

Protective Effect of Daidzin against D-Galactosamine and Lipopolysaccharide-Induced Hepatic Failure in Mice

Sung-Hwa Kim¹, Jeong-Haing Heo¹, Yeong Shik Kim², Sam Sik Kang², Jae Sue Choi³ and Sun-Mee Lee^{1*}

¹College of Pharmacy, Sungkyunkwan University, Suwon, Gyeonggi-do 440-746, Korea

²College of Pharmacy, Seoul National University, Seoul 151-747, Korea

³Faculty of Food Science and Biotechnology, Pukyong National University, Busan 608-737, Korea

This study examined the effects of daidzin, a major isoflavone from *Puerariae Radix*, on D-galactosamine (D-GalN) and lipopolysaccharide (LPS)-induced liver failure. Mice were given an intraperitoneal injection of daidzin (25, 50, 100 and 200 mg/kg) 1 h before receiving an injection of D-GalN (700 mg/kg)/LPS (10 µg/kg). Daidzin markedly reduced the elevated serum aminotransferase activity and the levels of lipid peroxidation and tumor necrosis factor- α . The glutathione content was lower in the D-GalN/LPS group, which was attenuated by daidzin. The daidzin pretreatment attenuated the swollen mitochondria observed in the D-GalN/LPS group. Daidzin attenuated the apoptosis of hepatocytes, which was confirmed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling method and a caspase-3 assay. Overall, these results suggest that the liver protection of daidzin is due to reduced oxidative stress and its antiapoptotic activity. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: apoptosis; daidzin; D-galactosamine; LPS; liver failure; oxidative stress; TNF- α .

INTRODUCTION

Acute hepatic failure is characterized by hepatic encephalopathy, severe coagulopathy, jaundice and hydroperitoneum, and is associated with high mortality if not treated with liver transplantation (Lee, 1994). A low dose of lipopolysaccharide (LPS) in combination with D-galactosamine (D-GalN) has been shown to induce an experimental liver injury that is similar to the acute hepatic failure observed clinically (Nakama *et al.*, 2001). It has been reported that LPS treated mice show a dysfunction of the liver microcirculation, metabolic changes, direct cytotoxicity and macrophage-mediated liver injury. D-GalN is a specific hepatotoxic agent that has been used to increase sensitivity to the lethal effects of endotoxin (Galanos *et al.*, 1979). Early studies on the mechanism of D-GalN/LPS hepatotoxicity suggested that the injury to hepatocytes is caused by the overproduction of several cytokines and inflammatory mediators such as tumor necrosis factor- α (TNF- α), interferon- γ and nitric oxide. Reactive oxygen species (ROS) are also generated from the liver macrophages (Wang *et al.*, 2007). TNF- α and ROS-induced hepatocyte apoptosis has been identified as an early and possible

causal event during D-GalN/LPS-induced hepatic failure (Xiong *et al.*, 1999).

Puerariae radix is one of the earliest and most important edible crude herbs used in traditional Korean, Chinese and Japanese medicines. It is widely used in Oriental medicine as an antipyretic and analgesic for the treatment of the common cold. It was reported that saponins from *Puerariae radix* showed inhibitory activity against an *in vitro* immunological injury of a rat hepatocyte culture (Arao *et al.*, 1997). *Puerariae radix* inhibits hydrogen peroxide-induced apoptosis through the induction of the antioxidative enzyme (Kang *et al.*, 2005). In addition, its water extract alleviates the adverse effect of ethanol ingestion by enhancing the lipid metabolism and the hepatic antioxidant defense system (Lee, 2004).

Daidzin is a major isoflavone from *Puerariae radix*, and is used to counteract problems associated with excessive alcohol consumption (Keung *et al.*, 1995). *In vitro*, daidzin is a potent and selective inhibitor of mitochondrial aldehyde dehydrogenase (ALDH-2) (Keung and Vallee, 1993), which is a major ALDH isozyme that catalyzes the detoxification of ethanol-derived acetaldehyde. In a recent study, daidzin suppressed the LPS-induced production of TNF- α in mice (Hasumuma *et al.*, 2007). Moreover, its antioxidative activity was reported (Cherdshewasart and Sutjit, 2008). According to these studies, it was assumed that daidzin might offer protection against D-GalN/LPS-induced acute hepatotoxicity.

