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• *BASIC RESEARCH* •

Effects of total glucosides of peony on immunological hepatic fibrosis in rats

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Abstract

AIM: To study the effects of *total glucosides of peony* (TGP) on immunological hepatic fibrosis induced by human albumin in rats.

METHODS: Sixty adult male Sprague-Dawley rats were randomly divided into: Normal group, model group, TGP (60 and 120 mg/kg) treatment groups and colchicines (0.1 mg/kg) treatment group. On the day before the rats were killed, those in TGP or colchicine groups received TGP or colchicine as above from the first day of tail vein injection of human albumin. The rats in normal and model groups were only administered with the same volume of vehicle. At the end of the 16th wk, rats in each group were killed. Blood and tissue specimens were taken. Levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), nitric oxide (NO), content of malondialdehyde (MDA), activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-px), were measured by biochemical methods. Serum procollagen type III (PC III) and laminin (LN) were determined by radioimmunoassay. Liver collagen level was determined by measuring hydroxyproline content in fresh liver samples. Hepatic tissue sections were stained with hematoxylin-eosin and examined under a light microscope.

RESULTS: Histological results showed that TGP improved the human albumin-induced alterations in the liver structure, alleviated lobular necrosis and significantly lowered collagen content. The antifibrotic effect of TGP was also confirmed by decreased serum content of LN and PCIII in TGP-treated group. Moreover, the treatment with TGP effectively reduced the hydroxyproline content in liver homogenates. However, the level of ALT and AST increased in fibrotic rat but had no significance compared with normal control, whereas the ratio of A/G decreased without significance. TGP had no effect on level of ALT, AST and the ratio of A/G. Furthermore, TGP treatment significantly blocked the increase in MDA and NO, associated with a partial elevation in liver total antioxidant capacity including SOD and GSH-px.

CONCLUSION: TGP has beneficial effects on hepatic fibrosis in rats by inhibition of collagen synthesis and decreasing oxidative stress.

Key words: Total glucosides of peony; Hepatic fibrosis; Rat; Oxidative stress

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INTRODUCTION

Hepatic fibrosis is traditionally defined as a progressive pathological process involving multiple cellular and molecular events that lead ultimately to deposition of excess matrix proteins in the extracellular space[1-3]. Chronic injury leading to fibrosis in liver occurs in response to a variety of insults, including viral hepatitis (especially hepatitis B in China), alcohol abuse, drugs, metabolic diseases, *etc*. When this injury process is combined with ineffective regeneration and repair, there is increasing distortion of the normal liver architecture, and the end result is cirrhosis. Current evidence indicates that hepatic fibrosis even cirrhosis is dynamic and can be bidirectional (involving phases of progression and regression)[4,5]. Thus, efforts to understand fibrosis focus primarily on events that lead to the early accumulation of scar in hopes of identifying therapeutic targets to slow its progression. Unfortunately, no effective hepatic antifibrotic therapies are available. Antifibrotic strategies might therefore be usefully targeting towards either reducing matrix synthesis or increasing matrix degradation[6,7].

Paeonia lactiflora pall root, a traditional Chinese herb, has been used to relieve the pain and been a component of effective prescriptions for treatment of liver disease[8]. The *total glucosides of peony* (TGP), a powder substance extracted from *Paeonia lactiflora* pall root, were composed of peoniflorin, hydroxypeoniflorin, peonin, albiflorin, benzoylpeoniflorin, *etc*. Peoniflorin, accounting for some 90%, is a main effective component of TGP. TGP have been recognized as the valuable traditional herbs used in the treatment of rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and hepatitis with a long history in traditional Chinese medicine[9-11]. The anti-inflammatory, anti-oxidative, anti-hepatic injury and immunoregulatory activities without evident toxic or side-effects of TGP have been extensively proved in our laboratory for many years[12-16]. These observations have led to an interest in the potential role of TGP as an antifibrotic agent.

