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Journal of Ethnopharmacology 87 (2003) 175–180
Journal of ETHNO-PHARMACOLOGY
www.elsevier.com/locate/jethpharm

Effect of *Holotrichia diomphalia* larvae on liver fibrosis and hepatotoxicity in rats

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Received 30 April 2002; received in revised form 28 March 2003; accepted 28 March 2003

Abstract

Holotrichia diomphalia larvae, one of the most widely used Korean folk medicinal preparations, have long been used for the treatment of chronic liver cirrhosis. The present study was undertaken to clarify whether extract of *Holotrichia diomphalia* larvae could prevent acute liver damage and liver fibrosis in rats. A single administration of *Holotrichia diomphalia* protected rats from acute liver damage induced by carbon tetrachloride (200 μl/kg, i.p.) and β-D-galactosamine (600 mg/kg, i.p.). This was evidenced by the lowered serum aminotransferase (ALT, AST) activities in rats treated with *Holotrichia diomphalia*. The hepatic cirrhosis was induced by 28 days of bile duct ligation/scission in rats. The four-week treatment with *Holotrichia diomphalia* reduced the serum ALT, AST, alkaline phosphatase activities, and hydroxyproline content in the liver and improved the histological appearance of the liver sections. The present results led us to conclude that *Holotrichia diomphalia* larvae can reduce the degree of hepatocellular damage and may become a promising antifibrotic agent for liver fibrosis/cirrhosis.

Keywords: *Holotrichia diomphalia* larvae; Acute hepatotoxicity; Liver cirrhosis

1. Introduction

Recently, the number of cases of liver disease has tended to increase year by year, and more than 15,000 deaths from this disease occur annually in Korea. Hepatic fibrosis is an important feature of, and a common sequel to, most forms of chronic liver disease and is an essential component in the development of cirrhosis (Friedman, 1993). Therefore, the prevention or suppression of fibrotic changes in the liver or protection from and treatment of cirrhosis is important. At present, there is no effective therapy to cure cirrhosis or to prevent its complications. Furthermore, there are no drugs to suppress fibrosis, therefore, it is important to prevent the development of hepatic fibrosis.

Holotrichia diomphalia (Coleoptera, Scarabaeidae) larvae (Scarab beetle) have been traditionally used in Korea for the treatment of liver cirrhosis, contusion, edema, furuncle and apoplexy. Recently, potent antibacterial proteins have been isolated from *Holotrichia diomphalia* larvae (Lee et al., 1994). Prophenoloxidase from the hemolymph of *Holotrichia diomphalia* larvae has also been purified and characterized (Kwon et al., 1997). Furthermore, our own recent studies have shown that *Holotrichia diomphalia* larvae augment macrophage function and stimulate the synthesis and release of cytotoxic mediators (Kang et al., 2002). However, the hepatoprotective effects of *Holotrichia diomphalia* larvae have not been investigated.

The present study was undertaken to investigate the hepatoprotective effect of *Holotrichia diomphalia* larvae against acute liver injury and liver fibrosis.

2. Materials and methods

2.1. Animals and treatments

Male, Sprague–Dawley rats weighing 240 ± 20 g were obtained from Jeil Animal Breeding Company of Korea and were acclimated to the laboratory conditions at Sungkyunkwan University for at least one week. During this period, food (Samyang Co., Korea) and tap water were supplied ad libitum. The experimental animals were kept in

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0378-8741/03/$ – see front matter
doi:10.1016/S0378-8741(03)00140-5

a temperature- and humidity-controlled room (25 ± 1 °C, 55 ± 5%, respectively) with a light–dark cycle of 12 h, and were fasted 18 h before the experiment.

2.2. Chemicals

Carbon tetrachloride, β-D-galactosamine hydrochloride, silymarin, *p*-dimethylaminobenzaldehyde, and *p*-toluene-sulfon-chloramide (chloramine T) were supplied by the Sigma Chemical Co., USA. All the other chemicals used in this study were reagent grades and are locally and commercially available.

