Prevention of *Cistanche salsa* Extract on Hepatic Fibrosis Induced by Carbon Tetrachloride in Rats

YANG Feng-ruï*, WEN Du-su, FANG Bu-wu, LOU Jian-shi, MENG Lin

Department of Pharmacology, Tianjin Medical University, Tianjin 300070, China

Abstract: **Objective** To explore the antifibrotic effect of echinacoside on carbon tetrachloride (CCl₄)-induced hepatic fibrosis in rats. **Methods** Male Wistar rats were randomly divided into normal control (n = 8), model (n = 14), and echinacoside treatment (n = 14) groups. The hepatic fibrosis model was induced by CCl₄ compositor. The rats were ig administered with echinacoside at a daily dose of 50 mg/kg. The anti-oxidant status, liver function parameters, and hepatic hydroxyproline content were detected by chromatometry. The serum levels of hyaluronic acid (HA), type IV collagen (CIV), type III procollagen (PIIIP), and laminin (LN) were assayed with radioimmunoassay. The hepatic injury was detected by haematoxylin-eosine staining. The deposition of collagen was observed with Masson staining. **Results** Echinacoside increased the superoxide dismutase activity and reduced the levels of malondialdehyde, aspartate aminotransferase, alanine aminotransferase, HA, CIV, PIIIP, and LN in serum. Echinacoside could also reduce the hydroxyproline content in liver, alleviate hepatic injury, and inhibit collagen deposition. **Conclusion** Echinacoside possesses antihepatic fibrosis effect.

Key words: carbon tetrachloride; echinacoside; extracellular matrix; hepatic fibrosis; lipid peroxidation

DOI: 10.3969/j.issn.1674-6348.2013.03.004

Introduction

A variety of pathological factors, including viral hepatitis (especially hepatitis B and C), alcohol and drug abuse, metabolic diseases due to overload of iron or copper, autoimmunity against hepatocytes or bile duct epithelium, and congenital abnormalities could cause hepatic injury (Friedman, 1993). Lifestyle changes (mainly exercise withdrawal and weight gain) have probably raised the prevalence of nonalcoholic fatty liver disease (NAFLD), which is the first cause of chronic liver disease in Western world. All these chronic hepatic diseases could cause hepatic fibrosis. If the hepatic fibrosis treatment was delayed, hepatic cirrhosis would be developed (Okazaki, 2001). Hepatic fibrosis, cirrhosis in particular, is associated with significant morbidity and mortality (Faouzi *et al.*, 1999). Hepatic fibrosis is characterized by imbalance between extracellular matrix synthesis and degradation. The extracellular matrix mainly results from hepatic stellate cells (HSCs) which could be transformed into myofibroblast initially (Li and Friedman, 1999). Transforming growth factor (TGF) β-Smad signal pathway plays an important role in this process (Gressner *et al.*, 2002), and it could activate HSCs and promote collagen synthesis.

Echinacoside (Fig. 1), a phenylethanoidglycoside, is isolated and purified from the stems of *Cistanche salsa* (C. A. Mey.) G. Beck, a parasitic plant native to northwest China, which is used as a traditional Chinese herbal medicine with antisenile and antifatigue effects (Liu, 2005). Previous studies (Wu *et al.*, 2007; 2008) suggested that echinacoside could provide a definite protective effect against acute hepatic injury caused by carbon tetrachloride (CCl₄) in rats. However, its effects on chronic hepatic injury, especially on extracellular matrix synthesis have not been studied. The present study aims at the observation of the effects of echinacoside on chronic hepatic injury and hepatic fibrosis.

* Corresponding author: Yang FR  Address: Tianjin Medical University, Qixiangtai Road, Heping District, Tianjin 300070, China  E-mail: yangfengrui_tj@126.com  Received: February 23, 2013; Revised: April 18, 2013; Accepted: May 18, 2013  Fund: Grants from Tianjin Medical University, China (2009ky42)  Online time: July 17, 2013  Online website: http://www.cnki.net/kcms/detail/12.11410.R.20130717.1619.005.html
Materials and methods

Materials

Echinacoside from *Cistanche salsa* (C. A. Mey.) G. Beck was kindly provided by Datian Fengtuo Pharmaceutical Company (Beijing, China). The purity of the compound was shown to be more than 60% on HPLC.

Experimental animals and CCl<sub>4</sub>-induced hepatic fibrosis

Male Wistar rats weighing 200—250 g were housed six per cage in a room temperature maintained at (22 ± 2) °C with an alternating 12 h light-dark cycle. The rats had food pellets and tap water *ad libitum* and were kept in these facilities for at least 1 week before the experiments. The rats were randomly divided into normal control (*n* = 8), model (*n* = 14), and echinacoside treatment groups (*n* = 14). Except for the normal control group, all the rats were sc injected with the solution of CCl<sub>4</sub> dissolved in peanut oil (CCl<sub>4</sub>-peanut oil 4:6), 5 mL/kg for the first time, and then 3 mL/kg twice a week for 8 weeks. In the former 2 weeks, rats were raised with feedstuff I (80% corn meal, 20% lard, and 0.5% cholesterol). After 2 weeks, they were raised with feedstuff II (corn meal and 0.5% cholesterol). Except for the normal control group, 30% alcohol solution was ig given to each rat every other day from the beginning, 1 mL for each rat. For the normal control group, the peanut oil was sc injected to each rat. Echinacoside was dissolved in water, and ig given by gavage at a daily dose of 50 mg/kg for therapeutic group, all the rats were anesthetized with ether. Blood was taken from the abdominal vein, centrifuged at 4 °C, 3000 r/min for 20 min, and the serum was kept at −20 °C for assay.

