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Hepatoprotection by *Semisulcospira libertina* against Acetaminophen-Induced Hepatic Injury in Mice

Tae Won Jeon[1], Young Sun Lee[2] and Hyo Jung Kim[3†]

[1]*Institute for Drug Research, College of Pharmacy, Yeungnam University, Gyungsan 712-749, Korea*
[2]*Efficacy and Safety Research Center for Traditional Oriental Medicines, Daegu Haany University, Kyongbuk Technopark, Daegu 706-060, Korea*
[3]*College of Oriental Medicine, Daegu Haany University, Daegu 706-060, Korea*

Abstract

Recently, we reported (*J Korean Soc Food Sci Nutr*, 31(3): 516-520, 2002) that *Semisulcospira libertina* (Marsh Snail) pretreatment has a hepatoprotective effect on CCl_4-induced liver damage in rats. The purpose of this study was to investigate the possible mechanisms of hepatoprotection by *S. libertina* (SL) on liver injury induced by acetaminophen (AA). Male ICR mice were pretreated with dehydrated powder of SL once daily for three consecutive days, given a single toxic dose of AA (450 mg/kg) and liver function determined 24 h later. Liver damage was assessed by quantifying serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH) activities, and by measuring hepatic lipid peroxidation. To confirm possible mechanism(s), the content of hepatic glutathione (GSH) and gene expression of tumor necrosis factor α (TNF α) mRNA by reverse transcription-polymerase chain reaction (RT-PCR) were also measured. Pretreatment with SL dramatically lowered AA-elevated ALT, AST and SDH activities. SL pretreatment decreased AA-produced lipid peroxidation by 11% and restored the AA-depleted hepatic GSH by 27%. Furthermore, SL markedly suppressed the expression of TNF α mRNA induced by AA. Our findings revealed that the possible hepatoprotective mechanisms of SL could be attributed, at least in part, to the glutathione-mediated detoxification as well as the regulation of TNF α mRNA expression.

Key words: *Semisulcospira libertina*, acetaminophen, tumor necrosis factor α mRNA, glutathione

INTRODUCTION

Semisulcospira libertina (*Gould*) (SL) has been used as a folk medicine for the treatment of various diseases such as hepatitis, jaundice, stomachache, dyspepsia and diarrhea in Japan, China and Korea. It is well known in Korea as 'Daslgi' and in English as Marsh Snail. We previously reported (1) that SL pretreatment had a hepatoprotective effect on CCl_4-induced liver damage in rats. Despite the fact that SL has shown promising effects for the treatment of various of diseases, few reports have definitively addressed the mechanism of hepatoprotection by SL on AA-intoxication. Its detailed mechanism remains to be elucidated.

Acetaminophen (N-acetyl-*p*-aminophenol, Paracetamol, AA), a widely used analgesic drug, at relatively high doses causes centrilobular hepatic necrosis, renal failure, and even death in several species including humans (2-4). AA is a clinically important over-the-counter drug, which has been used as a model hepatotoxicant in mechanistic studies for decades. AA-induced toxicity is thought to be produced by N-acetyl-*p*-benzoquinoneimine (NAPQI), a relatively minor metabolite that is generated by a cytochrome P-450 catalyzed reaction (5). This electrophilic metabolite is normally conjugated with various hepatocellular nucleophiles, particularly glutathione (GSH) and excreted in the urine as conjugates (6,7).

Normally GSH acts as a reducing agent, an antioxidant (8) and a reservoir for cysteine and participates in detoxification reactions for xenobiotics and the metabolism of numerous cellular compounds. Its intracellular abundance and potency as a reducing agent protects cells against chemical injury. The breakdown of the GSH-dependent antioxidant defensive system increases the intracellular flux of oxygen free radicals (9) creating an oxidative stress and initiating apoptosis. A GSH-dependent mechanism can protect the liver microsomal membrane against AA-induced damage (10). Overdoses of acetaminophen deplete glutathione stores, leading to accumulation of NAPQI, mitochondrial dysfunction (11), and the development of acute hepatic necrosis (12). Furthermore, depletion of GSH enhances the expression of tumor ne-

†Corresponding author. E-mail: hyokimm@yahoo.co.kr
Phone: +82-53-770-2299. Fax: +82-53-762-1263

crosis factor α (TNF α) (13).