Therefore, this study examined the hepatoprotective effect and mechanisms of daidzin from *Puerariae radix* against fulminant hepatic failure, particularly its effect on the extent of oxidative damage and apoptosis.

* Correspondence to: Dr Sun-Mee Lee, College of Pharmacy, Sungkyunkwan University, 300 Cheoncheon-dong, Jangan-gu, Suwon, Gyeonggi-do 440-746, Korea.

E-mail: sunmee@skku.edu

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MATERIALS AND METHODS

Extraction and isolation of daidzin. The dried roots of *Pueraria lobata* (12 kg) were refluxed with hot methanol (MeOH) twice. The MeOH extract was concentrated in vacuum to give a residue (2.5 kg) that was suspended in water and successively partitioned with dichloromethane (CH₂Cl₂), ethyl acetate (EtOAc) and *n*-butanol (BuOH), and then evaporated to yield a CH₂Cl₂ fraction (105 g), an EtOAc fraction (43 g) and *n*-BuOH fraction (1.1 kg). A portion of a *n*-BuOH (400 g) fraction was subjected to a Diaion HP-20 column chromatography (10 × 50 cm) eluted with a gradient of distilled water (H₂O) and MeOH (100: 0-0:100) and separated into four fractions: H₂O (B1, 175 g), 40% MeOH (B2, 276 g), 60% MeOH (B3, 17 g) and 100% MeOH (B4, 23 g). Repeated chromatography of fraction B3 (17 g) on a silica gel column eluted with a mixture solvent of CH₂Cl₂:MeOH:H₂O (26:14:4) to afford daidzin (3.1 g).

Daidzin [4,7-dihydroxyisoflavone 7-*O*-β-D-glucopyranoside, daidzioside] colorless needles; mp. 235–237 °C; UV, max (MeOH): 306 (sh 3.70), 270 (4.23), 248 (4.40); IR, imax (KBr) 3470, 1618, 1590, 1462, 1402, 1271, 1113 cm⁻¹; EI-MS *m/z* (%) 416. ¹H-NMR (400 MHz, DMSO-*d*₆): 9.56 (1H, s, OH-4'), 8.39 (1H, s, H-2), 8.05 (1H, d, *J* = 8.6 Hz, H-5), 7.41 (2H, d, *J* = 8.6 Hz, H-2', 6'), 7.23 (1H, d, *J* = 2.3 Hz, H-8), 7.14 (1H, dd, *J* = 2.3, 8.6 Hz, H-6), 6.82 (2H, d, *J* = 8.6 Hz, H-3', 5'), 5.45 (1H, d, *J* = 4.3 Hz, OH-2''), 5.17 (1H, d, *J* = 3.9 Hz, OH-3''), 5.11 (1H, d, *J* = 7.0 Hz, H-1''), 4.62 (1H, t-like, *J* = 5.5 Hz, OH-6''), 3.72 (1H, m, H-6''), 3.47 (2H, m, H-5'', 6''), 3.31 (2H, m, H-2'', 3''), 3.17 (1H, t, *J* = 9.0 Hz, H-4''); ¹³C-NMR (100 MHz, DMSO-*d*₆) δ: 174.74 (C-4), 161.39 (C-7), 157.25 (C-4'), 157.02 (C-9), 153.33 (C-2), 130.08 (C-2', 6'), 126.95 (C-5), 123.69 (C-3), 122.30 (C-1'), 118.46 (C-10), 115.57 (C-6), 114.97 (C-3', 5'), 103.37 (C-8), 99.97 (C-1''), 77.20 (C-2''), 76.47 (C-3''), 73.12 (C-4''), 69.61 (C-5''), 60.62 (C-6'').

