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Original Article

Hepatoprotective Activity of Licorice Water Extract against Cadmium-induced Toxicity in Rats

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Licorice is commonly used as a cure for digestive disorders and as a detoxification agent in East Asia. This study investigated the protective effect of licorice water extract against cadmium ($CdCl_2$, Cd)-induced liver toxicity in rats. To induce acute toxicity, Cd (4 mg/kg body weight) was dissolved in normal saline and intravenously (i.v.) injected into rats. The rats then received either a vehicle or licorice water extract (50, 100 mg/kg/day) for 3 days, and were subsequently exposed to a single injection of Cd 24 h after the last licorice/vehicle treatment. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were significantly increased by Cd treatment. In contrast, pretreatment with licorice reduced ALT, AST and LDH. In histopathological analysis, licorice decreased the central necrosis around central veins, the peripheral hemorrhage around portal triads, the percentage of degenerative hepatic regions (%/mm^2 hepatic parenchyma) and the number of degenerative hepatic cells (N/100 hepatic cells). Licorice also inhibited the increment of Bad (a BH3 domain-containing protein) translocation by Cd in liver cells. These results demonstrate that licorice could have a hepatoprotective effect by inhibiting the translocation of Bad to the mitochondria in Cd-intoxificated rats.

Keywords: Licorice – Cadmium – Protective Effect – Liver Toxicity – Bad Translocation

Introduction

Licorice (*Glycyrrhizae radix*) is one of the oldest and most frequently used botanical treatments in East Asia. Licorice has been recommended for its life-enhancing properties, detoxification and as a cure for digestive disorders and swelling (1). Herbal medicines containing licorice have shown stimulatory effects in immune systems (2,3). Licorice has also been reported to attenuate free radical-induced oxidative damage in the kidney (4) and prevent carcinogenesis induced by toxicants or hormones (5). Licorice contains flavonoids and pentacyclic triterpene saponins including liquiritigenin, liquiritin, isoliquiritigenin, liquiritin apioside and glycyrrhizin (6). Among these components, glycyrrhizin, which is the major constituent, comprises 4% to 13% of the dried root weight (1). Glycyrrhizin has antiviral (7–9), anticarcinogenic (10,11) and hepatoprotective (12–16) effects. Liquiritigenin, an aglycone of liquiritin, shows cytoprotective effects against cadmium (Cd)-induced toxicity (17) in a rat-derived hepatocyte cell line and hepatoprotective effects against acute injuries induced by acetaminophen (18).

There are approximately 35 heavy metals in our environment. Heavy metals become toxic when they are

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not metabolized, which allows them to accumulate in many organs (19,20). Cd, a common toxic heavy metal, is widely distributed in the environment due to its use in industry (21,22). Acute exposure to Cd causes dysuria, polyuria, chest pain, fatigue and headache (23). Chronic intake of Cd in contaminated food or air produces organ dysfunction as a result of cell death, resulting in pulmonary, hepatic and renal tubular diseases (24). The liver is the most important target organ when considering Cd-induced toxicity because Cd primarily accumulates in the liver (25–27).

We previously reported that licorice-inhibited Cd-induced toxicity *in vitro*, therefore in this study, we tested the effects of licorice *in vivo* by examining its protective effects against Cd-induced toxicity in rats.

Methods

Preparation of Licorice Water Extract

Licorice water extract contains high levels of liquiritin, liquiritin apioside, liquiritigenin, isoliquiritin, isoliquiritin apioside and glycyrrhizin (6). Licorice water extract was prepared by boiling 600 g of licorice (Wolsung Pharm., Daegu, Korea) in 51°C of water for 3 h, then filtering the solution through a 0.2 μm syringe filter (Nalgene, USA) and storing it at −20°C until use. The amount of licorice water extract was estimated based on its dried weight after being lypholized. The yield of lyophilized water extract from licorice was 13%.

Rats

Rat studies were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. Six-weeks-old Sprague-Dawley rats (140–160 g) were provided by Hyochang Science (Daegu, Korea) and acclimatized for 1 week. Rats were caged in an atmosphere of filtered, pathogen-free air, provided with commercial rat chow (Purina, Korea) and water *ad libitum* and maintained at a temperature between 20 and 23°C with a 12 h light/dark cycle and relative humidity of 50%. To induce acute liver injury, Cd ($CdCl_2$, 4 mg/kg body weight) was dissolved in normal saline and intravenously (i.v.) injected into the rats. This dose was selected because it severely elevated plasma alanine aminotransferase (ALT) levels in a previous experiment that evaluated the hepatoprotective activity of $GdCl_3$, (28). To evaluate the hepatoprotective effect of licorice, rats were administrated either a vehicle (tap water) or licorice water extract (50, 100 mg/kg) for 3 days and subsequently exposed to a single injection of Cd 24 h after the last licorice/vehicle treatment. Tissue and blood samples were obtained 24 h after Cd exposure.