The present study was designed to evaluate whether treatment with TGP exerts any beneficial effect on liver histopathology and liver function in an experimental model of immunological hepatic fibrosis, and the mechanism of its part were also investigated.

MATERIALS AND METHODS

Animal experiments and drug treatment

Sixty adult male Sprague-Dawley rats, weighing 120-150 g [provided by Shanghai BK Experimental Animal Center (Grade II, Certificate No D-65)] were employed in the study. The rats were randomly divided into five groups. A rat model of hepatic fibrosis was produced by immunologically attacking with human albumin, using the method introduced by Wang *et al*[17]. Ten male Sprague-Dawley rats were regarded as normal group. The other fifty healthy rats were randomly divided into four groups including model group, TGP (60 and 120 mg/kg) treated group and colchicines (0.1 mg/kg) treatment group with the experimental attacking as follows.

All rats were injected with 0.5 mL human albumin diluted with normal saline (0.5 mL equals 4 mg human albumin) and the same quantity of an incomplete Freund's adjuvant, once every 14 d for the first two times, then once every 10 d, twice. Ten days after the last injection, serum antibody was measured. Rats with positive serum antibody were chosen for experiment through tail vein injection of human albumin, twice a week, 2.5 mg each in the first week, with a gradual increase of 0.5 mg once each to 4.5 mg eventually, and this dose was maintained for 2 mo. All animals were killed under anesthesia with ether. Blood sample was collected from femoral arteries and veins, centrifuged (3 000 r/min for 10 min), and the serum stored at 4 ℃ until analysis. After this, the liver was quickly washed in situ with ice-cold isotonic saline, removed, and divided into two portions, one was for histological examination, the other immediately frozen in liquid nitrogen.

In this experiment, the animals were divided into five groups randomly which included normal group, model group, TGP (60 and 120 mg/kg) treatment groups and colchicines (0.1 mg/kg) treatment group. The rats in TGP or colchicine groups received TGP or colchicine as above using an 18-gauge stainless steel animal feeding needle from the first day of tail vein injection of human albumin to the day prior to killing the rats. The rats in normal and model groups were fed the same volume of vehicle.

Histopathological examination

Liver tissue sections were fixed in 4 g/L formaldehyde saline and embedded in paraffin. HE staining was performed according to the standard procedure. Histological grade of hepatic fibrosis was determined by a semi-quantitative method based on the criteria of the Knodell index, scoring as the following[18,19], no fibrosis: normal liver and absence of fibrosis; I, perivenular and/or pericellular fibrosis: A few collagen fibrils extended from the central vein and portal tract; II, septal fibrosis: collagen fibrils extension was apparent but had not yet encompassed the whole lobule; III, incomplete cirrhosis: collagen fibrils extended into and encompassed the whole lobule; IV, complete cirrhosis: diffuse extension of collagen fibrils and pseudo-lobule formed. Two pathologists who had no knowledge of their sources examined the stained slides independently. Each sample was observed at 100×magnification and every specimen was analyzed containing a centrilobular vein. The degree of fibrosis was expressed as the mean of 10 different fields in each slide.

Analysis of liver function

The serum activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and the bilirubin concentration were estimated by commercially available kits (Nanjing Jiancheng Institute of Biotechnology, China). Protein conce-ntration was measured according to Lowry *et al*, using serum bovine albumin as standard and then the ratio of albumin and globulin (A/G) was calculated.

Measurement of NO, LN and PC III in serum

Nitric oxide (NO) content in serum was measured by a microplate assay using Griess reagent, which produces a chromophore with the nitrite[20]. The serum levels of procollagen type III (PC III) and laminin(LN) were determined by radioimmunoassays (Shanghai Navy Medical Institute, China). The operations were performed according to the manufacturer's instructions.

