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Antifibrotic Activity of LCC, a Cerebroside of *Lycium chinense* Fruit, in Bile Duct-Ligated Rats

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Abstract – We previously reported that a novel cerebroside, LCC, isolated from the fruits of *Lycium chinense* (Solanaceae), significantly exerted hepatoprotective activity against both the carbon tetrachloride-induced and galactosamine-induced toxicities in primary cultures of rat hepatocytes. In the present study, we further attempted to determine the effect of LCC on hepatic fibrosis in animal model. Hepatic fibrosis was induced in rats by bile duct ligation/scission (BDL) for a period of 5 weeks. Treatment of BDL rats with LCC significantly reduced collagen deposition and the activities of serum alkaline phosphatase and γ-glutamyl transpeptidase. In addition, the LCC treatment of BDL rats significantly preserved the decreased hepatic glutathione as well as the activities of glutathione reductase and catalase in BDL rats. From the results, it can be speculated that LCC might exert antifibrotic activity in rats with BDL, in part, through the preservation of antioxidant enzymes and hepatic glutathione.
Keywords – LCC, Bile duct ligation/scission, *Lycium chinense*, Hepatic fibrosis, Antifibrotic activity, Antioxidative activity

Introduction

Hepatic fibrosis is one of the most frequent lesions in chronic liver diseases and has been characterized by an increased accumulation of extracellular matrix (ECM) protein including collagen (Hui and Friedman, 2003; Friedman, 2004). It is well recognized that hepatic fibrosis has been associated with oxidative stress. That is, lipid peroxidation has been observed in hepatic fibrosis caused by iron overload (Gualdi *et al*., 1994), ethanol (Kamimura *et al*., 1992; Niemela *et al*., 1995), carbon tetrachloride (CCl_4) (Tsukamoto *et al*., 1990) and bile duct ligation/scission (Fox *et al*., 1997; Pastor *et al*., 1997). Therefore, enhancement of antioxidative defense mechanism has been proposed as a mean to protect against some clinical manifestations of fibrosis (Hernandez-Munoz *et al*., 1997; Muriel and Moreno, 2004; Loguercio *et al*., 2007).

In our previous study, we reported that a novel cerebroside isolated from the fruits of *Lycium chinense* (Solanaceae), 1-*O*-(β-D-glucopyranosyl)-(2*S*,3*R*,4*E*,8*Z*)-2-*N*-palmityloctadecasphinga-4,8-diene (LCC) (Fig. 1), significantly exerted hepatoprotective activity against both the CCl_4-induced and galactosamine-induced toxicities in primary cultures of rat hepatocytes through maintaining the hepatic glutathione (GSH) redox system (Kim *et al*., 1997, 1999 and 2000). As a continuation of the study, we attempted to determine the antifibrotic activity of LCC in an animal model.

Experimental

Animal model for hepatic fibrosis – Female Wistar rats weighing around 200 g were obtained from the Laboratory Animal Center, Seoul National University. They were kept on standard rat chow with free access to tap water, in temperature- and humidity-controlled animal quarters under a 12-h light-dark cycle. All experiments were conducted according to the guidelines of the Committee on Care and Use of Laboratory Animals of

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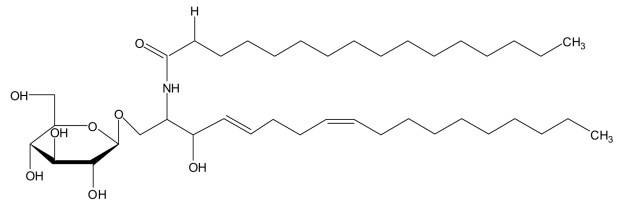

Fig. 1. Chemical structure of a cerebroside, LCC, isolated from *L. chinensis* fruits.

Seoul National University.