TNF α, a proinflammatory cytokine, is assumed to act as a mediator in toxic liver injury. Elevated levels of hepatic TNF α occurs in many acute and chronic liver diseases, including fulminant hepatic failure, viral hepatitis, alcohol abuse, metabolic disease, autoimmunity, and biliary obstruction, as well as following chemical-induced hepatotoxicity (14-19). Normally, TNF α participates in the regulation of inflammation and immunity to pathogens. However, unusually high amounts of TNF α can result in host cell damage through its proinflammatory or cytotoxic properties. TNF α can be directly cytotoxic to hepatocytes by inducing apoptosis and necrosis (20).

This study was designed to clarify the mechanisms underlying this hepatoprotection by SL pretreatment against AA-induced acute hepatotoxicity in ICR mice. Liver damage was assessed by quantitating activities of enzymes in serum that are liver function indices as well as by measuring hepatic lipid peroxidation. Protection against oxidative injury was evaluated by measuring hepatic GSH concentrations; protection against inflammatory damage was evaluated by determining the expression of TNF α mRNA.

MATERIALS AND METHODS

Preparation of dehydrated powder

Semisulcospira libertina (SL) used in this study were supplied by Korea Life Science Co., Ltd. (Seoul, Korea). SL were obtained from the upper stream of Mt. Chiri in Hamyang (Kyongnam, Korea). After washing, obtained SL were stored at -20°C prior to preparation. The SL were dried in a hot-air dryer at 100°C for 2~3 days. Dehydrated SL was pulverized by roll mill and cutting mill. This dehydrated powder was used for subsequent animal studies.

Animals and treatment

Specific pathogen-free male ICR mice weighing 30~32 g were purchased from Dae Han Bio Link Co., Ltd., Umsong, Korea and housed at 22±2°C and 50±5% relative humidity with a 12 h light/dark cycle. Rodent Chow (Samyangsa Co., Wonju, Korea) and tap water were provided *ad libitum*. The mice were acclimated for approximately one week. Mice were pretreated with dehydrated powder of *Semisulcospira libertina* (3.5 g/kg bw in 0.5 mL saline, *p.o.*; SL) once daily for three consecutive days and then given a single dose of AA (450 mg/kg bw in saline, *i.p.*) at 2 h after the last pretreatment. Control and AA treated groups were administered an equal volume of only saline instead of the SL pretreatment. All animals were sacrificed at 24 h after AA treatment. At the day of autopsy, each animal was anesthetized with ethyl ether and the maximum amount of blood taken by cardiac puncture. After the blood samples were removed, the abdominal cavity of each animal was opened and the liver removed and weighed. Serum was obtained after centrifugation of collected blood samples and stored at -80°C prior to analysis.

Hepatic lipid peroxide (LPO) and reduced glutathione (GSH) determination

For LPO and GSH determination, a piece of liver tissue was homogenized (Polytron PT-MR 2100, KINEMATICA, Switzerland) in 4 fold 0.25 M sucrose solution. A 20% (w/v) homogenate was obtained and LPO and GSH were measured according to methods of Ohkawa et al. (21) and Ellman (22), respectively. Briefly, LPO content was assayed by measuring malondialdehyde (MDA). Samples containing 0.2 mL of homogenates were combined with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid adjusted to pH 3.5 and 1.5 mL of 0.8% TBA. The mixture was brought to a final volume of 4.0 mL with distilled water and heated at 95°C for 60 min. After cooling to room temperature, 5.0 mL of a mixture of n-butanol and pyridine (15 : 1, v/v) was added to each sample and the mixture shaken vigorously. After centrifugation at 1,500 rpm for 10 min, the supernatant fraction was isolated and the absorbance was measured at 532 nm. Lipid peroxidation was expressed as nmoles MDA per g of tissue. GSH content was estimated by a colorimetric method using Ellman's reagent. Homogenized tissue (0.2 mL) was combined with 0.5 mL of 4.0% sulfosalicyclic acid. The mixture was brought to a final volume of 1.0 mL with distilled water and centrifugated at 3,000 rpm for 15 min. The supernatant (0.3 mL) mixed with 2.7 mL of 0.1 M sodium phosphate buffer (pH 8.0), containing 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid) and color was developed. The absorbance was measured at 412 nm. Final results were expressed as μmoles reduced glutathione per g of tissue.

