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Rhein inhibits liver fibrosis induced by carbon tetrachloride in rats

GUO Mei-Zi[1], LI Xiao-Sheng, XU Hai-Rong, MEI Zhe-Chuan, SHEN Wei, YE Xiu-Feng[2]

Department of Gastroenterology, Second Affiliated Hospital,
[2]Department of Pathology, Chongqing Medical University, Chongqing 400010, China

KEY WORDS rhein; fibrosis; carbon tetrachloride; rats

ABSTRACT

AIM: To investigate the effect of rhein on liver fibrosis induced by the exposure of carbon tetrachloride (CCl_4)/ethanol in rats. **METHODS:** Male Wistar rats were divided into four study groups (n=10 each group): healthy controls, CCl_4/ethanol-injured rats left untreated, and CCl_4/ethanol-injured rats treated with rhein of low-dose (25 mg·kg^{-1}) and high-dose (100 mg·kg^{-1}). Rhein was given once a day since rat received CCl_4/ethanol injury. After administration of rhein for 6 weeks rats were killed. The following parameters were determined: the activity of alanine aminotransferase (ALT), hyalauronic acid (HA) and procollagen type III (PC-III) concentrations in serum, liver malondialdehyde (MDA) level, the degree of liver fibrosis, and the expression of α-smooth muscle actin (α-SMA) and transforming growth factor-β1 (TGF-β1) in liver tissue. **RESULTS:** The treatment of rhein markedly reduced the ALT activity, HA and PC-III concentrations, and liver MDA level in CCl_4/ethanol-injured rats (P<0.01). It also improved significantly histological changes of fibrosis and decreased the expression of α-SMA and TGF-β1 in liver of these rats (P<0.05 or P<0.01). **CONCLUSION:** Rhein has protective effect on liver injury and can inhibit liver fibrosis induced by CCl_4/ethanol in rats. The mechanisms possibly contribute to its action of antioxidant and anti-inflammatory activity, also associated with its effect of inhibiting TGF-β1 and suppressing the activation of hepatic stellate cells.

INTRODUCTION

Fibrosis occurs as a result of initial liver injury, including hepatocyte damage, Kupffer cell activation, hepatic stellate cell (HSC) activation and proliferation. In hepatic fibrosis, the hepatocyte, Kupffer cell, and HSC communicate by way of oxygen stress, intracellular free calcium $[Ca^{2+}]_i$ increasing and cytokines imbalance. In theory, it should be possible to halt or prevent fibrosis if cell communication was blocked. The Chinese herbal medicines have been used for many years by Chinese investigators treating with liver cirrhosis in the advantage of their low toxicity. Herbal medicines have marked effects against liver injury and fibrosis by blocking cell communication is associated with their action of antioxidant or/and anti-inflammatory[1,2].

[1] Correspondence to Dr GUO Mei-Zi.
Phn 86-23-6384-9075, ext 2352. E-mail aguoyier@sina.com.cn
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Rhein (4,5-dihydroxyanthraquinone-2-carbxylic acid) is one of the most important active components of rhubarb (*Rheum officinale*), a traditional Chinese herb to treat chronic liver disease, shows broad-gauged pharmacological effects. It is reported that rhein blocked the IL-12 mRNA transcription through lowering down the $[Ca^{2+}]_i$ and inhibition of protein kinase C[3]. And recently, data showed rhein has a strong action to suppress the synthesis of some inflammation factors in macrophage, such as leukotriene C4, B4. It also suppresses the metabolism of arachidonic acid[4]. *In vitro* study, rhein has a protection in pancreatic cell injured by TNF-α[5]. It has shown that rhein dose-dependently inhibits superoxide anion production, chemotaxis and phagocytic activity of neutrophils, and macrophage migration and phagocytosis[6]. Data showed rhein can inhibit interleukin 1 beta (IL-1β) synthesis, also decrease the number of its receptors[7]. Studies have shown that expansion and activation of the HSC population follows that of the monocyte/macrophage population, and both cell types accumulate exclusively within areas of necrosis. It induces ECM deposition by simultaneously stimulating the synthesis of new matrix components, increasing the synthesis of enzymes that inhibit ECM degradation, and decreasing the synthesis of matrix-degrading proteases. Soluble factors, such as TNF-α, IL-1β, released by Kupffer cells lead to HSC proliferation and promote their synthesis of collagen, proteoglycans, and hyaluronate. Preventing the action of TNF-α and IL-1β can block the induction of collagen. Besides the antioxidant and anti-inflammatory effects, rhein has other effects such as antitumor and antibacterial. This paper firstly probed the effects of rhein on the liver fibrosis induced by CCl_4/ethanol and its possible mechanisms.