Treatment of animals. Male ICR mice (20–22 g) were fasted overnight but given access to water *ad libitum*. All the animals were treated humanely under the Sungkyunkwan University Animal Care Committee guidelines. In the daidzin treated group, mice were administered daidzin (suspended in 10% Tween 80-saline) 25, 50, 100 and 200 mg/kg intraperitoneally, while other groups received an equivalent volume of the vehicle as the control. All the animals (except for the normal control) were injected intraperitoneally with D-GalN (700 mg/kg; Sigma Chemical Co., St Louis, MO, USA) and LPS (10 µg/kg *Escherichia coli* 026:B6; Sigma) dissolved in phosphate-buffered saline. Six treatment groups were examined: (a) vehicle-treated control, (b) vehicle-treated D-GalN/LPS, and (c–f) daidzin (25, 50, 100 and 200 mg/kg)-treated D-GalN/LPS groups. The mice were killed by decapitation at different time points, and blood and liver samples were collected for further determination.

Determination of lethality. The survival rates of the mice were monitored over a 24 h period after D-GalN/LPS administration. The number of dead mice was counted at 8 h and 24 h after the D-GalN/LPS injection.

Serum alanine aminotransferase activity. The serum alanine aminotransferase (ALT) activity 8 h after the D-GalN/LPS injection was determined using a Hitachi 747 automatic analyser (Hitachi, Tokyo, Japan).

Histopathological analysis and detection of apoptotic cells. The liver specimens for the histopathological analysis were obtained 8 h after administering the D-GalN/LPS. The sample was fixed in 10% neutral-buffered formalin and embedded in paraffin. The samples were sliced into 5 µm sections, and stained with hematoxylin-eosin for a blinded histological assessment. The apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using an *in situ* apoptosis detection kit (TaKaRa Co., Shizu, Japan). The sections were evaluated in nonconsecutive, randomly chosen ×200 histological fields.

Hepatic lipid peroxidation levels and glutathione contents. The levels of hepatic lipid peroxidation and glutathione were measured 8 h after the D-GalN/LPS injection. The steady-state level of malondialdehyde, a lipid peroxidation end product, was analysed by measuring the level of thiobarbituric acid reactive substances spectrophotometrically at a wavelength of 535 nm according to the method reported by Buege and Aust (1978) using 1,1,3,3-tetraethoxypropane (Sigma) as the standard. The total glutathione level in the liver homogenate was determined spectrophotometrically at a wavelength of 412 nm using the method reported by Tietze (1969) with yeast glutathione reductase, 5,5'-dithio-bis(2-nitrobenzoic acid) and NADPH. The GSSG level was measured using the same method in the presence of 2-vinylpyridine (Griffith, 1980), and the GSH level was determined from the difference between the total glutathione and the GSSG levels.

Isolation of liver mitochondria. The level of mitochondrial swelling was determined by preparing a liver mitochondrial fraction 6 h after the D-GalN/LPS injection according to the method reported by Johnson and Lardy (1967). The isolated liver was placed in an ice-cold medium containing 250 mM sucrose, 10 mM Tris-HCl and 1 mM EDTA, pH 7.2 at 4 °C. The homogenate was centrifuged at 600 × g for 10 min, and the supernatant was centrifuged for 5 min at 15 000 × g to obtain a mitochondrial pellet. The mitochondrial pellet was then washed with a medium containing no added EDTA and centrifuged for 5 min at 15 000 × g. The resulting pellet contained approximately 50 mg protein/mL, which was determined using the Bradford method (1976).