Determination of anti-oxidant status

The parameters of anti-oxidation in the liver were determined by measuring the malondialdehyde (MDA) level of serum and the activity of superoxide dismutase (SOD) using commercially available kits (Jiancheng Inst. Biotechnology, Nanjing, China) according to the manufacturer’s instructions.

Liver laboratory tests

The serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) were measured using commercially available kits (Jiancheng Inst. Biotechnology, Nanjing, China) according to the manufacturer’s instructions.

Determination of hepatic hydroxyproline content in liver

Liver (0.5 g) was homogenised in 10 mL of 3% aqueous sulphosalicylic acid. The homogenate was filtered through Whatmann No. 2 filter paper. The filtrate (2 mL) was taken in a test tube to which 2 mL of acid ninhydrin was added and heated in the boiling water bath for 1 h. The reaction was terminated by placing the test tube in ice bath. Toluene (4 mL) was added to the reaction mixture and stirred well for 2 min. The Toluene layer was separated and warmed to room temperature. The intensity of red color was measured at 520 nm. The hydroxyproline content of the liver was expressed as μg/mg of wet weight.

Determination of hyaluronic acid, type IV collagen, type III procollagen, and laminin in sera

The serum levels of hyaluronic acid (HA), type IV collagen (CIV), type III procollagen (PIIIP), and laminin (LN) were determined by radioimmunoassay (RIA) using commercially available kits (Beifang Inst. Biotechnology, Beijing, China) according to the manufacturer’s instructions.

Histological examination

Liver tissues were taken from the left lobe of the liver of each rat, fixed in 15% buffered paraformaldehyde, and dehydrated in a graded alcohol series. The specimens were embedded in paraffin blocks, cut into 5 μm-thick sections, and placed on glass slides. The sections were stained with hematoxylin-eosin (HE) and Masson.

Statistical analysis

The data were analyzed using SPSS 12.0 version. The results were expressed as $\bar{x} \pm s$. The significance of the mean difference between the control group and treatment group was determined by Student’s t-test. The level of $P < 0.05$ was used as the criterion of statistical significance.

Results

Effect of echinacoside on lipid peroxidation

The serum level of MDA was increased in the
model group compared with that of the control group ($P < 0.05$), which indicated that CCl$_4$ triggered the lipid peroxidation reaction. Compared with the model group, the serum level of MDA was decreased in echinacoside therapeutic group (Fig. 2A). The serum level of SOD was decreased in the model group compared with that of the control group. Compared with the model group, the serum level of SOD was increased in echinacoside therapeutic group (Fig. 2B).

**Effect of echinacoside on liver function**

It is evident that CCl$_4$ produced a marked increase in the activities of serum ALT and AST in hepatic fibrosis rats ($P < 0.01$). The echinacoside group showed a significant decrease in the enzyme levels ($P < 0.05$, 0.01), but the levels were still higher than those of control group (Fig. 3).

**Fig. 2  Effect of echinacoside on serum level of MDA (A) and SOD activity (B)**

$^*P < 0.05$ vs control group  $^#P < 0.05$ vs model group

**Effect of echinacoside on hepatic hydroxyproline content**

The hydroxyproline content of the liver could be used as an indirect measure of tissue collagen content. Fig. 4 showed the content of hydroxyproline was increased in the model group compared with that of the control group ($P < 0.01$). Echinacoside could alleviate collagen deposition ($P < 0.01$).

**Fig. 4 Effect of echinacoside on hydroxyproline content in liver**

$^*P < 0.01$ vs control group; $^#P < 0.05$ vs model group

**Effects of echinacoside on serum levels of HA, CIV, PIIP, and LN**

As for the changing trend of fibrotic markers in serum, the serum levels of HA, CIV, PIIP, and LA were similar to those of the enzyme levels. The results showed the serum levels of HA, CIV, PIIP, and LA were significantly higher than those of normal control groups. Echinacoside could decrease them (Fig. 5).

**Effect of echinacoside on hepatic histopathological changes**

At the end of the study, in control group the liver of rats showed normal lobular architecture with central veins and radiating hepatic cords (Fig. 6A). Complete septa interconnecting with each other was formed, which divided the parenchyma into separate fragments and a great number of inflammatory cells were infiltrated in intralobules and interlobules, the cell degeneration and focal necrosis were found in rats with hepatic fibrosis (Fig. 6B), which were improved after echinacoside treatment (Fig. 6C).