MATERIALS AND METHODS

Reagents Rhein was obtained from Chinese Academy of Medical Sciences (purity >95 %), hyalauronic acid (HA) and procollagen type III (PC-III) RIA kits were purchased from Shanghai Navy Medical Research Institute. Polyclonal antibodies specific for α-smooth muscle actin (α-SMA) and transforming growth factor-β1 (TGF-β1) and immunohistochemical Streptavidin/Peroxidase (SP) kit were products of Santa Cruz Biotech Inc.

Animals and experimental design Male inbred Wistar rats weighing 180-220 g, were purchased from the Experimental Animal Center of Third Military Medical University (Grade II, Certificate No 24301050). They were divided into four groups (ten rats in each group): healthy controls, CCl_4/ethanol-injured rats left untreated, and CCl_4/ethanol-injured ratstreated with rhein of low-dose (25 mg·kg^{-1}) and high-dose (100 mg·kg^{-1}) respectively. Except for the healthy group, all the rats were treated with sc injection of 60 % CCl_4 (0.3 mL·kg^{-1}, but 0.5 mL·kg^{-1} at the first time), mixed with vegetable oil, twice a week for 6 weeks. Simultaneously, they received 5 % ethanol added to their drinking water. The rhein interfused in 0.5 % carmellose sodium (CMC-Na) solution was administrated by gastric gavage at a dose of 25 mg·kg^{-1} or 100 mg·kg^{-1} once a day for the 6-week period of toxin exposure, and the untreated rats only were given 0.5 % CMC-Na solution. All the rats were fed with standard rat chow and received humane care in accordance with the animal care provisions.

Methods At the end of experiment, rats were anesthetized with ether, and blood samples were collected before the rats were killed by decapitation. All the samples were kept at –20 ℃ until the assays were performed. Serum levels of alanine aminotransferase (ALT) were determined by standard hospital laboratory methods, the concentrations of HA and PC-III were detected by radioimmunoassay. The livers were rapidly removed, and divided into three portions, for histological study (stained with H&E, van Gieson's), malondialdehyde (MDA) estimation, and immunohistochemical studies respectively. Qualitative and quantitative histological analyses were performed blindly by light microscoping (Olympus, Japan) and computer image analysis system (CM2000B, Beijing University of Aeronautics & Astronautics). After homogenized and spun the liver tissue, MDA levels were determined according to the method previously described[8] with some modification. Immunohistochemistry was performed using an indirect SP technique. The following

histological grading system for α-SMA and TGF-β1 immunostaining was used[9,10].

Statistical analysis Data are presented as mean±SD. For parametric data, comparisons between groups were examined statistically using one-way ANOVA and *t*-test. For nonparametric data, a rank sum test was used. The level of significant difference was considered when $P<0.05$.

RESULTS

Animal No death in healthy control group. All rat death in other groups were caused by CCl_4/ethanol toxication: 4 rats died in CCl_4/ethanol-injured group, 3 in 25 mg·kg^{-1} rhein-treated group, 1 in 100 mg·kg^{-1} rhein-treated group. Irritability, aggression, and lose weights was present predominantly in the CCl_4/ethanol-injured rats.

Histopathology Liver sections taken from untreated CCl_4/ethanol-injured rats had more inflammatory infiltration, steatosis, hepatocyte coagulative necrosis and fibrous septa than healthy control group. Qualitative and quantitative histological analysis showed rhein markedly improved the degree of hepatic fibrosis in CCl_4/ethanol-injured rats (Tab 1, Fig 1A). Rhein groups displayed delicate fibrous septa, and lower collagen levels than untreated CCl_4/ethanol-injured group (Tab 2).

Tab 1. Degree of liver fibrosis in 4 groups of rats. $^bP<0.05$, $^cP<0.01$ *vs* CCl_4/ethanol.