Secondary biliary fibrosis was induced by BDL as described previously (Kountouras *et al*., 1984; Kim *et al*., 1993). The sham-operated rats and BDL rats served as control groups. LCC was daily given at 10 a.m. *per os* at a dose of 6.25 and 12.5 mg/kg body weight (in 5% Tween 80) to the BDL rats throughout the experimental period. After 5 weeks of biliary obstruction, sham-operated rats, BDL rats and BDL rats treated with LCC were sacrificed under urethane and blood was collected by heart puncture. A lobe of the liver was rapidly removed for the determination of the activity of antioxidant enzymes and the contents of GSH and 4-hydroxyproline. Liver sections fixed by 10% formalin and frozen were used to determine hepatic collagen content. Serum was obtained for the determination of alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (GGT).

Determination of hepatic collagen content – The collagen content in liver section was determined by the method of Lopez de Leon and Rojkind (1985), as validated by Jimenez *et al*. (1985).

Determination of 4-hydroxyproline content – The frozen middle lobe of liver (30 - 50 mg) was lyophilized overnight. This lyophilized tissue was ground into powder and subsequently extracted five times with 2 ml of diisopropylether to remove vitamin A. It was then hydrolyzed with 6 N HCl for 20 h at 100 °C. The hydrolysate was filtered through a 0.22-μm Millipore filter. The content of 4-hydroxyproline in the liver hydrolysate was determined as described by the method of Jamall *et al*. (1981).

Biochemical analysis of blood – The activity of ALT was measured according to the method of Reitman and Frankel (1957) using an assay kit obtained locally (Youngdong Pharmaceuticals, Seoul, Korea). ALP activity was measured according to the method of Tietz *et al*. (1983), and GGT activity was determined by the method of Glossman and Nevile (1972) using γ-glutamyl-*p*-nitroaniline as a substrate.

Determination of GSH content and antioxidant enzyme activity – The content of GSH was determined by the method of Hissin and Hilf (1976). GSH-*S*-transferase (GST), GSH peroxidase (GPx) and GSH disulfide reductase (GR) activities were determined as described by Carlberg and Mannervik (1975); Flohe and Gunzler (1984); Habig *et al*. (1974), respectively. Catalase activity was measured by the method of Beers and Sizer (1952). Protein concentration was determined by the Lowry method (Lowry *et al*., 1951).

Statistical analysis – All data are expressed as the mean ± S.D. Group means are evaluated for statistically significant differences using analysis of variance (one way ANOVA). Values of $p < 0.05$ were considered statistically significant differences.

Results and Discussion

The BDL rat model has been recognized as a representative experimental model for studying cholestasis and cirrhosis (Kountouras *et al*., 1984; Mullen and McCullough, 1989; Kim *et al*., 1993). Dueland *et al*. (1991) reported that after the BDL for 4 to 5 weeks, liver cirrhosis in rats occurred by the newly formed duct-like structure associated with collagen deposition, mainly type I and III, in liver tissue. It was also reported that cirrhotic liver contained more than 15 mg of collagen per gram tissue than normal tissue and the content of other ECM material was also increased (Rojkind *et al*. 1983). In this experiment, we found similar morphological changes in BDL rat after 5 weeks of BDL. In addition, BDL results in the increase in spleen weight, an indicator of portal hypertension, and liver weight because of pathological defense mechanism and collagen deposition (Kountouras *et al*., 1984). We also found similar histological changes in BDL rats after 5 weeks of BDL (Table 1). Weights of liver and spleen were significantly increased in BDL rats compared to the sham-operated control rats ($p < 0.001$). However, the treatment of BDL rats with LCC at a dose of 6.25 and 12.5 mg/kg body weight/day, respectively, significantly lowered the increased weights of liver and spleen ($p < 0.05$) in BDL rats. There were no significant differences in body weight between BDL rats and BDL rats treated with LCC.

Excessive production and deposition of ECM such as collagen are important characteristics of hepatic fibrosis (Hui and Friedman, 2003). Thus, we determined collagen deposition by the selective dye-binding method of collagen quantitative assay (Lopez de Leon and Rojkind, 1985). After 5 weeks of BDL, hepatic collagen content in BDL rats increased to 1.2-fold compared to that in sham-operated controls (Table 2). The treatment of BDL rats

Table 1. Weights of body, liver and spleen in BDL rats.