Mitochondrial swelling. The rate of mitochondrial swelling, which indicates the level of the mitochondrial permeability transition, was determined from the change in absorbance of a mitochondrial suspension at 520 nm according to the procedure reported by Elimadi *et al.* (2001). Briefly, the liver mitochondria (4 mg) were isolated from the animals in each experimental group, and 4 mL of a phosphate buffer containing 250 mM sucrose, 5 mM KH₂PO₄ and 1 µM rotenone, pH 7.2 at 25 °C was added. The resulting suspension (1.8 mL) was added to both the sample and reference cuvettes. Succinate (6 µM) was added to the sample cuvette only. The cuvettes were scanned at a wavelength of 520 nm.

Serum TNF- α levels and caspase-3 activity. The serum TNF- α level was determined 1 h after the D-GalN/LPS injection using an enzyme-linked immunosorbent assay (ELISA) with a commercial mouse TNF- α ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The caspase-3 activity was measured using an *in vitro* fluorogenic peptide substrate, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-cumarine (DEVD-AFC; BioMol, Plymouth Meeting, PA, USA), according to the procedure reported by Morin *et al.* (2004). Eight hours after the D-GalN/LPS treatment, a sample of liver tissue (1 g) was homogenized in 6 mL of a buffer containing 25 mM Tris, 5 mM MgCl₂, 1 mM EGTA and 50 μ L of a protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 600 \times g for 10 min. The supernatant was then centrifuged for 15 min at 40 000 \times g, and the resulting supernatant was collected to determine the caspase-3 activity. Dithiothreitol (10 mM) was added to the samples immediately before freezing. The caspase-3 activity was assayed in a total volume of 100 μ L. Thirty micrograms of the cytosolic protein was incubated in a buffer containing 30 mM HEPES, 0.3 mM EDTA, 100 mM NaCl, 0.15% Triton X-100 and 10 mM dithiothreitol. The samples were incubated at room temperature for 15 min. The reaction was started by adding 200 μ M of DEVD-AFC and the samples were incubated at 37 °C. The change in fluorescence (excitation at 400 nm and emission at 490 nm) was monitored after 120 min incubation.

Statistical analysis. The results are presented as the mean \pm SEM. The overall significance of the results was examined using one-way analysis of variance (ANOVA). The differences between the groups were considered statistically significant at a value of $p < 0.05$ with the appropriate Bonferroni correction made for multiple comparisons.

RESULTS

Lethality and serum ALT levels in mice with D-GalN/LPS-induced fulminant hepatic failure

The mice began to die at 6 h after the D-GalN/LPS injection (data not shown), and the mortality reached 73.3% at 24 h. However, a pretreatment with daidzin markedly decreased the mortality, as shown in Table 1. The serum ALT level, which is a serum marker of hepatocyte necrosis, in the control animals was 56.1 \pm 11.0 U/L. In the D-GalN/LPS-treated group, the serum ALT levels increased by approximately 80-fold of the

Table 1. Effect of daidzin on lethality induced by D-GalN/LPS

Group	Dose (mg/kg i.p.)	8 h	Dead/total 24 h	Lethality at 24 h (%)
Control	–	0/15	0/15	0
D-GalN/LPS	–	8/15	11/15	73.3
Daidzin + D-GalN/LPS	25	5/15	8/15	53.3
	50	3/15	7/15	46.7
	100	3/15	4/15	26.7
	200	4/15	4/15	26.7

Each group consisted of 15 mice. Mice were intraperitoneally injected with D-GalN (700 mg/kg)/LPS (10 μ g/kg). Daidzin (25, 50, 100 and 200 mg/kg) or vehicle were intraperitoneally administered at 1 h before D-GalN/LPS injection.

control at 8 h after the D-GalN/LPS injection, which indicates severe liver injury. This increase was suppressed by the administration of 50, 100 and 200 mg/kg daidzin (Table 2).

Histological changes of liver induced by D-GalN/LPS

The histological features shown in Fig. 2 indicate a normal liver lobular architecture and cell structure in the livers from the control animals. However, the livers exposed to D-GalN/LPS showed apparent broad hemorrhagic necrosis, extensive areas of portal inflammation and a moderate increase in inflammatory cell infiltration. These pathological alterations were ameliorated in the animals receiving the daidzin treatment.

