Antifibrotic Effects of a Polysaccharide Extracted from *Ganoderma lucidum*, Glycyrrhizin, and Pentoxifylline in Rats with Cirrhosis Induced by Biliary Obstruction

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For the past few years, we have been investigating polysaccharides from *Ganoderma lucidum* as antifibrotic agents. In a previous study, we discovered that polysaccharides extracted from *G. lucidum* lowered the collagen content in liver but had no effect on serum biochemical parameters in rats subjected to bile duct ligation and scission-induced fibrosis. In this study, we changed the extraction method and obtained polysaccharides extracted from *G. lucidum*. The polysaccharide from *G. lucidum* reduced the serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and total bilirubin and also reduced the collagen content in liver and improved the morphology. Pentoxifylline, which is reported to exhibit an antifibrotic effect in pigs with fibrosis induced by yellow phosphorus, did not have any antifibrotic effects in fibrosis induced by biliary obstruction. Glycyrrhizin, which is used in the treatment of hepatitis, reduced serum ALT and AST values but there was no significance. It had no effect on liver hydroxyproline content which implies that glycyrrhizin has no antifibrotic effect in the rats with fibrosis induced by bile duct ligation and scission. These data suggest that the polysaccharide from *Ganoderma lucidum* could be a promising antifibrotic agent. However, further study is needed to understand the inhibition mechanism of collagen deposition of polysaccharides from *Ganoderma lucidum* and its clinical applicability remains to be established.

Key words *Ganoderma lucidum*; glycyrrhizin; pentoxifylline; antifibrotic agent; bile duct ligation/scission

Liver cirrhosis is a chronic progressive disease characterized by increased connective tissue synthesis and deposition in the interstitial space with parenchymal cell injury, resulting in formation of structurally abnormal nodules and liver failure.¹⁾ Whatever the etiology may be, chronic injury leads to liver fibrosis and cirrhosis.²⁾

Until recently, there has been no specific treatment for hepatic fibrosis (cirrhosis) although it is one of the commonest causes of all deaths.^{2–4)} It has been reported that activated mesenchymal cells seem to play an important role in connective tissue synthesis and deposition, although the mechanism of fibrosis has not been fully explained yet.⁵⁾ However, it is evident that pharmacological inhibition of new connective tissue formation and deposition appears to be a promising therapeutic approach.

In the past few years, we have screened a number of antifibrotic agents. During this we have noticed that the aqueous extract of *Ganoderma lucidum* had a hepatoprotective effect in CCl₄-intoxicated mice.⁶⁾ In addition, polysaccharide extracted from the mycelium of *Ganoderma lucidum* (PG) reduced the hydroxyproline content of liver when liver fibrosis was induced by bile duct ligation/scission in rats.⁷⁾ It has been reported that glycyrrhizin (GL) has a γ -interferon-inducing ability in mice⁸⁾ which is responsible for inhibition of collagen production in fibroblasts and it inhibits hepatic lipocyte activation.^{9,10)} It also has a hepatoprotective effect in hepatocytes.¹¹⁾ In *in vitro* testing, pentoxifylline (PF), a methylxanthine, reduces the platelet-derived growth factor (PDGF)-driven proliferation of fibroblasts.¹²⁾ Also, it has been reported that long-term administration of pentoxifylline to pigs with fibrosis induced by yellow phosphorus prevented the histological changes associated with fibrosis and elevation of γ -glutamyl transferase and alkaline phosphatase.¹³⁾

These findings led us to investigate if PG, GL and PF could protect the liver from the fibrosis and cirrhosis induced by biliary obstruction.

MATERIALS AND METHODS

Female Sprague–Dawley rats (initial body weight: 200–250 g) were used. They received normal chow and water *ad libitum* and were maintained under 12-h light-dark cycles throughout the experiment. Rats were anesthetized with Ketamine/Rompun and the double ligations were performed on the common bile duct with a section between the ligatures.⁶⁾ In sham rats, only an incision was made in the abdomen which was then closed without any treatment. The number of rats used in each group is shown in Table 1.

