fibrosis.

METHODS

Drugs and reagents

Berberine chloride was purchased from Sigma (St Louis, MO, USA). Rabbit anti-rat TGF-β1 and mouse anti-rat α-SMA antibodies were obtained from Zhongshan Golden Bridge Biotechnology (Beijing, China) and Boster Biotechnology (Wuhan, China), respectively. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD) and hydroxyproline (Hyp) assay kits were purchased from Biotechnology (Wuhan, China), Thio-barbituric acid test (TBA) and other chemicals and reagents were of analytical grade.

Animal models and treatments

Male Wistar rats, weighing 220–250 g, were obtained from the Experimental Center of Medical Scientific Academy of Hubei (Wuhan, China). Following 1 week acclimatization after arrival, 40 Wistar rats were randomly separated into five groups: a normal control group, a fibrotic control group and fibrotic groups treated with three different doses of berberine (50, 100 and 200 mg/kg). With the exception of the normal control group, all animals were fed with a high-fat diet (89.5% corn flour, 10% lard and 0.5% cholesterol) and were given 10% ethanol to drink for 4 weeks. On Day 8 after starting the high-fat diet, rats were injected with an initial dose of 5 mL/kg, s.c., of 40% CCl4 in olive oil (v/v), followed by 3 mL/kg twice a week for 3 weeks. Normal control rats received the same volume of olive oil with normal diet and free access to water. In the beginning, to induce the liver fibrosis models, the three berberine groups were administered 50, 100 or 200 mg/kg berberine, intragastrically, daily for 4 weeks. The three concentrations of berberine were dissolved in 0.5% sodium carboxymethyl cellulose at 5, 10 and 20 mg/mL, respectively, and were given in a volume of 1 mL/100 g bodyweight. Rats were killed at the end of the 4th week, blood samples were obtained immediately before rats were killed and livers were excised. The right lobes of the livers were fixed in 0.4 g/L paraformaldehyde solution and embedded in paraffin prior to processing for histopathology. The study protocol was in accordance with the guideline for animal research and was approved by the Ethical and Research Committee of Medical School of Wuhan University.

Biochemical analysis

Serum levels of ALT and AST, hepatic SOD activity and hepatic Hyp content were analysed using standard spectrophotometric methods with commercially available test kits. Malondialdehyde (MDA) in liver tissue was measured by the thio-barbituric acid-reactive substances (TBARS) method. In brief, the reaction mixture, containing 0.1 mL tissue sample (10% liver homogenate), 1.5 mL of 1% (v/v) phosphoric acid and 0.5 mL of 0.6% TBA, was boiled at 100°C for 45 min. After cooling, 2 mL n-butanol was added to the medium and mixed moderately. The butanol phase was separated by centrifugation (10min, 25°C, 750 g). Absorbance was measured at 535 and 520 nm. The difference in absorbance was used to calculate MDA values (μmol/g liver weight).

Histopathology

For histopathological examination, liver specimens were fixed overnight in 0.4 g/L phosphate-buffered paraformaldehyde and processed by the paraffin slice technique. Sections (5 μm) were stained with haematoxylin and eosin (HE) for routine histology and with Masson’s trichrome for collagen. The collagen content was quantified using a Medical Color Image Analysis System (HMIAS-2000; Guangzhou Longest Technology, Guangzhou, China). Briefly, a field containing a portal vein at its centre that was approximately 100 μm in diameter was selected under a magnification of ×200. Digitalized images of the field and five random fields of the same size were captured for computer analysis by a digital camera. Collagen was delineated in the images and its area was scored. The average of five fields was calculated for assessment of the degree of fibrosis in each case.

Immunohistochemistry

Fresh livers were fixed in 0.4 g/L paraformaldehyde in phosphate-buffered saline (PBS) for 24 h. Immunohistochemical staining for α-SMA and TGF-β1 was performed using the routine immunohistochemistry streptavidin peroxidase (SP) method, with a monoclonal antibody against α-SMA and a polyclonal antibody against TGF-β1 (diluted to 1 : 100 and 1 : 200, respectively). An interstitial brown stellate structure was taken as positive expression for α-SMA, whereas a cytoplasmic brown granule was taken as positive expression of TGF-β1. At least five random fields from each section were examined at a magnification of ×400 and analysed using the HMIAS-2000. The formula positive content (PC) = mean optical density × positive area was used.