Measurement of MDA, SOD and GSH-px level in liver homogenates

Livers were thawed, weighed and homogenized with Tris-HCl (5 mmol/L containing 2 mmol/L EDTA, pH 7.4). Homogenates were centrifuged (1 000 r/min, 10 min, 4 ℃) and the supernatant was used immediately for the assays of MDA,GSH-px and SOD. They were determined following the kit instructions. In brief, MDA in liver tissue was determined by the thiobarbituric acid method[21]. The assays for total SOD and GSH-px were based on their ability to inhibit the oxid-ation of oxyamine by the xanthine-xanthine oxidase system.

Measurement of hydroxyproline content in liver

Liver collagen concentration was determined by measuring hydroxyproline content in fresh liver samples using a modif-ication of the method of Jamall *et al*[22,23], after digestion with acid, as previously reported. Briefly, liver samples were homogenized and hydrolyzed in 6 N HCl at 110 ℃ for 18 h. After filtration of the hydrolysate through a 0.45-mm milli-pore filter, chloramine T was added to a final concent-ration of 2.5 mmol/L. The mixture was then treated with 410 mmol/L paradi-methyl-amino-benzaldehyde and incub-ated at 60 ℃ for 30 min. After cooling to room temper-ature, samples were read spectrophotometrically at 560 nm against a reagent blank containing no tissue and compared with a standard curve of known amount of hydroxyproline. The hydroxyproline content of the liver was expressed as mg/g wet weight.

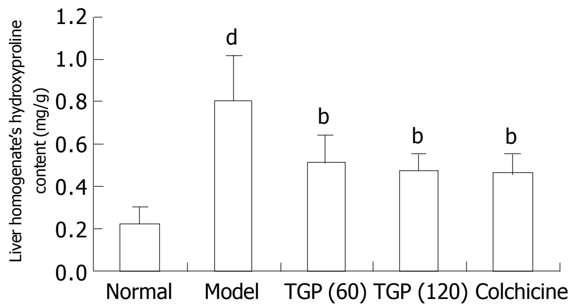

Figure 1 Effects of TGP on contents of hydroxyproline of human albumin-induced fibrotic liver in rats (n = 8, mean±SD) ^{b}P<0.01 *vs* model group; ^{d}P<0.01 *vs* normal control group.

Statistical analysis

mean±SD were calculated for quantitative data. Significant differences between means were evaluated by analyses of variance and in the case of significance, frequency data were compared using Ridit procedure. A difference was considered significant at P<0.05.

RESULTS

Effect of TGP on liver function of immunological hepatic fibrosis

In model group, the level of ALT and AST increased but had no significant difference compared with normal group, whereas the ratio of A/G decreased also with no significant difference. Transaminase activities in TGP or colchicine treated group tended to decrease, whereas the ratio of A/G had an increasing tendency and both had no significance compared with model group (Table 1).

Effect of TGP on hydroxyproline content in liver homogenates

Hepatic fibrosis was quantified by the measurement of hepatic hydroxyproline. It was found that the hydroxyproline content of the model group was significantly higher than that of the normal group. Treatment with TGP or colchicine effectively prevented the immunological hepatic fibrosis induced by human albumin by reducing the hydroxyproline content in liver homogenates (Figure 1).

Histological results

From Table 2, we can see the significant difference of pathologic grading between the normal and model groups. The pathologic grading was significantly decreased in TGP or colchicine treated group.

As shown in Figure 2, the structure of liver tissues was normal in control group (Figure 2A). In liver tissues from rats with immunological hepatic fibrosis, hyperplasia of the lattice fibers and collagenous fibers was observed in portal area and extended outwards. Hyperplasia surrounding the central vein observed was distributed along hepatic sinuses and associated with each other. The hepatic lobules were encysted and separated by collagen bundles. The normal structure of lobules was destroyed and pseudolobules formed. Infiltration of small numbers of inflammatory cells was found around the portal area and central vein (Figure 2B). TGP alleviated lobular necrosis and significantly lowered collagen content. The structure of liver tissues was almost normal (Figure 2C). In colchicine-treated group, hyperplasia of the lattice fibers and collagenous fibers was also observed in portal area, but they were alleviated compared with model group (Figure 2D).