Blood Chemistry

At the end of the experimental period, blood was collected by means of a heart puncture and serum was separated by centrifugation. Alanine aminotransferse (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) in serum, were assayed using an analysis kit for each respective enzyme according to the manufacturer's instructions (Pointe Scientific Inc., Canton, MI, USA). Assays were read using an automated blood chemistry analyzer (Photometer 5010, Robert Riele GmbH & Co KG, Berlin, Germany).

Histopathology

The left lateral lobe of the liver was sliced (three slices per rat), and tissue slices were fixed in 10% neutral buffered formalin for 6 h, embedded in a paraplast automatic tissue processor (Citadel 2000, Shandon Scientific, Cheshire, UK), sectioned (4 μm) and stained with hematoxylin and eosin (H&E) stain. The percentage of the degenerative liver region showing central necrosis and peripheral hemorrhage was calculated using image analysis (SIS, Germany) under microscopic examination at 50× magnification (Zeiss, Germany) with the results expressed as $\%/mm^2$ of hepatic parenchyma. Additionally, the number of degenerative cells showing vacuolation or any necrotic process was also calculated using an automated image analysis under microscopic examination at 200× magnification and expressed as $N/100$ hepatic cells.

Mitochondrial Bad Assay

Under death friendly condition, Bad (a BH3 domain-containing protein) translocates to mitochondria and induces apoptosis. To examine the effect of licorice on apoptosis in liver tissue, Bad protein in mitochondria was determined by western blot analysis. Mitochondria isolation was conducted using a mitochondria isolation kit (Pierce, USA) according to the manufacturer's instructions. Briefly, 150–200 mg of liver tissue was subjected to Dounce homogenization on ice, and then centrifuged after the addition of the mitochondria isolation reagent provided in the kit. The supernatant was then centrifuged again at 3000g for 15 min at 4°C. Next, the precipitated mitochondrial pellet was washed and the protein content analyzed by BCA protein assay. The expression of Bad was immunochemically monitored using antimouse Bad antibody (Santa Cruz, USA). Bands corresponding to Bad and actin were visualized using ECL western blotting detection reagents (Amersham Biosciences, USA) according to the manufacturer's instructions.

Table 1. Effects of licorice on the values of ALT, AST and LDH in serum of experimental animals

Treatment	Survival rate† (%)	Weight†† (Liver/Body)	ALT (IU/L)	AST (IU/L)	LDH (IU/L)
Control	100	0.0510 ± 0.00244	111.9 ± 12.6†††	228.7 ± 22.9	911.2 ± 229.8
Cd alone	60	0.0488 ± 0.00078	1536.0 ± 279.5*	2739.0 ± 346.0*	8564.0 ± 825.8*
Cd + licorice (50 mg/kg)	90	0.0508 ± 0.00141	845.0 ± 112.0**	2236.0 ± 276.8	4027.7 ± 922.3**
Cd + licorice (100 mg/kg)	100	0.0505 ± 0.00149	510.8 ± 144.9**	1253.5 ± 303.9**	3420.7 ± 1168.9**

†Survival was recorded 24 h after Cd exposure following the consecutive licorice pretreatment for 3 days. Ten rats per group were used at the beginning. ††Each liver weight was divided by body weight. †††Values represent the mean ± SE (significantly different from vehicle-treated control, *$p < 0.01$ and significantly different from Cd alone, **$p < 0.01$).

Data Analysis

A one-way analysis of variance procedure was used to assess significant differences among the treatment groups. The Newman–Keuls test was used for comparisons of multiple group means for each treatment for which a significant effect was observed. The criterion for statistical significance was set at $p < 0.05$ or $p < 0.01$.

Results

Survival Rates

All control rats were maintained safely for the duration of the experiment. Rats in the licorice treated groups showed increased survival rates of 60%, 90% and 100% when treated with Cd alone, Cd plus 50 mg/kg of licorice and Cd plus 100 mg/kg of licorice, respectively (Table 1). The liver weights of the Cd-treated rats and licorice treated rats were not significantly different from the controls (Table 1).