Statistical analysis

Results are shown as the mean±SD. Data were analysed using one-way ANOVA using spss 14.0 (SPSS, Chicago, IL, USA). P < 0.05 was taken to indicate statistical significance.

RESULTS

Liver index and liver function

Compared with the normal control group, the bodyweight of rats in the fibrotic control group decreased approximately 4%, but liver weight increased 111%. Thus, the liver index (liver weight/bodyweight ratio) was markedly increased in the fibrotic control group (Table 1). Compared with the fibrotic control group, both bodyweight and liver weight in the berberine-treated groups were slightly decreased (P > 0.05). However, the liver index of rats treated with 200 mg/kg berberine was significantly reduced compared with the fibrotic control group (P < 0.05; Table 1). In the fibrotic control group, serum levels of ALT and AST were increased significantly, 1.75- and 1.35-fold, respectively (P < 0.01), compared with the normal control group. However, treatment with 50, 100 and 200 mg/kg berberine resulted in a 38, 48 and 55% decrease in serum ALT, respectively, and a 37, 41 and 52% decrease in serum AST, respectively, compared with the fibrotic control group (Table 2).
Malondialdehyde and SOD

Hepatic MDA content was increased markedly and hepatic SOD activity was reduced in fibrotic control group compared with the normal control group ($P < 0.05$ and $P < 0.01$, respectively). Compared with the fibrotic control group, treatment of rats with 200 mg/kg berberine resulted in a significant reduction in liver MDA content ($P < 0.05$) and an increase in hepatic SOD activity ($P < 0.01$; Fig. 1).

Histopathology and Hyp

The extent of liver fibrosis was evaluated using two histological staining methods, namely HE staining and Masson’s trichrome staining. Both methods showed the same pattern. Histological analysis of livers from normal control rats indicated normal architecture (Fig. 2a,d). Treatment of rats with multiple hepatotoxic factors for 4 weeks caused liver extensive fibrosis, as indicated by qualitative and quantitative histopathological examinations of rats in the fibrotic control group (Fig. 2b,e). In contrast with normal liver, treatment of rats with the multiple hepatotoxic factors resulted in severe histopathological alterations such as steatosis, macrophage infiltration and myofibroblast proliferation and liver fibrosis (i.e. disruption of tissue architecture, extension of fibres, formation of large fibrous septa, pseudolobe separation and collagen accumulation). However, these changes were prevented by 50, 100 and 200 mg/kg berberine. Figure 2c,f shows that the intensity of liver fibrosis was reduced in the group treated with 200 mg/kg berberine, resulting in only marginal fibrosis and weak portal inflammation. Semiquantitative analysis confirmed that liver fibrosis was significantly increased in the fibrotic control group compared with the normal control group ($P < 0.01$; Fig. 3). However, berberine treatment significantly reduced both the area and percentage of collagen in the liver ($P < 0.01$; Fig. 3). The hepatic Hyp content was significantly increased in the fibrotic control group compared with the normal control group ($P < 0.01$). Treatment of rats with 50, 100 and 200 mg/kg berberine resulted in 26, 29 and 33% reductions in Hyp content, respectively (Fig. 4). These results are consistent with the qualitative examination and semiquantitative analysis of collagen.

Immunohistochemistry for $\alpha$-SMA and TGF-$\beta$1

Expression of $\alpha$-SMA and TGF-$\beta$1 was detected using immunohistochemical methods. The PC value was scored. $\alpha$-Smooth muscle actin was detectable in normal control rats (PC $= 0.015 \pm 0.013$; Fig. 5a). In fibrotic control rats, high expression of $\alpha$-SMA was found in Disse’s space and around the perportal fibrotic band areas, central vein and fibrous septa (PC $= 1.355 \pm 0.062$; Fig. 5b). There was a significant difference between these two groups ($P < 0.01$). Expression of $\alpha$-SMA in the 50, 100 and 200 mg/kg berberine-treated
groups was significantly reduced (PC = 1.070 ± 0.078, 0.668 ± 0.129 and 0.466 ± 0.129, respectively; P < 0.01 compared with the fibrotic control; Figs 5c,6).