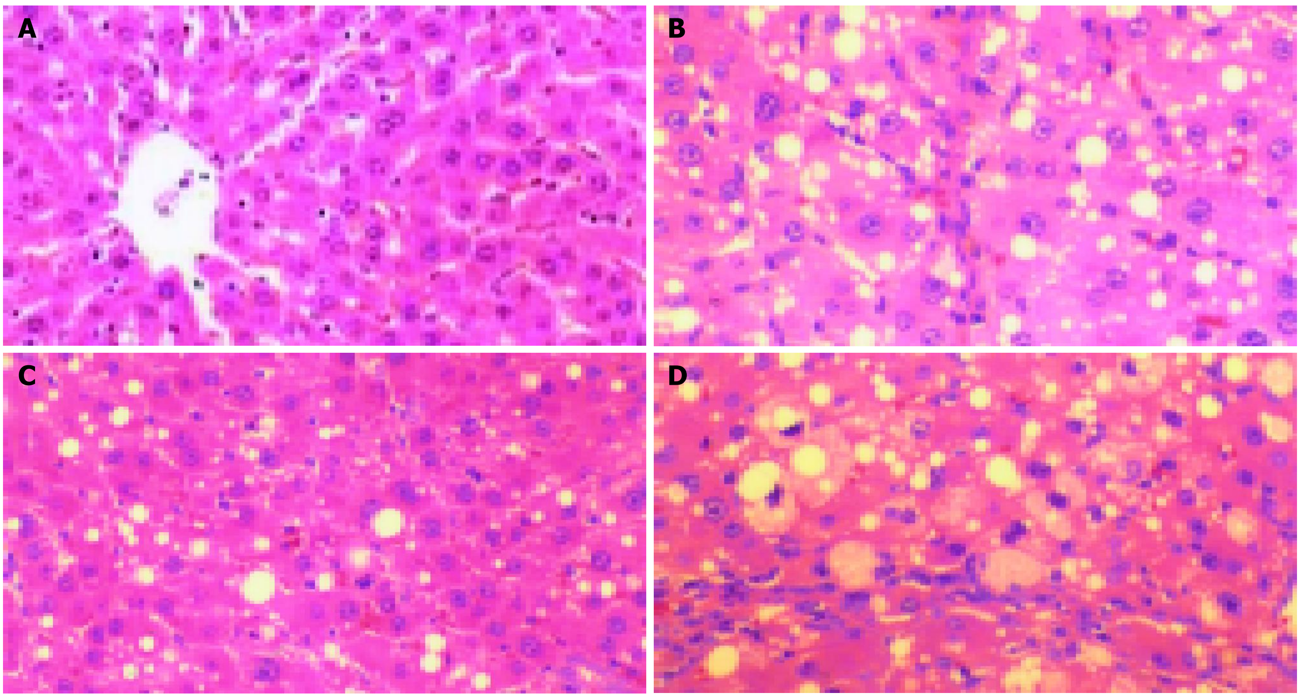

Figure 2 Histological results of tissues stained with HE under light microscope. A: Normal group; B: Model group; C: TGP-treated group; D: Colchicine-treated group.

Table 1 Effect of TGP on serum ALT, AST activities and A/G value in immunological hepatic fibrosis rat induced by human albumin. (*n* = 8, mean±SD)

Groups	Doses (mg/kg)	ALT (U/L)	AST (U/L)	A/G
Normal	--	54.63±22.50	61.47±27.81	1.17±0.31
Model	--	78.79±15.03	94.51±37.30	0.90±0.19
TGP	60	75.67±22.50	82.25±17.33	0.92±0.21
	120	70.61±24.62	75.43±26.94	0.96±0.17
Colchicine	0.1	77.71±27.64	78.99±22.24	0.93±0.18

Table 3 Effect of TGP on plasma LN and PC III level in immunological hepatic fibrosis rat induced by human albumin. (*n* = 8, mean±SD)

Groups	Doses (mg/kg)	LN (μg/L)	PC III (μg/L)
Normal	---	106.9±25.4	74.4±25.9
Model	---	228.0±76.2[d]	294.1±99.2[d]
TGP	60	142.1±39.8[a]	174.7±69.5
	120	127.1±26.8[b]	148.5±68.6[a]
Colchicine	0.1	112.6±27.7[b]	121.6±32.6[b]

[a]$P<0.05$, [b]$P<0.01$ *vs* model group; [d]$P<0.01$ *vs* normal control group.