Hepatic lipid peroxidation and reduced glutathione levels in mice liver with D-GalN/LPS

As indicated in Table 2, the hepatic malondialdehyde level in the control group was low. However, the administration of D-GalN/LPS increased the hepatic malondialdehyde level to approximately double that of the control animals. This elevation was attenuated by 25, 50 and 100 mg/kg of daidzin. In contrast, the GSH content decreased significantly 8 h after the D-GalN/LPS injection, and was attenuated by 25, 50, 100 and 200 mg/kg daidzin (Table 2).

Mitochondrial swelling induced by D-GalN/LPS

Figure 3 shows the initial rates of mitochondrial swelling in each experimental group. The mitochondria in

Table 2. Effect of daidzin on ALT activity, lipid peroxidation and reduced glutathione levels in mice after D-GalN/LPS-treatment

Group	Dose (mg/kg i.p.)	ALT (U/L)	Malondialdehyde (nmol/mg protein)	GSH (μ mol/g liver)
Control	–	56.1 \pm 11.0	0.35 \pm 0.02	5.9 \pm 0.4
D-GalN/LPS	–	4335.1 \pm 660.0 ^{aa}	0.61 \pm 0.05 ^{aa}	2.2 \pm 0.3 ^{aa}
Daidzin + D-GalN/LPS	25	3975.3 \pm 1709.7 ^{aa}	0.38 \pm 0.06 ^b	4.7 \pm 0.5 ^b
	50	1724.8 \pm 600.7 ^{aa,b}	0.39 \pm 0.02 ^{bb}	4.1 \pm 0.2 ^b
	100	994.3 \pm 298.2 ^{aa,bb}	0.40 \pm 0.02 ^b	5.0 \pm 0.4 ^{bb}
	200	1108.1 \pm 609.1 ^{aa,b}	0.45 \pm 0.02	4.5 \pm 0.3 ^b

Each value is the mean \pm SEM of 8–10 animals per group. Significantly different (^{aa} $p < 0.01$) from controls. Significantly different (^b $p < 0.05$, ^{bb} $p < 0.01$) from D-GalN/LPS.

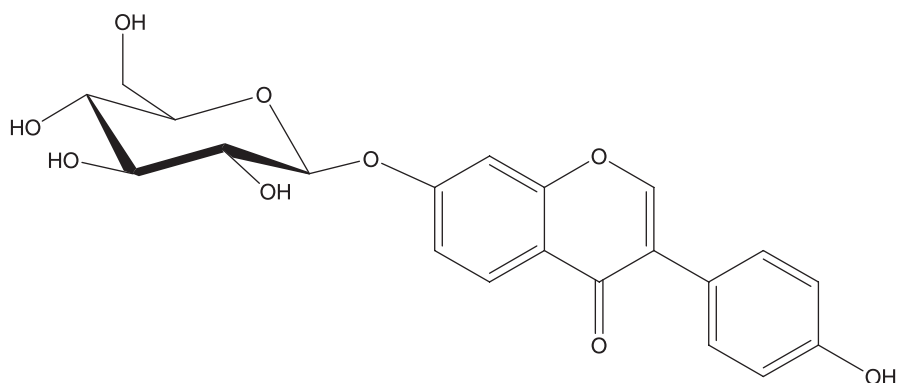


Figure 1. Chemical structure of daidzin.