Determinations of hepatotoxicity parameters

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a spectrophotometric enzyme assay kit (#505-P and #505 respectively, Sigma Chemical Co., St. Louis, USA) by the methods suggested by the manufacturer. Specific activities were expressd as Sigma-Frankel (SF) units per mL of serum. Sorbitol dehydrogenase (SDH) activity was determined by the method of Gerlach (23) with a slight modification. Briefly, 0.25 mL of serum and 25 μL of 12 mM NADH were added into 0.1 M Trizma buffer (pH 7.5) and incubated at 25°C for 10 min. Then, 0.25 mL of 4 M fructose was added as substrate. Standard spectrophotometric procedure, which involved monitoring of the conversion of NADH to NAD over time, was used to

measure SDH activity at 340 nm (DU 530, Beckman, USA). Specific activity was expressd as mU per mL of serum.

Total RNA isolation

Mouse liver in Trizol (Gibco BRL) reagent was well homogenized and vortexed after 1/10 volume of chloroform was added. After incubating the mixture on ice for 15 min, the samples were centrifuged at 12,000 rpm, 4°C for 15 min. The aqueous phase was transferred to a new 1.5-mL micro centrifuge tube. RNA from the aqueous phase was precipitated by mixing with the same volume of isopropyl alcohol and centrifuged at 12,000 rpm for 15 min at 4°C following 30 min of incubation on ice. Precipitated RNA pellets were washed once with 70% ethyl alcohol, and were re-dissolved in DEPC-treated water (Quality Biological Inc., Gaithersburg, MD).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT reaction of 4 mg of RNA was performed in a 20 mL RT reaction mixture containing 0.5 mL of MMLV reverse transcriptase (200 U/mL, Promega), 4.0 mL of 5× MMLV RT buffer (Promega), 2.0 mL of dNTP mixture (10 mM, BM), 0.5 mL of RNasin (RNase inhibitor, 40 U/mL, Promega), and 2.0 mL of oligo dT (50 mM) in DEPC-treated water. The reaction was performed at 42°C for 1 h and at 95°C for 5 min. PCR was carried out with the use of 1.0 mL of RT products as templates: 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec, and elongation at 72°C for 45 sec. The last cycle was followed by a 10 min extension step at 72°C. The amplified products were analyzed by ethidium bromide-stained agarose gel electrophoresis. The PCR primer sequences are shown in Table 1.

Statistical analysis

All data are expressed as the mean±SE. Significance of differences were evaluated by one-way analysis of variance (ANOVA). Differences between groups were evaluated by Duncan's multiple range *post-hoc* analysis (SPSS program, ver 10.0), and considered statistically significant if the p value was less than 0.05.

RESULTS AND DISCUSSION

We reported previously that SL pretreatment definitely protected hepatic injury on CCl_4-induced liver damage in rats (1), but the mechanisms underlying this hepatoprotection is not clear. Acetaminophen has been used as a model hepatotoxin in mechanistic studies for decades and acetaminophen-induced hepatotoxicity in rodents is a widely used animal model to assess hepatoroptective efficacy and safety of new compounds (24,25). Consequently, to identify the possible mechanisms of the hepatoprotection by SL, male ICR mice were pretreated with SL once daily for 3 consecutive days, given a single toxic dose of AA (450 mg/kg) at 2 h after the last pretreatment, and biochemical and RT-PCR assay were performed 24 h later.

Table 1. Primer sequence used for detection of TNF α mRNA gene expression

	Oligonucleotide sequence
G3PDH	5′-CCA CCC AGA AGA CTG TGG ATG GC-3′
	5′-CAT GTA GGC CAT GAG GTC CAC CAC-3′
TNF α	5′-AAC TAG TGG TGC CAG CCG AT-3′
	5′-CTT CAC AGA GCA ATG ACT CC-3′

Effect of SL pretreatment on the hepatotoxicity of AA

Hepatic enzyme release is commonly regarded as a toxic response due to an altered membrane permeability of hepatocytes, and is widely used in the detection and evaluation of liver injury in laboratory animals and in man. The rise in serum levels of ALT, AST and SDH has been attributed to the damaged structural integrity of the liver (26-28) because these are cytoplasmic in location and are released into circulation after cellular damage (29).