Group	n	Body weight (g)		Liver weight (g)	Spleen weight (g)
		0 wks	5 wks		
Sham-operated	12	220 ± 19	240 ± 14	8.9 ± 1.1	0.65 ± 0.07
BDL	10	223 ± 12	259 ± 26	17.5 ± 3.3	1.52 ± 0.27
LCC 6.25 + BDL	13	223 ± 17	242 ± 22	13.4 ± 2.1*	1.26 ± 0.24*
LCC 12.5 + BDL	14	212 ± 18	239 ± 21	12.5 ± 2.8*	0.95 ± 0.17**

LCC was given at a dose of 6.25 or 12.5 mg/kg body weight/day for 5 weeks. Sham-operated rats were given vehicle (5% Tween 80). Data are expressed as the mean ± S.D.
*, ** Significantly different from BDL control at $p < 0.05$ and 0.01, respectively.

Table 2. Effects of LCC on the contents of hydroxyproline and collagen in liver tissue of BDL rats.

Groups	Hydroxyproline (μg/g liver)	Collagen (μg/mg total protein)
Sham-operated	54.5 ± 8.7	102.81 ± 5.19
BDL	108.9 ± 13.6	119.08 ± 6.44
LCC 6.25 + BDL	115.1 ± 19.7	111.45 ± 5.62*
LCC 12.5 + BDL	88.7 ± 11.8*	105.84 ± 8.11*

LCC was given at a dose of 6.25 or 12.5 mg/kg body weight/day for 5 weeks. Sham-operated rats were given vehicle (5% Tween 80). Collagen content in liver sections was measured by the selective binding of Sirius red and Fast Green to collagen and non-collagen protein, respectively. Hydroxyproline content in liver hydrolysates was determined spectrophotometrically as described in "Experimentals". Data are expressed as the means ± SD. * Significantly different from BDL rats at $p < 0.05$.

Table 3. Effects of LCC on activities of serum and cholestatic enzymes in BDL rats.

Group	ALT (U/L)	ALP (U/L)	GGT (U/L)
Sham-operated	18.1 ± 6.81	102.3 ± 29.0	0.00 ± 0.01
BDL	72.4 ± 7.52	184.6 ± 30.8	17.64 ± 4.57
LCC 6.25 + BDL	69.3 ± 6.07	141.2 ± 23.8	8.65 ± 2.19*
LCC 12.5 + BDL	65.7 ± 6.43	90.3 ± 11.5*	6.82 ± 1.24**

LCC was given at a dose of 6.25 or 12.5 mg/kg body weight/day for 5 weeks. Sham-operated rats were given vehicle (5% Tween 80). Data are expressed as means ± SD. *,** Significantly different from BDL control at $p < 0.05$ and $p < 0.01$, respectively.

with LCC at a dose of 12.5 mg/kg body weight/day significantly reduced the increased collagen content induced by BDL (Table 2). The result was consistent with the reduction of 4-hydroxyproline content in liver tissues of BDL rats treated with LCC. The 4-hydroxyproline content in liver tissues increased 2-fold after 5 weeks of BDL. However, the treatment of BDL rats with LCC at a dose of 12.5 mg/kg body weight/day decreased the 4-hydroxyprolin content to 18.5% of that in BDL rats ($p < 0.05$). These results suggested that LCC inhibit the collagen synthesis at the translational level because it significantly reduced the hydroxyproline content. It was also speculated that antifibrotic effect of LCC might be involved in the inhibition of prolyl-4-hydroxylase, a key enzyme of collagen fibril production that catalyzes the hydroxylation of peptide-bound proline residues to 4-hydroxyproline, which needs to be further clarified (Bickel *et al*., 1998; Aoyagi *et al*., 2002).