2.3. Preparation of crude extracts

Holotrichia diomphalia larvae (1 kg) were purchased at the herbal drug market in Cheju-Do, Korea, in August 1999 and identified by Dr. B.G. Lee of the Institute for Traditional Medicine, Sungkyunkwan University, Suwon, Korea. A voucher specimen (SKK-H001) is deposited in the College of Pharmacy at Sungkyunkwan University. Air-dried and chopped *Holotrichia diomphalia* larvae (1 kg) were refluxed with 70% ethanol (2 l) two times for 8 h. The materials were filtered, and the clear supernatant was then concentrated under reduced pressure at 40 °C with a vacuum rotary evaporator. The concentrated ethanol extract (100 g) was partitioned between water (1 l) and *n*-hexane (0.5 l × 2, 20 g). After removing the *n*-hexane fraction, the aqueous layer was partitioned again with methylene chloride (0.5 l × 2, 10 g), followed by *n*-butanol (0.5 l × 2, 40 g). The extract was evaporated and the residue was used for the experiment.

2.4. Acute hepatotoxicity

Five groups of animals were studied. All animals were treated humanely under Sungkyunkwan University Animal Care Committee guidelines. The rats in group I (vehicle) received only olive oil (2 ml/kg, i.p.). In groups II–V, carbon tetrachloride (CCl_4) was dissolved in olive oil (1:9) (v/v), then intraperitoneally administered (final concentration: 200 μl/kg). Four hours after the CCl_4 treatment, groups I (vehicle) and II (control) were treated with saline (10 ml/kg, p.o.), and groups III–V were treated with silymarin (positive control, 300 mg/10 ml/kg, p.o.) and *Holotrichia diomphalia* (100, 300 mg/10 ml/kg, p.o.). Following 24 h CCl_4 administration, blood was taken from the abdominal aorta for the assay of serum aminotransferase activity.

In order to induce hepatitis, five other groups of rats were given an intraperitoneal injection of 600 mg/kg of β-D-galactosamine dissolved in saline. Group I (vehicle) was given an intraperitoneal injection of saline (1 ml/kg). Four hours after the β-D-galactosamine treatment, group II (control) was treated with saline (10 ml/kg, p.o.), and groups III–V were treated with silymarin (300 mg/10 ml/kg, p.o.) and *Holotrichia diomphalia* (100, 300 mg/10 ml/kg, p.o.). After 48 h β-D-galactosamine administration, blood was taken from the abdominal aorta for the assay of serum aminotransferase activity.

2.5. Liver cirrhosis

Secondary biliary cirrhosis was induced by double ligation and division of the common bile duct (Kountouras et al., 1984; Gross et al., 1987). Under ether anesthesia, a midline incision was made to the abdomen, the common bile duct was isolated, and the proximal bile duct was held with two silk sutures and then dissected between the double ligations. In sham-operated group, the bowel and mesentery were manipulated and replaced. After operation, saline (10 ml/kg), silymarin (12.5 mg/10 ml/kg) or *Holotrichia diomphalia* (6.25, 12.5 mg/10 ml/kg) were fed orally to each group of rats once a day for four weeks. At four weeks, blood was taken from the abdominal aorta for the assay of serum aminotransferases and alkaline phosphatase activities. The medium and left lobes of the liver were removed and used for histology and hydroxyproline measurements.

2.6. Assay of serum ALT, AST and ALT activities and hydroxyproline content

The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities were determined by spectrophotometric procedures, using Sigma Kits (Sigma Chemical Co., St. Louis, MO, USA). The hydroxyproline (HOPro) contents in the liver were measured according to the method of Jamall et al. (1981). Briefly, the liver tissue was homogenized in 6N hydrochloride (HCl) and then hydrolyzed at 110 °C for 18 h. After cooling, chloramine T was added to the hydrolysate. After 5 min, *p*-dimethylaminobenzaldehyde was added and the mixture was incubated for 30 min at 60 °C. The samples were read at 560 nm against a reagent blank, which contained the complete system without added tissue, using a spectrophotometer (Milton Roy Spectonic 1201, USA).