Effect of TGP on serum LN and PC III

As expected, serum levels of LN and PC III, the surrogate markers of liver fibrogenesis, increased significantly in hepatic fibrotic rats in model group. However, in TGP-treated group they were lower compared with model group. These data confirmed the histological findings that TGP could inhibit hepatic fibrogenesis (Table 3).

Effect of TGP on MDA content and SOD, GSH-px activities in liver homogenates

Hepatic lipid peroxidation, measured as thiobarbituric acid reactive substance (MDA), was significantly increased in fibrotic rats while liver SOD and GSH-px activities decreased. TGP treatment significantly blocked the increase in MDA and was associated with a partial elevation in liver total antioxidant capacity including SOD and GSH-px (Table 4). In colchicine-treated group, only MDA content was lower than model group.

Effect of TGP on NO production in serum

As shown in Figure 3, when the rats were challenged with human albumin, the level of NO was elevated significantly. TGP obviously decreased the NO level while colchicine had no effect.

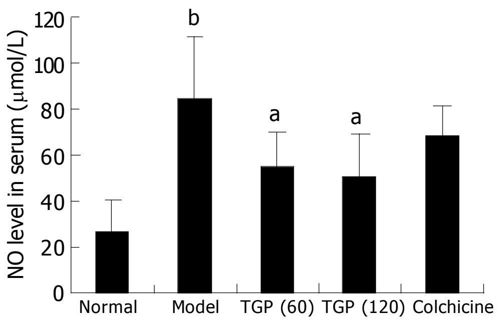

Figure 3 Effects of TGP on NO level in immunological hepatic fibrotic rats (*n* = 8, mean±SD), [a]*P*<0.05 *vs* model group; [b]*P*<0.01 *vs* normal control group.

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 effective ways to inhibit fibrogenesis and prevent the development of cirrhosis[24]. Unfortunately, no effective hepatic antifibrotic therapies are available. Colchicine has been commonly used for anti-fibrosis, but its side effect is severe and its clinical application is limited. Medicinally useful plants including traditional Chinese herbs are well known for their cheap prices and negligible side effects and have particular potential in the treatment of hepatic fibrosis[25,26]. In this study we firstly established the animal model of immunological hepatic fibrosis. The histological results showed that the normal structure of lobules was destroyed and pseudolobules formed, which were similar to the pathology of chemical hepatic fibrosis induced by long-term administration of carbon tetrachloride. Moreover, LN and PC III are known to be good serum markers of hepatic fibrogenesis, thus the increased hydroxyproline content in liver and serum LN and PC III also confirmed the hepatic fibrogenesis in rats. Unlike the severe hepatocyte necrosis and inflammation induced by CCl_4 toxicity, the ALT or AST released from hepatocytes did not increase and less immigration of inflammatory cells in liver indicated the mild inflammation in rat with immunological hepatic fibrosis induced by human albumin. Those results are in accordance with the findings of immunological hepatic fibrogenesis[17]. The present study demonstrated that administration of TGP was effective in

Table 2 Effect of TGP on the pathologic grading of immunological hepatic fibrosis rat induced by human albumin. (*n* = 10, mean±SD)

Group	Dose (mg/kg)	Pathologic grading of hepatic fibrosis 0	I	II	III	IV	*P*
Normal	–	10	0	0	0	0	–
Model	–	0	0	3	3	4	0.000[d]
TGP	60	0	2	5	2	1	0.043[a]
	120	0	4	4	2	0	0.006[b]
Colchicine	0.1	0	3	4	3	0	0.016[a]

[a]$P<0.05$, [b]$P<0.01$ *vs* model group; [d]$P<0.01$ *vs* normal control group.