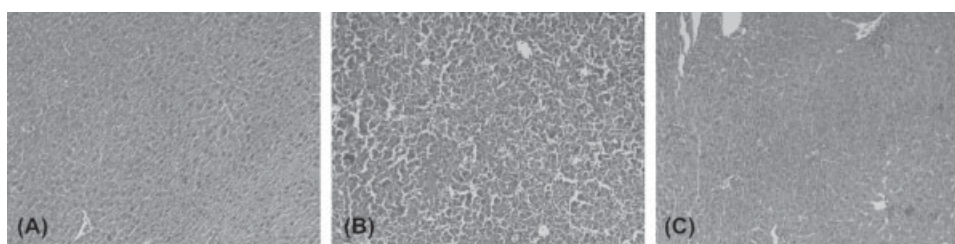


Figure 2. Histological analysis of the livers after D-GalN/LPS administration. Typical images were chosen from the different experimental groups (original magnification $\times 200$). (A) The control group: normal lobular architecture and cell structure; (B) D-GalN/LPS group: multiple and extensive areas of portal inflammation and hepatocellular necrosis, and a moderate increase in inflammatory cell infiltration; (C) daidzin (100 mg/kg)-treated D-GalN/LPS group: minimal hepatocellular necrosis and inflammatory cell infiltration, and mild portal inflammation.

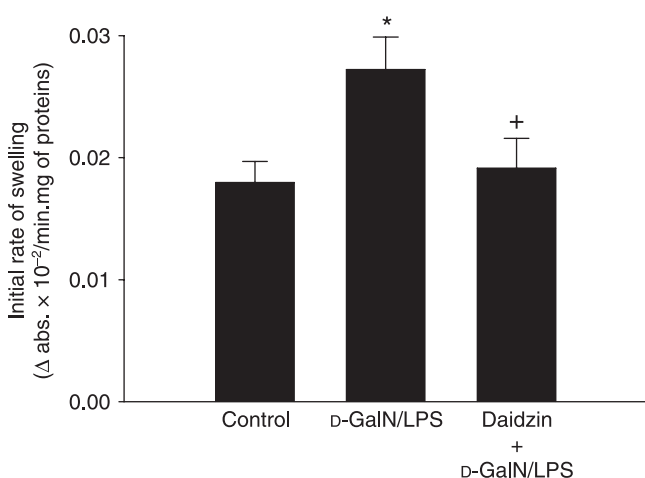


Figure 3. Effect of daidzin on the rate of mitochondrial swelling in the liver from the mice after D-GalN/LPS. Each value is reported as the mean \pm SEM of 8–10 animals per group. Significantly different (* $p < 0.05$) from the controls. Significantly different (⁺ $p < 0.05$) from the D-GalN/LPS group.

all groups swelled after being energized with succinate. The rate of mitochondrial swelling at 6 h after the D-GalN/LPS injection was significantly higher than that in the control group. This rapid increase in the mitochondrial volume was attenuated by daidzin.

Hepatocyte apoptosis in mice treated with D-GalN/LPS

Apoptotic hepatocytes were detected by TUNEL staining. A large number of TUNEL-positive hepatocytes were

Table 3. Effect of daidzin on serum TNF- α levels and caspase-3 activities in mice after D-GalN /LPS

Group	TNF- α (pg/mL)	Caspase-3 (% of control)
Control	76.3 \pm 2.5	100.0 \pm 15.4
D-GalN/LPS	1233.3 \pm 105.2 ^{aa}	460.6 \pm 74.1 ^{aa}
Daidzin + D-GalN/LPS	475.4 \pm 50.9 ^{aa,bb}	172.8 \pm 71.6 ^{bb}

Each value is the mean \pm SEM of 8–10 animals per group. Significantly different (^{aa} $p < 0.01$) from controls. Significantly different (^{bb} $p < 0.01$) from D-GalN /LPS.

observed in the mouse liver tissues obtained 8 h after the D-GalN/LPS treatment. However, few TUNEL-positive hepatocytes were observed in the livers obtained from the animals pretreated with daidzin (Fig. 4).

Serum TNF- α levels and caspase-3 activity induced by D-GalN/LPS

As shown in Table 3, the serum TNF- α levels and caspase-3 activity in the control animals were quite low. In contrast, the serum TNF- α level increased 16.2-fold 1 h after the D-GalN/LPS treatment. The caspase-3 activity in the cytosol fraction of the liver isolated 8 h after the D-GalN/LPS treatment was significantly higher than that observed in the control animals. These changes were attenuated by a pretreatment with daidzin.