Group	*n*	Fibrosis grade 0	I	II	III	IV
Healthy control	10	10	0	0	0	0
CCl_4/ethanol	6	0	1	1	3	1
Rhein 25 mg·kg^{-1}	7	0	4	2	1	0
100 mg·kg^{-1}	9	0	6	2	1	0

Liver cirrhosis was graded as 0, no fibrosis and normal liver architecture; I, fibrosis localized only in portal zone and had the inclination of proliferation into lobule; II, fibrosis increased into the lobule of its 2/3 and had the same changes as I; III, fibrosis proliferated into lobule to the periphery of its central vein; IV, fibrosis increased into whole lobule, pseudolobule formation and had the same changes as III.

Tab 2. Distribution of the area of fibrosis in 4 groups of rats. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* CCl_4/ethanol. $^fP<0.01$ *vs* healthy control.

Group	*n*	Area of fibrosis /μm^2	Area of fibrosis/%
Healthy control	10	33±21	0.12±0.06
CCl_4/ethanol	6	2640±1478 ^{f}	9±5^f
Rhein 25 mg·kg^{-1}	7	1375±1263^b	5±4^b
100 mg·kg^{-1}	9	1078±971^c	4±3^c

The area of 29339.36 μm^2 were as the standard views(general area), every tissue sample (aim views) was selected randomly 5 views under the light microscope (× 100), and calculated the percent of area.

The expression of a -SMA and TGF-b 1 protein Both α-SMA and TGF-β1 positive staining of immunohistochemistry was localized in the cytoplasm or membrane. Specimens from untreated CCl_4/ethanol-injured rats contained more α-SMA positive stellate cells than healthy control rats, and also more TGF-β1 expression in the perisinusoidal space. Both α-SMA and TGF-β1 expressing changes had significant difference in rhein groups compared with untreated CCl_4/ethanol-injured group ($P<0.05$ or $P<0.01$, Tab 3, Fig 1B, 1C).

Tab 3. Protein expression of a -SMA, TGF-b 1 in liver in 4 groups of rats. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* CCl_4/ethanol.

Group	*n*	α-SMA	TGF-β1
Healthy control	10	1.0±0.0	1.0±0.0
CCl_4/ethanol	6	5.0±2.5	4.8±2.0
Rhein 25 mg·kg^{-1}	7	3.2±1.9^b	3.0±1.4^b
100 mg·kg^{-1}	9	2.7±1.3^c	2.6±1.6^c

Serum ALT, HA, PC-III levels and MDA tissue concentration As shown in Tab 4, serum ALT