Clinical Chemistry

An increase in liver ALT, AST and LDH indicates liver damage. The blood biochemistry showed a protective effect of licorice on Cd-induced liver toxicity. The levels of ALT, AST and LDH activities in the plasma of rats were increased 24 h after a single Cd treatment. Licorice pretreatments at a dose of 50 mg/kg and 100 mg/kg for three consecutive days inhibited the plasma ALT and LDH activities in rats challenged with Cd (Table 1). Licorice at a dose of 100 mg/kg reduced the Cd-increased plasma AST activity (Table 1).

Histopathological Evaluation

To verify the liver protective effects of licorice against Cd-induced toxicity, the extent of liver damage was examined histopathologically. Healthy control rats showed no pathological changes (Fig. 1A, a), however severe central lobular necrosis around central veins and peripheral hemorrhage around portal triads was observed in the livers of Cd-treated rats (Fig. 1A, b). These Cd-induced histological changes were reduced by licorice treatment in a dose dependant manner (Fig. 1A, c and d). The percentage of degenerative hepatic regions (%/mm^2 hepatic parenchyma) following Cd treatment was increased to 83.30% compared with the vehicle-treated control rate of 1.42%. In contrast, these regions were decreased to 54.62% and 46.07% in the rats treated with 50 mg/kg and 100 mg/kg of licorice, respectively (Fig. 1B). An increased number of degenerative hepatic cells compared to that of the control samples were also detected after Cd treatment. The numbers of degenerative hepatic cells (N/100 hepatic cells) in the control and Cd treatment groups were 3.67 and 51.50, respectively, however, the number of cells in the Cd treatment group was reduced to 36.17 and 32.67 in rats pretreated with 50 mg/kg and 100 mg/kg of licorice, respectively (Fig. 1B).

Inhibition of Bad Translocation into Mitochondria by Licorice

Bad is localized in the cytosol under normal conditions. In the presence of death-inducing stimuli, Bad translocates to mitochondria and binds to Bcl-2, Bcl-xL and Bcl-w inhibiting their antiapoptotic actions. It promotes apoptosis through the cytochrome *c* release from mitochondria (29,30). Thus, mitochondrial translocation of Bad is an important apoptotic regulatory mechanism. Previously we reported that cell death, as a result of Cd, occurred due to apoptosis involving Bad translocation, and licorice reduced apoptosis by inhibiting translocation of Bad in hepatocyte cell lines (17). To investigate this effect of licorice *in vivo*, we isolated the mitochondrial fraction and conducted western blot analysis. As shown in Fig. 2, we found that Bad translocation into mitochondria was reduced by licorice.

Discussion

Many toxicants, including chemicals and drugs, induce liver injuries. Some components of licorice have been reported to exhibit hepatoprotective activities. CCl_4-induced hepatotoxicity is a common model for screening hepatoprotective compounds (31). Glycyrrhizin, a major