To study the antifibrotic effect of *Ganoderma lucidum*, we extracted protein-bound polysaccharide with components of molecular weight typically 5000–20000 kDa by the following method: culture medium of the mycelium of *Ganoderma lucidum* was centrifuged and the precipitate was extracted using sodium hydroxide (final concentration: 2 N NaOH) at room temperature for 24 h. After extraction, it was neutralized to pH 7.0 using acetic acid and centrifuged at 3000 $\times g$. The supernatant underwent ultrafiltration (M.W. 10000) and was then freeze dried. The resulting sample consisted of polysaccharides, mostly with a (1 $\rightarrow$ 3) D glucan bond. The freeze-dried sample (polysaccharides from *Ganoderma lucidum*) was suspended in distilled water, and given orally to rats from the beginning of the experiment up to 28 d, at a dose of 5 mg/rat/d. PF was purchased from Sigma Chemical Co. (U.S.A.), and GL from Junsei Chemical Co., Ltd. Each drug was either dissolved or suspended in distilled water

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and was given orally to rats at a dose of 6 mg/rat/d up to 28 d. The control groups received the equal amounts of solvent.

The rats were weighed weekly during the experiment. After 28 d of treatment, rats were anesthetized with ether and blood was obtained by cardiac puncture for serum biochemical testing. Immediately, the liver was removed and weighed. It was then kept at -20°C for hydroxyproline determination and a portion was stored in 10% neutralized formalin for morphological examination.

The liver hydroxyproline content was determined as described by Jamall *et al.*¹⁴⁾ Serum biochemical testing, which included measurement of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and total bilirubin, was carried out on a clinical chemistry analyzer (Gilford 400E, U.S.A.) using diagnostic kits (Ciba-Corning, U.S.A.). For morphological examination, sections of paraffin-embedded liver were stained with hematoxylin and eosin.

Results were expressed as means $\pm$ S.D. Statistical differences were determined between groups by ANOVA. Then, Tukey's multiple comparison test was performed. Values of $p < 0.05$ were considered to indicate a significant difference.

RESULTS

Effects of PG, GL and PF on Body and Liver Weight All the rats with cirrhosis induced by bile duct ligation and scission (BDL/S) showed a slight decrease in body weight due to the operation during the first week and then returned to normal weight afterwards. Between control BDL/S rats and treated BDL/S rats, there was no

significant difference in body weight. In BDL/S rats, the liver weight increased dramatically 28 d after biliary obstruction ($p < 0.01$). The liver to body weight ratio of treated BDL/S rats was slightly lower than that of the control BDL/S rats, but there was no significant difference (Table 1). The spleen was significantly enlarged in BDL/S operated rats, whether rats were treated or not, due to inflammation and liver failure ($p < 0.05$).

Hydroxyproline Content of Liver As shown in Fig. 1, BDL/S increased the hydroxyproline content about 3-fold ($p < 0.01$). Compared with the control BDL/S group,

Fig. 1. Hydroxyproline Content of Liver from Cirrhotic Rats with BDL/S Treated with PG, GL or PF

* Significantly different from control sham group ($p < 0.01$). # Significantly different from control BDL/S group ($p < 0.01$). Number of rats in each group is the same as in Table 1.

Table 1. Body Weight and Liver Weight Changes in Rats with Cirrhosis Induced by BDL/S Treated with PG, GL and PF