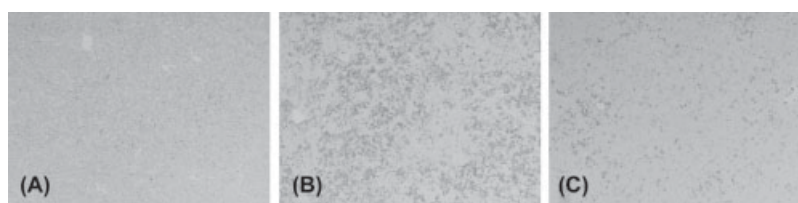


Figure 4. Detection of apoptotic hepatocytes in the liver from the mice after D-GalN/LPS injection (original magnification $\times 200$). (A) Control group; (B) D-GalN/LPS group; (C) daidzin (100 mg/kg)-treated D-GalN/LPS.

DISCUSSION

This study examined the reducing activity of daidzin, a major isoflavone of *Puerariae radix*, on D-GalN/LPS-induced liver injury. *Puerariae radix* contains several isoflavonoids, including puerarin, daidzin, daidzein, formononetin and their C- and O-glycosides (Prasain *et al.*, 2003). These compounds have been associated with antioxidant, antidipsotropic, and other pharmacological activities (Chueh *et al.*, 2004; Lee, 2004). It was reported recently that daidzin and daidzein offer protection against carbon tetrachloride-, D-GalN- and *t*-butyl hydroperoxide-induced hepatotoxicity *in vitro* (Park *et al.*, 2007).

In this study, the mice in the D-GalN/LPS-treated group began to die at 6 h, and the mortality rate reached 73.3% (11 of 15) by 24 h. Eight hours after the D-GalN/LPS injection, severe necrosis occurred as a result of D-GalN/LPS-induced fulminant hepatic failure. This was indicated by the significant increase in the serum ALT levels. These results show that daidzin protects mice from D-GalN/LPS-induced fulminant hepatitis, as evidenced by the significant decrease in the mortality and serum ALT levels in a dose-dependent manner. Indeed, the liver histopathology clearly showed that the D-GalN/LPS-induced hemorrhage, necrosis and hepatocyte degeneration were improved dramatically in the daidzin-pretreated mice. Although Hasumuma *et al.* reported that daidzin had no protective effect against D-GalN/LPS-induced lethality, the dose selected in their study was lower than the range of effective doses used in the present study.

The ROS are important cytotoxic and signaling mediators in the pathophysiology of inflammatory liver damage (Jaeschke, 2000) and play a major role in the onset of hepatic damage in the pathogenesis of D-GalN/LPS-induced fulminant hepatic failure (Xiong *et al.*, 1999). An ROS attack on the biological membranes can lead to the oxidative destruction of the polyunsaturated fatty acids in the membrane through lipid peroxidation. Lipid hydroperoxides and their breakdown products are involved in the deactivation and degradation of the mitochondrial enzymes and transport proteins (Chen and Yu, 1994). In order to counteract these deleterious effects of ROS, hepatocytes contain a small number of antioxidant systems represented mainly by GSH (Fernández-Checa *et al.*, 1998). Hence, conditions that reduce the GSH content may increase the level of oxidative injury associated with D-GalN/LPS-induced hepatic failure. In the D-GalN/LPS group, the hepatic GSH level was significantly lower than the controls as a result of its consumption caused by the elevated oxidative stress. In contrast, the level of hepatic lipid peroxidation 8 h after the D-GalN/LPS injection was significantly higher than in the control. This suggests that the ROS produced in the D-GalN/

LPS treated livers causes cell damage directly through GSH depletion and subsequent lipid peroxidation. The daidzin treatment attenuated the decrease in the hepatic GSH content and lipid peroxidation, which suggests that it increases the hepatic pool of GSH and reduces the level of oxidative stress.