In this study, the effects of SL pretreatment on AA hepatotoxicity in mice are shown in Fig. 1. AA (450 mg/kg, p.o.) produced severe liver injury in mice, as indicated by marked elevation of serum ALT, AST and SDH activities (about 54-, 9- and 56-fold, respectively). SL pretreatment dramatically reversed the AA induced increase in serum ALT activity by about 94% (1278±336 vs 75±18 unit/mL, $p<0.05$). AST and SDH were also increased by AA (366±95 and 885±231 mU/mL, respectively), but pretreatments with SL dramatically lowered AA-elevated activities by about 88% and 93%, respectively (44±8 and 64±16 mU/mL, respectively, $p<0.05$).

The reversal of increased serum transaminase and SDH enzymes in acetaminophen-induced liver damage by SL pretreatment may be due to the prevention of leakage of the intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes (30).

Hepatic LPO and GSH contents

In the present study, as shown in Fig. 2, treatment with AA significantly increased the hepatic content of lipid peroxide by 32% ($p<0.05$) compared to control. On the contrary, SL pretreatment decreased AA-produced lipid peroxidation by 11%, but without statistical significance.

The hepatic GSH level of AA-treated mice (3.35±0.63

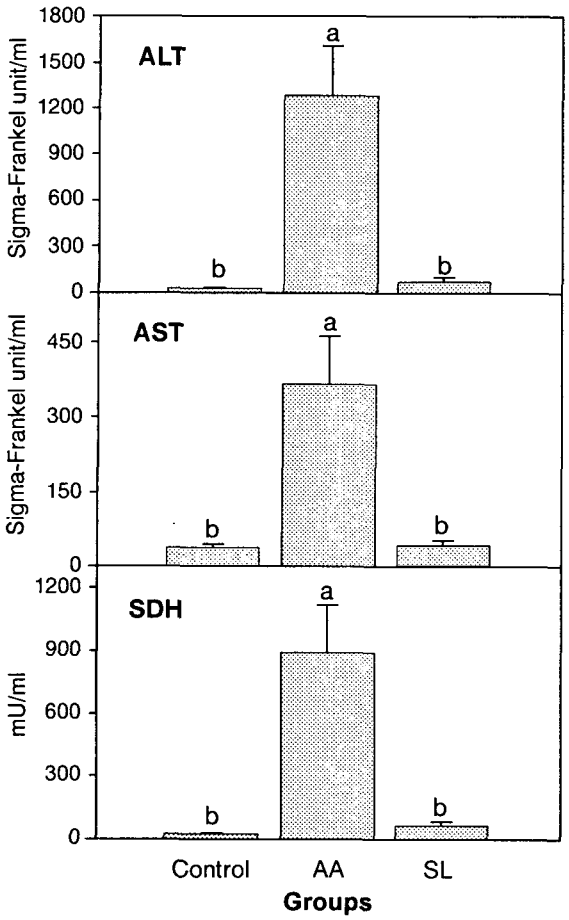

Fig. 1. Effects of dehydrated powder of *Semisulcospira libertina* on serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH) activity in acetaminophen-intoxicated rats. Each bar represents the mean ± SE for groups of 5 mice. Control, saline; AA, acetaminophen treated; SL, dehydrated powder of *S. libertina* pretreated plus AA treated. Means which are not significantly different are followed by the same letter ($p<0.05$).

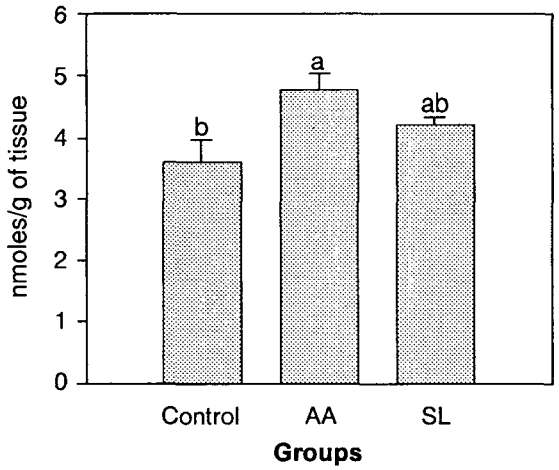

Fig. 2. Effect of dehydrated powder of *Semisulcospira libertina* (SL) on hepatic lipid peroxide content in acetaminophen (AA)-intoxicated rats. Each bar represents the mean ± SE for groups of 5 mice. Means which are not significantly different are followed by the same letter ($p<0.05$).