Group	n	Body weight (g)		Liver weight (g)	Liver wt./body wt.	Spleen weight (g)
		0 wk	4 wk			
Control sham	16	232 $\pm$ 19	246 $\pm$ 17	8.24 $\pm$ 1.97	3.34 $\pm$ 0.51	1.23 $\pm$ 0.59
PG sham	8	230 $\pm$ 26	254 $\pm$ 26	9.07 $\pm$ 1.92	3.57 $\pm$ 0.26	1.21 $\pm$ 0.26
GL sham	4	220 $\pm$ 17	250 $\pm$ 50	8.03 $\pm$ 0.82	3.55 $\pm$ 0.46	1.23 $\pm$ 0.40
PF sham	4	238 $\pm$ 9	243 $\pm$ 16	8.05 $\pm$ 0.71	3.31 $\pm$ 0.17	1.25 $\pm$ 0.63
Control BDL	18	236 $\pm$ 22	254 $\pm$ 28	19.51 $\pm$ 3.36**	7.76 $\pm$ 1.41**	2.56 $\pm$ 0.58**
PG BDL/S	13	229 $\pm$ 14	259 $\pm$ 24	19.89 $\pm$ 3.74**	7.65 $\pm$ 0.97**	2.18 $\pm$ 0.60**
GL BDL/S	9	244 $\pm$ 10	266 $\pm$ 13	20.36 $\pm$ 3.28**	7.69 $\pm$ 1.03**	2.10 $\pm$ 0.77*
PF BDL/S	9	252 $\pm$ 13	269 $\pm$ 19	19.37 $\pm$ 3.51**	7.21 $\pm$ 1.34**	2.38 $\pm$ 0.56**

* Significantly different from control sham group ($p < 0.05$). ** Significantly different from control sham group ($p < 0.01$). n, number of rats.

Table 2. Serum Biochemical Values in Rats with Cirrhosis Induced by BDL/S Treated with PG, GL and PF

Group	n	ALT (IU/l)	AST (IU/l)	ALP (IU/l)	Total bilirubin (mg/dl)
Control sham	16	62.7 $\pm$ 16.5	167.5 $\pm$ 38.0	184.5 $\pm$ 80.3	0.45 $\pm$ 0.36
PG sham	8	66.3 $\pm$ 12.5	136.3 $\pm$ 23.1	165.9 $\pm$ 34.1	0.26 $\pm$ 0.18
GL sham	4	50.3 $\pm$ 11.9	211.5 $\pm$ 84.4	267.7 $\pm$ 100.2	0.20 $\pm$ 0.20
PF sham	4	57.8 $\pm$ 18.9	213.0 $\pm$ 60.8	301.3 $\pm$ 128.5	0.51 $\pm$ 0.47
Control BDL/S	18	136.4 $\pm$ 40.7**	715.0 $\pm$ 126.2**	499.4 $\pm$ 138.7**	13.43 $\pm$ 3.46**
PG BDL/S	13	101.1 $\pm$ 33.5	434.4 $\pm$ 148.3**,*	301.5 $\pm$ 151.3**,*	9.43 $\pm$ 1.98**,*
GL BDL/S	9	109.1 $\pm$ 45.6**	515.7 $\pm$ 117.9**,*	668.1 $\pm$ 214.0**,*	9.60 $\pm$ 1.78**,*
PF BDL/S	9	130.4 $\pm$ 56.1**	394.0 $\pm$ 138.6**,*	798.2 $\pm$ 217.1**,*	10.71 $\pm$ 2.49**,*

* Significantly different from control sham group ($p < 0.05$). ** Significantly different from control sham group ($p < 0.01$). # Significantly different from control BDL group ($p < 0.05$). ** Significantly different from control BDL group ($p < 0.01$). n, number of rats.

treatment with PG reduced the hydroxyproline content in the liver by up to 76% ($p < 0.01$). In contrast, PF and GL had little or no effect on the hydroxyproline content of cirrhotic rat liver. In GL-, PG- or PF-treated sham rats, there were no significant changes in hydroxyproline content compared with that of control sham rats (data not shown).

Serum Biochemical Testing Serum biochemical parameters are shown in Table 2. Levels of serum AST, ALT, ALP and total bilirubin were elevated significantly in control BDL/S rats ($p < 0.01$). In BDL/S rats treated with PG, serum ALT and ALP levels were reduced to 74% and 60% that of control BDL/S rats, respectively, but the differences were not significant. Serum AST and total bilirubin levels were reduced to 61% and 70% that of control BDL/S rats, respectively, and this difference was statistically significant ($p < 0.01$). In GL-treated rats, the serum AST and total bilirubin levels were significantly reduced ($p < 0.01$), but the ALP level was significantly increased when compared with control BDL/S rats ($p < 0.01$). In BDL/S rats treated with PF, serum levels of AST ($p < 0.01$) and total bilirubin ($p < 0.05$) were significantly reduced, but the serum ALP level increased to 160% that of control BDL/S rats ($p < 0.01$).