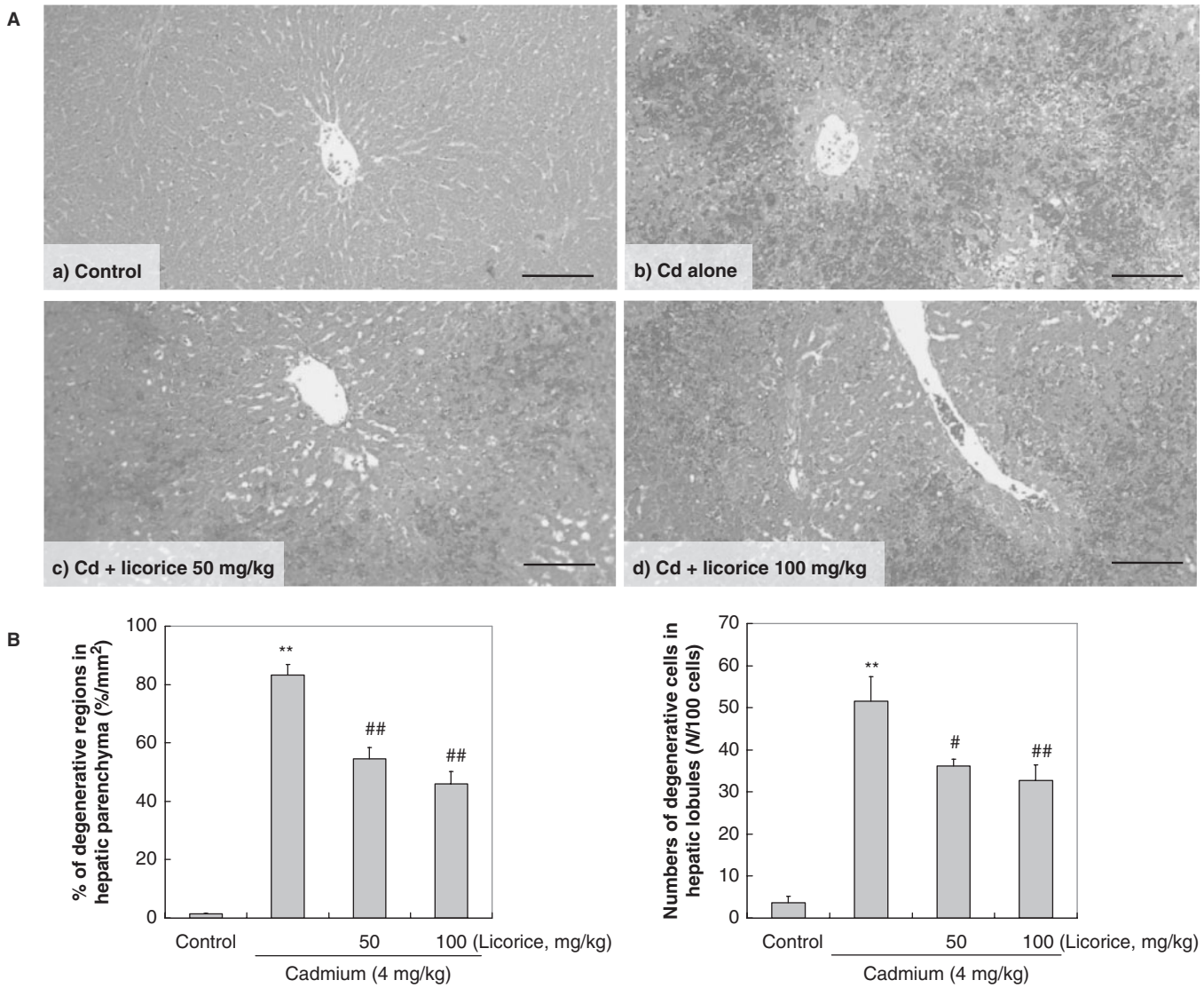

Figure 1. Inhibition of hepatic injuries in rats pretreated with licorice. Rats were orally pretreated with licorice (50, 100 mg/kg body weight for 3 days) and exposed to a single injection of Cd (i.v., 4 mg/kg body weight) 24 h after the last licorice/vehicle treatment. A) Hepatic histopathology: The liver sections from healthy control rats (a), Cd alone (b), Cd + licorice (50 mg/kg) (c) and Cd + licorice (100 mg/kg) (d) were stained with H&E (100× or 200×). Scale bars = 20 μm. B) Percentage of the liver degenerative region showing centrilobular necrosis and peripheral hemorrhage was calculated as%/mm^2 of hepatic parenchyma. The number of degenerative cells showing vacuolation or any necrotic process was calculated as N/100 hepatic cells. Values represent the mean ± SE ($n = 6$, significantly different from vehicle-treated control, $^{**}p < 0.01$ and significantly different from Cd alone, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

component of licorice, is a well-known hepatoprotective compound against CCl_4-induced liver injury in rats (14–16). 18β-Glycyrrhetinic acid, the aglycone found in glycyrrhizin, is also a potent hepatoprotective compound in CCl_4-induced hepatotoxicity (12,16). Pretreatment with 18β-Glycyrrhetinic acid reduced ALT and AST in serum, and also reduced hepatic lipid peroxidation caused by CCl_4. Acetaminophen overdose can also induce liver toxicity through CYP2E1-mediated oxidative metabolism. Accumulation of acetaminophen with buthionine sulfoximine leads to severe liver injuries that result in death. Liquiritigenin pretreatment significantly reduced ALT and LDH activities induced by acetaminophen with or without buthionine sulfoximine in plasma, and also reduced liver necrosis in rats (18).

Heavy metals such as cadmium and lead, which are liver toxicants, are widely distributed in the environment due to their use in industry. Oxidative stress, an important mechanism associated with toxic effects of lead, has been implicated in liver injury associated with lead (32,33). Cd, one of the most common toxic heavy metals, can induce and bind to metallothionein, which concentrates Cd up to 3000-fold (34). Free Cd, which has not complexed with metallothionein, changes the enzyme