2.7. Histology

A small piece of liver tissue from the anterior portion of the left lateral lobe was taken for light microscopy. Paraffin blocks were prepared after fixation in 10% neutral formalin and stained with hematoxylin and eosin. The degree of necrosis and fibrosis was assessed according to Frei et al. (1984). The severity of liver alteration was semi-quantitatively graduated on a scale of 0 to IV (0: absent; I: minimal; II: mild; III: modest; IV: severe), and separate scores were obtained for each of the following: cell necrosis, inflammatory cell infiltration, fibrosis and steatosis. The bile duct proliferation was rated as present or absent.

2.8. Statistical analysis

All results were expressed as means ± S.E.M. The unpaired Student's *t*-test was used to analyze the difference between groups. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Acute hepatotoxicity

The serum levels of ALT and AST in the vehicle-treated rats were 46 ± 4 U/l and 74 ± 6 U/l, respectively, which were similar to those of the normal rats. In the CCl_4-treated control group, the ALT and AST increased to 871 ± 71 U/l and 1310 ± 146 U/l, respectively. These higher levels were markedly suppressed in a dose-dependent manner by *Holotrichia diomphalia*. The increase in ALT and AST was also attenuated by silymarin (Table 1). In the galactosamine-treated control group, the ALT and AST increased to 1638 ± 282 U/l and 2342 ± 408 U/l, respectively. *Holotrichia diomphalia* and silymarin treatments prevented the elevations of serum aminotransferases. The hepatoprotective effect of *Holotrichia diomphalia* was stronger than that of silymarin (Table 2).

3.2. Liver cirrhosis

The liver of the sham-operated rats was normal. Histology of the bile duct ligation/scission (BDL/S) rat liver showed excessive bile duct proliferation, inflammation and

Table 1
Effect of *Holotrichia diomphalia* on serum aminotransferase activities in carbon tetrachloride-induced acute hepatic injury

Group	Dose (mg/kg)	ALT (U/l)	AST (U/l)
Vehicle		46 ± 4	74 ± 6
CCl_4			
Control		871 ± 71**	1310 ± 146**
Holotrichia diomphalia	100	402 ± 156+	577 ± 45**,++
	300	187 ± 31*,++	410 ± 51**,++
Silymarin	300	286 ± 64*,++	778 ± 144**,+

Each value is the mean ± S.E.M. for six to ten rats per group.
(*, **) significantly different ($P < 0.05$, $P < 0.01$) from vehicle-treated group.
(+, ++) significantly different ($P < 0.05$, $P < 0.01$) from control group.

Table 2
Effect of *Holotrichia diomphalia* on serum aminotransferase activities in β-D-galactosamine-induced hepatitis

Group	Dose (mg/kg)	ALT (U/l)	AST (U/l)
Vehicle		46 ± 4	74 ± 6
β-D-Galactosamine			
Control		1638 ± 282**	2342 ± 408**
Holotrichia diomphalia	100	704 ± 82**,+	694 ± 204*,+
	300	368 ± 99*,++	552 ± 132*,++
Silymarin	300	615 ± 75**,+	1266 ± 124**

Each value is the mean ± S.E.M. for six to ten rats per group.
(*, **) significantly different ($P < 0.05$, $P < 0.01$) from vehicle-treated group.
(+, ++) significantly different ($P < 0.05$, $P < 0.01$) from control group.

Table 3
Quantitative summary of histological observation on *Holotrichia diomphalia* protection of BDL/S-induced liver cirrhosis

Histological changes (%)	Sham	BDL/S			
		Control	*Holotrichia diomphalia*		Silymarin
			6.25 mg/kg	12.5 mg/kg	
Necrosis					
No change	100	0	0	0	0
Grades I–II	0	20	60	70	40
Grades III–IV	0	80	40	30	60
Inflammatory infiltration					
No change	100	0	0	0	0
Grades I–II	0	30	60	70	50
Grades III–IV	0	70	40	30	50
Fibrosis					
No change	100	0	0	0	0
Grades I–II	0	20	50	60	40
Grades III–IV	0	80	50	40	60
Bile duct proliferation	Absent	Present	Present	Present	Present

Scores are the numerical values of individual 10 rats per group. Necrosis and fibrosis were assessed according to Frei et al. (1984). Inflammatory infiltration grading was made according to five severity grades (0: absent, I: minimal, II: mild, III: modest and IV: severe). Bile duct proliferation was rated as present or absent.