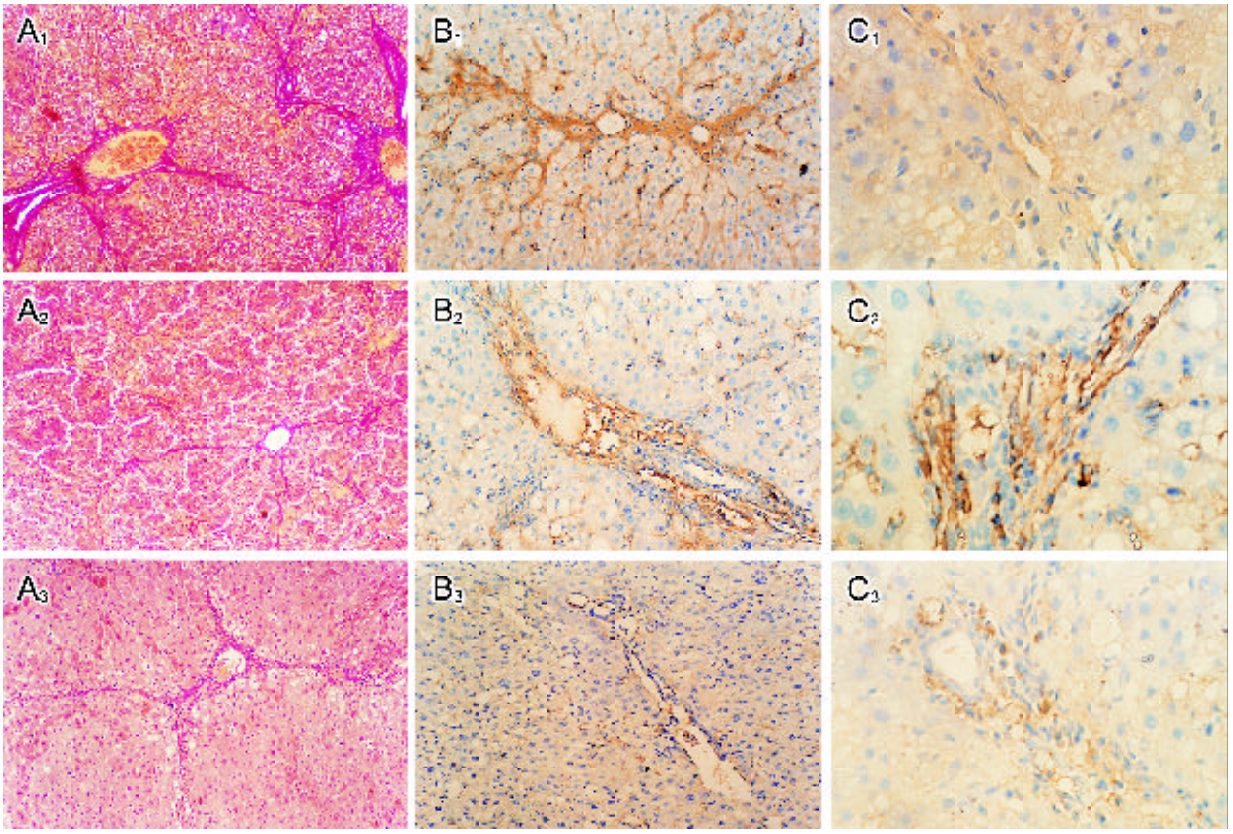

Fig 1. Rhein inhibited liver fibrosis induced by carbon tetrachloride in rats. VG & HE stain, ×100 (A); the expression of a -SMA, ×200 (B); and the expression of TGF-b 1 protein, ×400 (C). 1: CCl_4/ethanol group; 2: rhein 25 mg/kg group; 3: rhein 100 mg/kg group.

Tab 4. Activity of ALT and the level of HA and PC-III in serum and liver MDA concentration in 4 groups of rats. Mean±SD. $^cP<0.01$ *vs* CCl_4/ethanol. $^fP<0.01$ *vs* healthy control.

Group	*n*	ALT/U	HA /μg·L^{-1}	PC-III /μg·L^{-1}	MDA /μmol·g^{-1} protein
Healthy control	10	43±7	141±48	9±3	0.9±0.3
CCl_4/ethanol	6	150±16^f	321±97^f	31±14^f	3.7±0.7^f
Rhein 25 mg·kg^{-1}	7	105±25^c	267±90^c	20±8^c	2.6±0.5^c
100 mg·kg^{-1}	9	78±18^c	217±75^c	16±7^c	1.9±0.3^c

activity was significantly elevated in rats of 6-week CCl_4/ethanol exposure. Compared with the untreated CCl_4/ethanol-injured group, the ALT activity of rhein groups was decreased. Serum HA and PC-III concentrations were approximately twice and three times higher in CCl_4/ethanol-injured group than that in healthy group, and there was a significant decrease in rhein groups ($P<0.01$ *vs* CCl_4/ethanol-injured group). Liver MDA levels were increased in 6-week CCl_4/ethanol exposure rats. The MDA levels in untreated CCl_4/ethanol-injured group were higher than that in the rhein groups ($P<0.05$).

DISCUSSION

Liver fibrosis is characterized by proliferation of HSC and excessive deposition of extracellular matrix (ECM). In our study, we have detected the serum concentrations of PC-III and HA, routinely used to assess the degree of liver inflammation and fibrosis respectively, and simultaneously investigated the change of collagen using computer image analysis, a technique that has been shown to correlate well with the chemical determination of liver hydroxyproline and a histological semi-qualitative scoring system[11,12]. Further more, we as-

sayed for levels of expression of the proteins involved in hepatic fibrosis, namely, TGF-β1 and α-SMA. Our study showed the serum levels of ALT, PC-III, and HA and the fibrosis area decreased significantly in rhein groups than those in CCl_4/ethanol-injured group. It also showed treatment with rhein lowered the mortality of rats and reduced inflammatory infiltration, steatosis, and hepatocyte coagulative necrosis in liver. The results indicated that rhein decreased the synthesis of ECM and suggested that the effect of rhein may be cytoprotective and/or anti-inflammatory in nature.