**Effect of echinacoside on hepatic collagen deposition**

The rat liver was stained with Masson, and the collagen fiber was shown green. The collagen deposition was markedly increased in the model group compared with the control group. Compared with the model group, collagen deposition was significantly decreased in the echinacoside treatment groups (Fig. 7).
Discussion

Hepatic fibrosis is the common consequence of chronic liver injury of any etiology. Advanced hepatic fibrosis disrupts the normal liver architecture, causing hepatocellular dysfunction and portal hypertension. It is of great significance to search for the effective ways to inhibit fibrogenesis and prevent the development of cirrhosis. Unfortunately, no effective hepatic antifibrotic therapies are available. Colchicine has been commonly used for antifibrosis, but its side effect is severe and its clinical application is limited. Medicinally useful plants are well known for their cheap prices and negligible side effects and have particular potential in the treatment of hepatic fibrosis. In this study, we established the animal model of chemical hepatic fibrosis by long-term administration of CCl4 (Kokko et al., 1992). The histological results showed that the normal structure of lobules was
destroyed and the pseudolobules were formed. Moreover, PIIP, CIV, LN, and HA were known to be good serum markers for hepatic fibrogenesis (Hirata et al., 2001), thus the increased hydroxyproline content in liver and serum levels of PIIP, CIV, LN, and HA also confirmed the hepatic fibrogenesis in rats. The ALT or AST released from hepatocytes were also increased and more immigration of inflammatory cells in liver indicated the severe inflammation in rats with chemical hepatic fibrosis induced by CCl⁴. Those results are in accordance with the findings of CCl⁴-induced hepatic fibrogenesis (Giannini et al., 1999). The present study demonstrated that the administration of echinacoside was effective in treating hepatic fibrosis in rats based on both histological examination and functional analysis. The results obtained provide a basis for further studies on the potentially protective effect of echinacoside on liver function in cirrhotic patients.

The increasing experimental evidence suggests that the reactive oxygen species (ROS) such as H₂O₂, O₂⁻, and OH•, are implicated in the development and pathological progress of hepatic fibrosis. Under normal conditions, low amounts of ROS are produced as by-products of the aerobic respiration. At high doses, ROS are noxious to the cells leading to impaired metabolic functions, growth inhibition, and ultimately cell death. The cells therefore employ several anti-oxidative enzyme systems to maintain low levels of ROS, which plays a key role in hepatic fibrosis. The increased ROS and resulting oxidative stress are commonly detected in livers from patients with alcohol abuse, hepatitis C virus infection, iron overload, or chronic cholestasis, as well as in most types of experimental liver fibrogenesis (Bedossa et al., 1994). The oxidative stress, in particular, lipid peroxidation of cytomebrane, plays an important role in the early stage of hepatic injury, which could activate lipocyte HSCs (Jaeschke, 2000). The activation of HSCs “induced by some critical cytokines” is considered to be of great importance during the long period of hepatic fibrosis (Wu and Ma, 2000). This activated HSCs then become the main source of most cytokines and collagen proteins. In addition to being a product of lipid peroxidation, oxidative stress may result from the derangement of anti-oxidative defenses including SOD and decreased GSH-Px activities. It also acts as a signaling mediator for TGF-β, and plays a major role in hepatic fibrosis. HSCs produced and responded to TGF-β in an autocrine manner with increased collagen expression. Consequently, anti-oxidants, particularly those of plant origin, have emerged as potent anti-fibrotic agents. The previous and recent findings on the antifibrotic potential of plant-derived anti-oxidants showed that they could attenuate hepatic fibrosis in rodents and may exert beneficial effects in the patients with chronic liver diseases.

In the experimental model of CCl⁴-induced fibrosis, hepatic injury occurs with an increased generation of ROS which could cause lipid peroxidation. The beneficial effects and diminished hepatic fibrosis by echinacoside treatment may be partially related to the preservation of anti-oxidative enzyme defenses and reduction of lipid peroxidation.

*C. salsa* is a medicinal plant native to northwest China and used as a crude drug for the treatment of insomnia, renal insufficiency, neurasthenia, and senile constipation. Echinacea is probably the most widely used herbal medicine in the English-speaking world. The phenylethanoid glycosides are the major components of these two kinds of herbs. As one of them, echinacoside is found to have hepatic protective effects recently. Echinacoside was reported to have anti-oxidative stress effects and could decrease caspase-3 activity and TNF-α level. This may provide basis for its antifibrotic effects.

In conclusion, echinacoside could greatly retard the progression of the experimental chemical hepatic fibrosis through the inhibition of collagen synthesis and decreasing oxidative stress. Therefore, it is a potentially new antifibrotic drug for clinical application.

**References**


---

**Latest Progress on *Chinese Herbal Medicines (CHM)***

Since foundation of *Chinese Herbal Medicines* (CHM) in 2009, it has been included in China Academic Journals Integrated Online Database, and Chemical Abstracts Service (CAS) in USA, Index of Copurnicus (IC) in Poland, and Ulrich's Periodicals Directory (UPD) in USA domestically and abroad. In 2011, it can also be cited in Global Health and CAB Abstract. In 2013, CHM can also be indexed in EMBASE in Holland. The CAS report revealed that of all the 49 articles published in CHM in 2012, 31 papers were included in CAS.

We are working hard to make CHM more accessible online and more influential all over the world!