Table 4
Serum biochemical values in rats with cirrhosis induced by BDL/S treated with *Holotrichia diomphalia*

Group	Dose (mg/kg)	ALT (U/l)	AST (U/l)	ALP (U/l)
Sham		24 ± 4	177 ± 39	157 ± 10
BDL				
Control		308 ± 11**	665 ± 52**	929 ± 31**
Holotrichia diomphalia	6.25	141 ± 26**,++	318 ± 55++	576 ± 87**,++
	12.5	126 ± 11**,++	356 ± 65*,++	670 ± 20**,++
Silymarin	12.5	241 ± 33**	592 ± 35**	750 ± 39**,++

Each value is the mean ± S.E.M. for seven to ten rats per group.
(∗, ∗∗) significantly different ($P < 0.05$, $P < 0.01$) from sham-operated group.
(++) significantly different ($P < 0.01$) from control group.

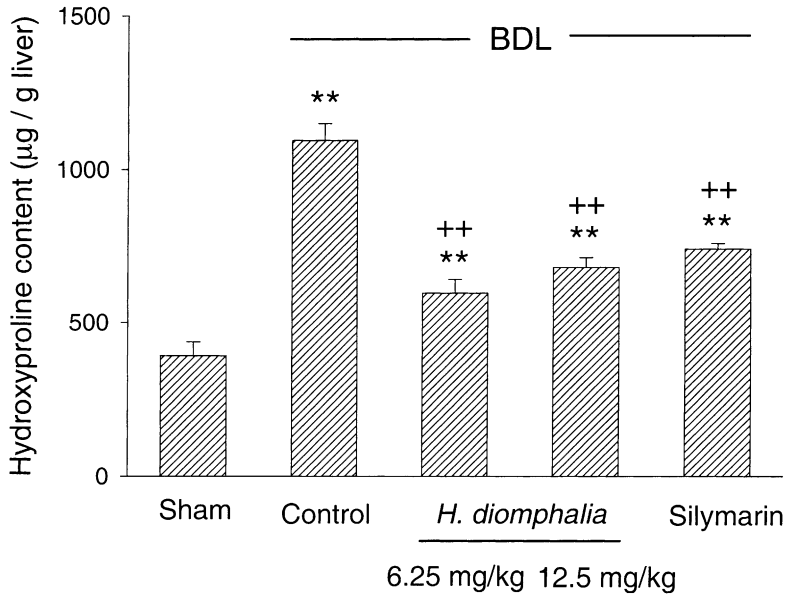

Fig. 1. Hydroxyproline content of liver from cirrhotic rats with BDL/S treated with *Holotrichia diomphalia*. (∗∗) significantly different ($P < 0.01$) from sham-operated group. (++) significantly different ($P < 0.01$) from control group. Values are means ± S.E.M. for seven to ten rats per group.

connective tissue deposition resulting in destruction of the lobular architecture. In the *Holotrichia diomphalia*-treated rats, there was a tendency towards less pronounced destruction of the liver architecture. In the silymarin-treated BDL/S rats, the liver histology was similar to that of the BDL/S rats (Table 3). The serum biochemical parameters of the BDL/S rats are shown in Table 4. The levels of serum ALT, AST and ALP were significantly elevated in the control BDL/S rats. In the *Holotrichia diomphalia*-treated rats, the serum ALT, AST and ALP levels were significantly lower relative to the control BDL/S rats. In the BDL/S rats treated with silymarin, the serum level of ALP was significantly reduced, and there were no significant changes in the serum ALT and AST levels compared with those of the control BDL/S rats. BDL/S increased the hydroxyproline content about three-fold. Compared with the control BDL/S group, treatments with *Holotrichia diomphalia* and silymarin reduced the hydroxyproline content in the liver by up to 64 and 68%, respectively (Fig. 1).