The CCl_4/ethanol-injured rat is frequently used as an experimental model to study hepatic fibrosis. CCl_4 treatment generates free radicals that trigger a cascade of events that result in hepatic fibrosis. Collagen synthesis in HSC is directly enhanced by oxygen free radicals. Ethanol metabolism induces oxidative stress by increasing the production of free radicals capable of initiating lipid peroxidation. It has been shown in human HSC that the end-product of lipid peroxidation, MDA, stimulates collagen type I gene transcription. Importantly, MDA-adducts has also been observed in quiescent cells in early primary culture, suggesting that lipid peroxidation might activate HSC as well as stimulating their fibrogenesis. Antioxidant and radical scavenging effects of rhein have been studied in rat hepatocytes induced by t-butyl hydroperoxide. Rhein showed high inhibitory activity against peroxidation and was an effective free radical scavenger[13]. Also, rhein blocked lipid peroxidation by suppressing MDA[14]. In our studies, the levels of MDA in the rhein groups were lower than those in CCl_4/ethanol-injured group. It suggested that rhein could inhibit the peroxidation induced by CCl_4/ethanol.

There is now overwhelming evidence that HSC are the principal cells involved in hepatic fibrogenesis. Thus, to control and prevent liver fibrosis depends greatly on controlling activation of HSC. In our study, activated HSC were identified immunohistochemically with polyclonal antibodies raised against α-SMA, and the expression of α-SMA in rhein group rats decreased significantly compared with the CCl_4/ethanol-injured rats. The result suggests that rhein can reduce the activation of HSC. *In vitro* studies demonstrated that rhein inhibited the proliferation of smooth muscle cell[15]. As well known, the smooth muscle cell is a subtype of myofibroblast. In the early pathogenesis of atherosclerosis, vascular smooth muscle cells migrate and proliferate results in atherosclerotic generation. Whether or not rhein can inhibit the proliferation of HSC, the derived myofibroblast, needs further studies.

TGF-β1 plays a central role in liver inflammation and fibrotic pathophysiological state. So far, there are many data demonstrating that rhein reversed the effect of TGF-β1 which play a central role in the synthesis of ECM such as fibronectin, collagen type I, and the proliferation of mesangial cell[16,17]. To address the way in which rhein results in a significant reduction in fibrosis, we have investigated the expression of TGF-β1. The levels of expression of TGF-β1 protein in rhein groups were significantly different from those in CCl_4/ethanol-injured group, including less distribution and thinner staining, which indicated treatment with rhein reduced TGF-β1 expression in rats of fibrosis.

In conclusion, in our first-step study, the biochemical and histological protocol demonstrated that rhein, administrated in a safe dosage with minimal side effects, effectively prevented both the biochemical and histological changes associated with liver fibrosis in CCl_4/ethanol-injured rats. But the precise mechanism involved remains unclear. The hypothesis is that the main mechanism of anti-fibrosis of rhein is related to its action of anti-inflammatory and antioxidant activity, also associated with its decreasing the synthesis of ECM by inhibiting TGF-β1 and suppressing the activation of HSC.

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大黄酸抑制四氯化碳诱导的大鼠肝纤维化形成

郭美姿[1]，李孝生，徐海荣，梅浙川，沈 薇，叶秀峰[2]
（重庆医科大学附属第二医院消化内科，[2] 重庆医科大学病理科，重庆 400010，中国）

关键词 大黄酸；纤维变性；四氯化碳；大鼠

目的： 观察大黄酸对实验性肝纤维化的影响. **方法：** 采用 60 %的四氯化碳(CCl_4)及 5 %的乙醇制备肝纤维化动物模型，分别用小剂量、大剂量大黄酸(25 mg/kg，100 mg/kg 体重)干预，测定血清丙氨酸氨基转移酶(ALT)、透明质酸(HA)、III 型前胶(PC-III)及肝组织丙二醛(MDA)含量，免疫组化方法观察转化生长因子 β1 (TGF-β1)、α 平滑肌肌动蛋白(α-SMA)的表达情况，并观察肝组织胶原面积及病理变化. **结果：** 大黄酸组较模型组：(1)血清 ALT、HA、PC-III 水平及肝组织中 MDA 含量显著降低($P<0.01$)；(2)肝组织中 TGF-β1，α-SMA 的表达显著减少($P<0.05$ 或 $P<0.01$)；(3)肝组织胶原面积明显减少，纤维化程度明显改善($P<0.05$ 或 $P<0.01$). **结论：** 大黄酸具有保肝作用和抑制肝纤维化作用，其作用机制可能与其抗炎、抗氧化作用及抑制 HSC 活化、抑制 TGF-β1 作用有关.

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