Serum GGT is an enzyme embedded in the canalicular domain of the liver. The liberation of this enzyme to serum indicates damage to the plasma membrane of hepatocyte by lipid peroxidation and thus injury to the liver as mentioned in elsewhere (Bulle *et al*., 1990; Muriel, 1998). As shown in Table 3, the level of serum GGT increased from 0.0 ± 0.01 in sham-operated to 17.64 ± 5.87 in BDL rats, indicating that the level of serum total bilirubin was increased after BDL. However, the treatment of BDL rats with LCC at a dose of 6.5 and 12.5 mg/kg body weight/day, respectively, significantly reduced the elevated level of serum GGT to 6.82 ± 1.24 ($p < 0.05$) and 8.65 ± 2.19 ($p < 0.01$), respectively. In BDL rats treated with LCC at a dose of 12.5 mg/kg body weight/day, serum ALP activity was significantly reduced to 51% of those of BDL rats ($p < 0.05$) (Table 3). These results indicated that the effective dose of LCC in treatment of liver cholestasis in BDL rats is 12.5 mg/kg body weight/day. There was no significant difference between BDL rats and BDL rats treated with LCC in serum ALT activities.

In our previous study, we found that LCC could protect primary cultured rat hepatocytes from oxidative stress induced by CCl_4 by maintaining hepatic GSH level (Kim *et al*., 1999). It is well known that obstruction of bile flow by BDL causes the accumulation and retention of hydrophobic bile salts in the liver, which leads to increase in oxygen radical production and remarkably decline in antioxidant defenses such as GSH (Krahebuhl *et al*., 1995; Benedetti *et al*., 1997; Roeb *et al*., 2003). The present study clearly showed that BDL for 5 weeks in rats profoundly reduced in the activity of antioxidant enzymes

Table 4. Effects of LCC on GSH content and antioxidant enzyme activities in liver tissue of BDL rats.

Group	GSH	GST	GPx	GR	Catalase
Sham-operated	24.78 ± 3.97	65.8 ± 3.60	0.829 ± 0.145	14.652 ± 1.682	311.6 ± 69.1
BDL	12.22 ± 1.94	70.6 ± 20.6	0.486 ± 0.095	1.815 ± 0.376	84.2 ± 12.3
LCC 6.25 + BDL	14.99 ± 1.41	67.0 ± 25.4	0.430 ± 0.096	4.286 ± 0.546*	174.9 ± 24.3*
LCC 12.5 + BDL	17.66 ± 3.19*	88.8 ± 16.2	0.458 ± 0.098	5.403 ± 0.987*	210.7 ± 49.9*

LCC was given at a dose of 6.25 or 12.5 mg/kg body weight/day for 5 weeks. Sham-operated rats were given vehicle (5% Tween 80). GSH: nmol/mg protein/min, GST: nmol/mg protein/min, GPx: μmol/mg protein/min, GR: nmol/mg protein/min, catalase: μmol/mg protein/min. Data are expressed as means ± SD.
* Significantly different from BDL control at $p < 0.05$.

and the reduced GSH content. As shown in Table 4, the total GSH content in liver tissue of BDL rats was decreased to the half of that in the sham operated control rats. However, the reduced GSH content in BDL rats was significantly increased by the treatment of LCC at a dose of 12.5 mg/kg body weight/day. In addition, the decreased activity of some antioxidant enzymes such as GR and catalase in BDL rats increased 2-fold by the LCC treatment. No significant differences in the activities of GPx and GST were observed by the treatment of LCC in BDL rats. The GSH depletion by BDL was significantly protected by the treatment of LCC although the decreased activities of GPx and GST were not restored ($p < 0.05$), suggesting that LCC might be involved in GSH biosynthesis. This statement should be clarified with further study.

In conclusion, these findings indicate that LCC might exert antifibrotic activity in BDL rats, preserving antioxidant enzymes and hepatic GSH, in part. Based on our results, we could suggest that LCC might be of significant therapeutic potential in the prevention of hepatic fibrosis, although the antifibrotic effects of LCC in *in vitro* should be further investigated using hepatic stellate cells which are responsible for liver fibrosis.

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