4. Discussion and conclusions

In the present study, the hepatoprotective effect of *Holotrichia diomphalia* larvae in three experimental liver injury models was investigated. The sequence of events after CCl_4 administration has been well characterized by many investigators (Slater, 1966; Recknagel, 1967). After administering CCl_4, the histological changes of the liver include ballooning degeneration, fatty metamorphosis in the adjacent hepatocyte, cell necrosis, cell inflammation and the infiltration of lymphocytes and Kupffer cells around the central vein.

The mechanism of CCl_4-induced liver damage is considered to be due to the enzymatic activation (cytochrome P450) of CCl_4 into the trichloromethyl free radical ($^{\bullet}CCl_3$) within the membrane of the endoplasmic reticulum. This is followed by chloromethylation, saturation, peroxidation and progressive destruction of the unsaturated fatty acid of the endoplasmic reticulum membrane phospholipids (Reynolds and Moslen, 1979). These processes are known as lipid

peroxidation, leading to its functional and structural disruption (Recknagel, 1983). In the CCl_4-treated control of the present study, the serum aminotransferases levels were elevated significantly. The hepatoprotective effects against CCl_4-induced hepatic injury were clearly demonstrated in the rats treated with *Holotrichia diomphalia*. This effect may be attributable to the inhibition of cytochrome P450 activity as well as the prevention of lipid peroxidation.

β-D-Galactosamine-induced acute liver injury was considered in an experimental model of hepatitis. Decker and Keppler (1974) have shown that the metabolite of β-D-galactosamine, uridindiphosphogalactosamine (Rasenack et al., 1980), may deplete several uracil nucleotides including UDP-galactose, UDP-glucose and UTP, impairing mRNA and glycoprotein synthesis and altering the composition of the cellular membranes. These phenomena may lead to cellular damage and cellular inflammation resulting in a histological and biochemical picture closely resembling viral hepatitis (Lin et al., 1996).

The results presented here show a significant increase in ALT and AST activities after administration of β-D-galactosamine. In contrast to the control rats, the elevated serum aminotransferase levels were reduced by treatment with *Holotrichia diomphalia*. Furthermore, the hepatoprotective effect of *Holotrichia diomphalia* seemed to be higher than that of silymarin, which has been used as a potent hepatoprotective agent. The present results of the CCl_4- and β-D-galactosamine-induced liver injuries suggest that *Holotrichia diomphalia* may have potential clinical application for treating liver diseases.

Therapy for hepatic fibrosis should affect the production of hepatic connective tissue proteins in particular (Schuppan, 1991). The best therapeutic strategies can be designed only with a full understanding of the mechanism involved in fibrogenesis, which has yet to be completed. Nonetheless, any intervention that blocks collagen deposition will probably be effective in reducing hepatic fibrosis, regardless of the mechanisms involved.

In this study, we used BDL/S cirrhotic rats. Cirrhosis induced by 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 the control BDL/S rats and *Holotrichia diomphalia*-treated BDL/S rats, there was no significant difference in body weight. In the BDL/S rats, the liver weight increased markedly 28 days after biliary obstruction. The liver-to-body weight ratio of the *Holotrichia diomphalia*-treated BDL/S rats was slightly lower than that of the control BDL/S rats, but the difference was not significant (data not shown).

We were able to show that this procedure resulted in biliary fibrosis, as demonstrated by the histology and moderately increased serum aminotransferases and alkaline phosphatase. Furthermore, a significant increase in the hydroxyproline content in the liver was observed. Treatment with *Holotrichia diomphalia* reduced the serum levels of ALT, AST, ALP and also lowered the hydroxyproline in the liver, and improved its morphology.

In conclusion, treatment using *Holotrichia diomphalia* prevents hepatocellular damage and improves liver fibrosis. Our results suggest that *Holotrichia diomphalia* could be a promising antifibrotic agent. Further study is needed to fully understand the mechanism by which *Holotrichia diomphalia* larvae inhibit collagen deposition and to establish its clinical applicability.

Acknowledgements

This work was supported by research grant (99-B-5-2) from the Kyunggi Pharmaceutical Research Center, Korea.

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