Table 4 Effects of TGP on MDA levels, SOD and GSH-px activities liver homogenates in of immunological hepatic fibrotic rats (*n* = 10, mean±SD)

Groups	Doses (mg/kg)	MDA (nmol/mg pr)	SOD (U/mg pr)	GSH-Px (U/mg pr)
Normal	-	1.88±0.73	142.33±41.00	101.00±28.70
Model	-	4.34±1.12[d]	81.95±26.48[d]	50.64±15.28[d]
TGP	60	2.41±0.85[b]	116.95±32.18	73.70±16.60
	120	2.21±0.89[b]	136.53±36.15[a]	83.64±26.16[a]
Colchicine	0.1	3.02±0.68[a]	101.19±44.47	69.80±23.65

[a]$P<0.05$, [b]$P<0.01$ *vs* model group; [d]$P<0.01$ *vs* normal control group.

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 TGP on liver function in cirrhotic patients.

Increasing experimental evidence suggests that reactive oxygen species (ROS) such as H_2O_2, O_2^-, and OH•, are implicated in the development and pathological progress of hepatic fibrosis[27-30]. 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. Cells therefore employ several antioxidant enzyme systems to maintain low levels of ROS, which plays a key role in hepatic fibrosis[29]. 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[31]. Oxidative stress, in particular, lipid peroxidation, induces collagen synthesis. It also acts as a signaling mediator for transforming growth factor (TGF)-β, and plays a major role in hepatic fibrosis. Hepatic stellate cells produce and respond to TGF-β in an autocrine manner with increased collagen expression. Consequently, antioxidants, particularly those of plant origin, have emerged as potent antifibrotic agents. Previous and recent findings on the antifibrotic potential of plant-derived antioxidants could attenuate hepatic fibrosis in rodents and may exert beneficial effects in patients with chronic liver diseases.

Our previous study showed that TGP attenuated inflammation and ROS and TGP inhibited hydrogen peroxide (H_2O_2) were released from peritoneal macrophages in adjuvant arthritis (AA) rats[15]. It was also found that treatment of AA rats with TGP (50 mg/kg) ig (14-28 d) could counteract the elevated level of MDA and NO and the lowered activities of SOD and GSH-px. The hemolytic action of H_2O_2 is related to the induction of lipid perox-idation and glutathione depletion in human erythrocytes. TGP (0.5-2.5 mg/L) could significantly inhibit hemolysis, lipid peroxidation, and glutathione depletion induced by H_2O_2. *In vitro*, TGP could scavenge OH· and O_2^-[9-15]. In the experimental model of human albumin-induced fibrosis, hepatic injury occurs with an increased generation of ROS that causes lipid peroxidation. In addition to being a product of lipid peroxidation, oxidative stress may result from deran-gement of antioxidant defenses including SOD and decr-eased GSH-px activities. Inverse correlations between antioxidant enzymes and pathology scores and/or lipid pero-xidation have been found in rats with CCl_4-induced cirrhosis, biliary obstruction, or alcoholic liver disease[30-32]. Beneficial effects and diminished hepatic fibrosis by TGP may be partially related to the preservation of antioxidant enzymes defenses and reduction of lipid peroxidation, but colchicine treatment only significantly inhibited malondialdehyde[33]. Changes in nitric oxide (NO) production may also play a role in the TGP-induced prevention of liver damage in cirrhotic rats. NO has been shown to react with superoxide generating the strong oxidant peroxynitrite, which can initiate lipid peroxidation or cause a direct inhibition of the mitoch-ondrial respiratory chain[34]. TGP was found to inhibit prod-uction of NO in this human albumin induced immun-ological hepatic fibrotic rats.

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

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