In the normal control group, TGF-β1 was mainly expressed in the portal area and interstitial cells (PC = 0.17 ± 0.10; Fig. 5d). In the fibrotic control group, a significant increase of TGF-β1 expression was seen in the portal area and interstitial cells and diffusely in the sinusoidal wall, perisinusoidal cells, inflammatory necrosis zone and connective tissue collagen bundles (PC = 16.77 ± 3.63; P < 0.01; Fig. 5e). In the 0, 100 and 200 mg/kg berberine-treated groups, the expression of TGF-β1 (PC = 11.68 ± 2.10, 8.36 ± 0.84 and 3.84 ± 0.73, respectively) was significantly reduced compared with that in the fibrotic control group (P < 0.01; Figs 5f,6).

**DISCUSSION**

The multiple hepatotoxic factor-induced liver fibrosis rat model was successfully established in the present study, as evaluated by the measurement of cytosol enzymes and the amount of collagen, as well as on the basis of histological observations. Cytotoxicity was shown as significant increases in ALT and AST. Liver fibrosis was shown as significant increases in Hyp as well as pathological histological changes. Following berberine treatment, the release of cytosolic enzymes and the amount of collagen decreased and, in addition, the status of the impaired liver was also attenuated, indicating that berberine has a direct inhibitory effect on fibrogenic potential in multiple hepatotoxic factor-induced fibrosis.

Oxidative stress plays an important role in the aetiopathogenesis of liver fibrosis via lipid peroxidation and HSC activation. Lipid...
Hepatic stellate cells, a principal cellular source of ECM during chronic liver injury, undergo a transition into α-SMA-expressing myofibroblast-like cells. Activation of HSC is associated with cell proliferation, increased contractility and enhancement of matrix production. Cassiman et al.33 and Ramm et al.34 have demonstrated that α-SMA is mainly retained in the portal ducts and fibre septa, accompanied by proliferating tubercles, corresponding to the distribution of collagen. Okazaki et al.35 showed that ROS-induced HSC activation can be inhibited by anti-oxidants. Consistent with these findings, the present study demonstrated that, during the development of liver fibrosis, α-SMA levels were markedly elevated and α-SMA was expressed in the cells of the portal ducts, fibre septa and perisinuses accompanied by proliferating bile ducts. The area of α-SMA expression in rats with liver fibrosis was larger than that in normal control rats. Following berberine treatment, α-SMA expression was significantly reduced compared with that in the untreated fibrotic control. This indicates that berberine has a potential action to suppress HSC activation, which may be through inhibition of ROS production.

Transforming growth factor-β1 has multiple pharmacological actions. A balance among these actions is required to maintain tissue homeostasis. Aberrant expression of TGF-β1 is involved in a number of disease processes in the liver.5 It is known that TGF-β1 is a crucial cytokine during HSC activation and formation of liver fibrosis. Oxidative production triggers TGF-β1,18 the latter stimulating production of ECM molecules and inducing phenotypic transdifferentiation.18 Thus, one strategy in the development of antifibrotic...
drugs could be to search for an effective TGF-β1 inhibitor. Our observations showed that berberine downregulates TGF-β1 expression, corresponding with a reduction in α-SMA expression, indicating that berberine is an effective inhibitor of TGF-β1.

It is well known that liver fibrosis is a result of increased collagen synthesis. Hydroxyproline is a characteristic compound collagen that is secreted by activated HSC. Therefore, the amount of collagen can be determined by measuring Hyp content and can be used as an indication of the degree of fibrosis. In the present study, we observed that the Hyp content was markedly increased in fibrotic control rats compared with normal control rats, but was decreased in all berberine-treated groups compared with the untreated fibrotic control. These results were validated by Masson’s trichrome staining and immunohistochemistry for α-SMA, indicating that berberine can reduce collagen secretion, which is consistent with decreased ROS production suppressing excess matrix deposition in the liver, further preventing liver fibrosis.

In conclusion, the findings of the present study provide direct evidence that berberine can prevent experimental liver fibrosis in rats by protecting hepatocytes, decreasing the secretion of TGF-β1 and inhibiting HSC activation, which may be through the regulation of the anti-oxidant system and lipid peroxidation.

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REFERENCES