Morphological Changes in Liver Histology of BDL/S rat liver showed excessive bile duct proliferation, inflammation and connective tissue deposition resulting in destruction of the lobular architecture. In PG-treated rats, there was a tendency towards less pronounced destruction of the liver architecture, although neither bile duct

proliferation nor inflammation was reduced when compared with control BDL/S rat liver. In both GL- and PF-treated BDL/S rats, the liver histology was similar to that of BDL/S rats, showing that GL and PF have no antifibrotic effects in BDL/S rats.

DISCUSSION

Therapy for hepatic fibrosis should interfere specifically with the production of hepatic connective tissue proteins.¹⁵ The best therapeutic strategies can be designed only with a full understanding of the mechanisms involved in fibrogenesis, an understanding which is still incomplete. Nonetheless, any intervention that blocks collagen deposition will probably be effective in reducing hepatic fibrosis, regardless of the mechanisms involved.

There has been a lot of effort devoted to finding the effective drugs for hepatic fibrosis and it has been reported that corticosteroids,¹⁶ penicillamine,¹⁷ colchicine,^{17,18} proline hydroxylase inhibitors,¹⁹ prostaglandins,¹⁷ γ -interferon,^{5,9,10} proline analogues (azetidine carboxylic acid),⁵ retinoids¹⁷ and malotilate²⁰ have antifibrotic effects, but there are few promising drugs that are effective and safe in clinical situations.⁵

For the past few years, we have been investigating polysaccharides from *Ganoderma lucidum* as antifibrotic agents. In a previous study, we discovered that polysaccharides extracted from *Ganoderma lucidum* lowered the collagen content of liver but it raised the serum biochemical values in BDL/S rats.⁷ In this study, we

Fig. 2. Microscopic Appearance of Liver of Rats with Cirrhosis Induced by BDL/S (Hematoxylin & Eosin, 63 $\times$)

A: BDL/S control. B: Polysaccharides from *Ganoderma lucidum* treated BDL/S. C: Glycyrrhizin treated BDL/S. D: Pentoxifylline treated BDL/S.

changed the extraction method and obtained the protein-bound polysaccharide fraction (M.W. 5000—20000 kDa) of *Ganoderma lucidum*, which is known to have β -1,3 bonds. It reduced the serum AST, ALT, ALP and total bilirubin and also lowered the collagen content in liver and improved its morphology. This fraction also produced a marked reduction in serum biochemical parameters in acute liver damage models such as CCl_4 and thioacetamide. However, we failed to improve the liver fibrosis in the BDL/S rats treated with pentoxifylline or glycyrrhizin, agents which have been reported to have a hepatoprotective effect.

Recently, the absorption, tissue distribution, metabolism and the excretion of this polysaccharide (^{14}C -labeled) have been studied in rats. Polysaccharide was labeled by adding D-[U- ^{14}C]glucose to the culture broth of *Ganoderma lucidum*, and about 10.5% of the added D-[U- ^{14}C]glucose was incorporated into the polysaccharide. The oral dose (25 mg/kg) pharmacokinetic study showed that the $t_{1/2}$ was approximately 41 h and the compound was excreted in urine, faeces, and bile. Following oral administration, the greatest amount of radioactivity in the gastrointestinal tract was measured within 7 h of dosing. The greatest concentration of radioactivity in the liver were measured at 7 and 24 h, at which times the liver contained about 2% of the administered radioactivity. The radioactivity in liver was always greater than in plasma, increasing 2-fold at 0.5 h to about 6-fold at 168 h.

The results of this study suggest that the polysaccharide from *Ganoderma lucidum*, could be a promising antifibrotic agent. Further study is needed to understand the mechanism by which this polysaccharide inhibits collagen deposition and its clinical applicability remains to be established.

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