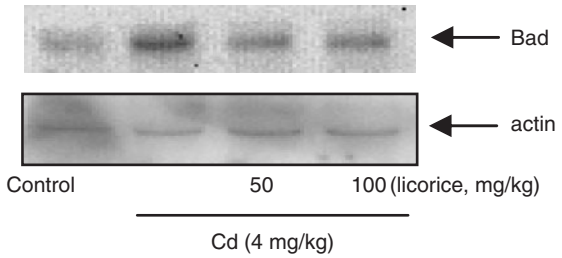

Figure 2. The effect of licorice on the levels of Bad protein associated with apoptosis in liver. Pretreatment with licorice inhibited Bad translocation induced by Cd in rats' livers. Rats were orally pretreated with licorice (50, 100 mg/kg body weight for 3 days) and exposed to a single injection of Cd (i.v., 4 mg/kg body weight) 24 h after the last licorice/vehicle treatment. The mitochondrial fractions were isolated using a kit and immunoblotted using Bad antibody.

activity and membrane structure by reacting with the sulfhydryl group of the membrane, resulting in liver injury (35). Liqiritigenin showed hepatoprotective effects against Cd-induced toxicity in a rat-derived hepatocyte cell line (17) and against acute injuries induced by acetaminophen (18). Glycyrrhizin is also beneficial against liver toxicity induced by CCl_4 (14–16), lead acetate (13) and acetaminophen (36). However, glycyrrhizin had no protective effects against Cd-induced hepatotoxicity in rats. Shaikh *et al*. reported that a Japanese drug containing glycine, glycyrrhizin and cystein has hepatoprotective effects against Cd toxicity and the hepatoprotective effect of this drug is due to glycine not glycyrrhizin (37). In this study, we confirmed the hepatoprotective activity of licorice against Cd-induced toxicity. Based on previous studies, the reported beneficial effects of licorice may be partially due to liquiritigenin, not glycyrrhizin, therefore the protective effects of liquiritigenin on Cd-induced hepatotoxicity needs to be elucidated.

Upon clinical examination, the increase of ALT and AST levels in plasma is considered a biomarker of liver injury (38). When exposed to Cd, the levels of ALT and AST were usually increased, which indicated liver injury. LDH is another index of hepatotoxicity, although the plasma LDH level is relatively insensitive (39). In our study, licorice significantly reduced Cd-induced ALT, AST and LDH levels in plasma.

Generally, Cd-induced hepatopathy shows central lobular necrosis and peripheral hemorrhage (40–42) and the hepatoprotective effects of various agents have been evaluated based on these histopathological changes (43–45). In this study, we confirmed that licorice could dose-dependently inhibit the severe central lobular necrosis around central veins and peripheral hemorrhage around portal triads in Cd-treated rat livers. Licorice also reduced the percentage of degenerative hepatic regions (%/mm^2 hepatic parenchyma) and the number of Cd-increased, degenerative hepatic cells (N/100 hepatic cells).

Oxidative stress is a major cause of Cd-induced toxicity. Dong *et al*. reported that toxic metals, such as $CdCl_2$ and V_2O_5, stimulate inflammatory cytokines in hepatocytes through an oxidative stress mechanism (46). Exposure to $CdCl_2$ leads to a decrease in the activities of antioxidant enzymes, such as superoxide dismutase and catalase (47), and to an increase in the activity of glutathione *S*-transferase in the liver (48). At the cellular level, Cd depletes glutathione and protein-bound sulfhydryl groups, leading to increased lipid peroxidation and enhanced intracellular oxidized states (49).

In certain pathophysiologic situations, Cd also causes apoptosis and necrotic cell death. Micromolar Cd induces apoptosis irrespective of sulfhydryl deficiency, whereas submolecular Cd, in conjunction with sulfhydryl deficiency, causes non-apoptotic cell death (50). Licorice and liquiritigenin prevented both apoptotic and non-apoptotic cell death induced by Cd (10 μM) only or Cd (0.3 μM) coupled with buthionine sulfoximine treatments. Specifically, licorice reduced apoptosis via inhibition of Bad protein translocation from cytosol to the mitochondrial membrane (17).

Bad is a pro-apoptotic Bcl-2 protein. That protein locates in cytosol combined with 14–3–3 protein in live cells. When the cells go to death program, what we call apoptosis, the Bad protein is dephosphorylated and translocates into mitochondrial membrane (29,30). This translocation of Bad into mitochondria induces cytochrome *c* release into cytosol from mitochondria, and released cytochrome *c* activates caspase pathway, an important step in apoptotic cell death. In this study, mitochondrial Bad was intensively increased by Cd treatment *in vivo*, implicating the involvement of apoptosis in rat liver injuries. However, Bad translocation into the mitochondria was blocked by licorice.

In conclusion, we clearly confirmed the *in vivo* hepatoprotective effects of licorice against Cd-induced injuries. Inhibition of Bad translocation contributes to the liver protection afforded by licorice.

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