Current biochemical and pathological studies suggest that hepatocyte apoptosis plays an important role in the development of fulminant hepatic failure. Moreover, the regulation of undesired hepatocyte apoptosis is an attractive strategy for the treatment of fulminant hepatic failure. In this model, apoptosis of hepatocytes was observed (Nakama *et al.*, 2001). Previous reports suggested that TNF- α plays an important role in the inflammatory and apoptotic response to a D-GalN/LPS injury (Enomoto *et al.*, 2003). TNF- α is a potent inflammatory cytokine produced by the monocyte-macrophage lineage, including Kupffer cells. TNF- α is essential for D-GalN/LPS-induced fulminant hepatic failure because it not only causes the production of reactive oxygen species but also activates the caspase-8-dependent apoptotic signals by binding to the TNF receptor on the surface of hepatocytes. Caspase-8 then triggers the activation of caspase-3, a downstream cysteine proteinase, through multiple apoptosis signal pathways. This study confirmed the dramatic increase in the serum TNF- α level 1 h after the administration of D-GalN/LPS. These changes were attenuated by the daidzin pretreatment. These results are similar to those reported by Hasumuma *et al.* (2007) in that daidzin suppressed the LPS-induced production of TNF- α . Therefore, the protective effects of daidzin against D-GalN/LPS-induced fulminant hepatic failure may be due to the inhibition of TNF- α production.

In recent years, the role of the mitochondria in apoptotic and necrotic cell death has attracted considerable attention. In apoptosis and necrosis, the mitochondrial permeability transition, which leads to a disruption of the mitochondrial membranes and mitochondrial dysfunction, is believed to be one of the key events, even though its precise role in cell death is not completely understood. The mitochondria permeability transition is quite sensitive to the redox state of the mitochondria. Oxidative stress triggers permeability transition pore opening, which ultimately leads to a loss of mitochondrial integrity resulting in the release of cytochrome *c* into the cytoplasm. This in turn activates the caspase activity and initiates apoptotic cell death. Recent reports suggest that permeability transition pore opening is responsible for the release of cytochrome *c* and other apoptogenic factors, which lead to the activation of the caspase activity that initiates apoptotic cell death (Bernardi *et al.*, 2001). Cytochrome *c* plays an important role in the apoptotic cascade. The release of cytochrome *c* can also be caused by opening of the mitochondrial permeability transition. The measurement

of the release of cytochrome *c* into the cytosol from mitochondria at both 6 h and 8 h after GalN/LPS injection, the typical time-points of hepatocyte apoptosis, was attempted by Western blot assay. Unfortunately, the cytochrome *c* levels could not be quantified due to massive congestion in the liver at these time-points. In this study, there was a significant increase in the swelling rate at 6 h after the D-GalN/LPS treatment, which then declined at 8 h (data not shown). This suggests that D-GalN/LPS cause an obvious disruption of the mitochondrial structure, which is characterized by swelling and pore opening. This disruption was prevented by daidzin. In accordance with these results, Soriano *et al.* (2004) reported that a treatment with cyclosporine A, an immunosuppressive cyclic oligopeptide that specifically blocks the conductance of ions through the mitochondria permeability transition pore, before or in conjunction with the D-GalN/LPS offered protection against hepatotoxicity. These increases in caspase-3 activity and TUNEL staining were measured to confirm the possible involvement of the mitochondria

in the apoptotic pathway. There is increasing evidence suggesting that an injection of D-GalN/LPS increases the activity of caspase-3 and the number of apoptotic hepatocytes, which leads to the death of the animal (Nakama *et al.*, 2001). These results show that a daidzin pretreatment attenuates the increase in caspase-3 activity caused by the D-GalN/LPS injection. These biochemical parameters of apoptotic cell death paralleled the morphological changes observed by TUNEL staining.

In conclusion, daidzin may prevent D-GalN/LPS-induced fulminant hepatic failure by suppressing oxidative stress and apoptosis of hepatocytes. This study provides evidence that daidzin may be an alternative treatment for the prevention of hepatic failure.

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