μmoles/g of tissue) was decreased by about 9% compared with control (3.66±0.22 μmoles/g of tissue) 24 h after AA administration, but SL pretreatment restored the AA-depleted GSH approximately 27% (4.26±0.19 μmoles/g of tissue), but not found to be statistically different at 24 h post sacrifice (Fig. 3).

GSH, being the most important biomolecule against chemicals-induced toxicity, can participate in the elimination of reactive intermediates by reduction of hydroperoxides in the presence of glutathione peroxidase. Detoxification of acetaminophen can be mediated by glutathione S-transferase-catalyzed conjugation with GSH in the liver (10). The protective role of GSH against cellular lipid peroxidation has been well documented (31). An increase in hepatic lipid peroxidation in liver homogenate and its suppression by SL pretreatment supports the view that the SL prevents acetaminophen-induced depletion of GSH. Therefore, the increased hepatic GSH content induced by SL pretreatment even at 24 h post sacrifice may reduce the acute AA hepatotoxicity.

Gene expression of TNF α mRNA

TNF α is produced by Kupffer cells, the resident macrophages of the liver, and may be involved in the manifestation of hepatotoxicity caused by chemicals, such as acetaminophen (32) and carbon tetrachloride (33-36). TNF α is a pleotropic cytokine associated with a variety of physiological and pathological conditions including cytotoxicity, differentiation, growth stimulation, immune-modulation and proinflammatory activity (19).

The aim of present study was to confirm whether TNF α is involved in hepatoprotection by SL on AA-induced liver damage. As shown Fig. 4, AA treatment markedly induced

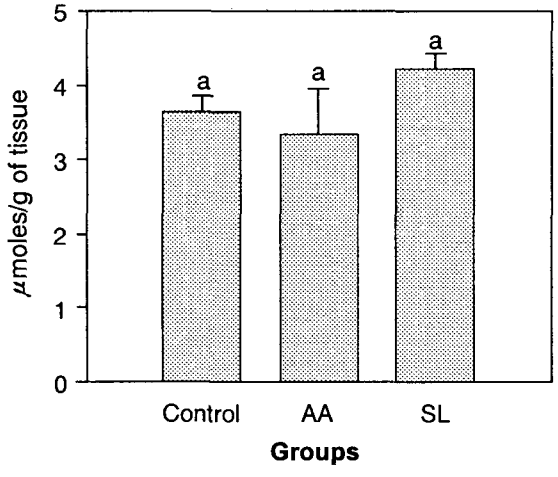

Fig. 3. Effect of dehydrated powder of *Semisulcospira libertina* (SL) on hepatic glutathione content in acetaminophen (AA)-intoxicated rats. Each bar represents the mean ± SE for groups of 5 mice. Means which are not significantly different are followed by the same letter ($p<0.05$).

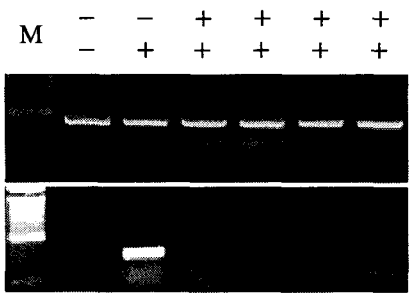

Fig. 4. Effect of dehydrated powder of *Semisulcospira libertina* (SL) on the TNF α mRNA expression in acetaminophen (AA)-treated mice. Total RNA was prepared from liver, and the expression of TNF α mRNA was analyzed by RT-PCR. G3PDH was used as control gene.

gene expression of TNF α mRNA, but SL pretreatment dramatically suppressed TNF α mRNA expression induced by AA in mouse liver tissue. This result clearly supports a role of TNF α in hepatotoxicity produced by AA and demonstrates that suppression of this cytokine can be involved in the mechanism of hepatoprotection by SL pretreatment against AA-induced liver damage.

In conclusion, SL pretreatment significantly alleviated AA-elevated serum enzymes, restored hepatic GSH reduction, and dramatically suppressed expression of TNF α mRNA induced by AA in mouse liver tissue. Therefore, we demonstrate that SL has a potent hepatoprotective action against AA-induced liver damage in mice, and that this protection is facilitated by GSH-mediated detoxification as well as the inhibition of TNF α mRNA expression.

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