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# Anti-apoptotic and Hepatoprotective Effects of Gomisin A on Fulminant Hepatic Failure Induced by D-Galactosamine and Lipopolysaccharide in Mice

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**Abstract.** This study examined the effects of gomisin A, a lignan compound from Schisandra fructus, on D-galactosamine (GalN) and lipopolysaccharide (LPS)-induced hepatic apoptosis and liver failure. Mice were given an intraperitoneal injection of GalN (700 mg/kg) / LPS (10 $\mu$g/kg). Gomisin A (25, 50, 100, and 200 mg/kg) was administered intraperitoneally 1 h before the GalN/LPS injection. The liver injury was assessed biochemically and histologically. GalN/LPS increased the serum aminotransferase levels and lipid peroxidation but decreased the reduced glutathione level. The pretreatment with gomisin A attenuated these changes in a dose-dependent manner. The survival rate of the gomisin A group was significantly higher than that of the control. The mitochondria isolated after the mice had been injected with GalN/LPS were swollen, which was attenuated by the gomisin A pretreatment. The elevation of serum tumor necrosis factor-$\alpha$ and activation of caspase-3 were observed in the GalN/LPS group, which was attenuated by gomisin A. The gomisin A–pretreated groups showed significantly fewer apoptotic (TUNEL-positive) cells and DNA fragmentation as compared with the GalN/LPS mice. The liver protection afforded by gomisin A is the result of the reduced oxidative stress and its anti-apoptotic activity.

***Keywords***: apoptosis, D-galactosamine (GalN), lipopolysaccharide (LPS), gomisin A, oxidative stress, tumor necrosis factor (TNF)-$\alpha$

## Introduction

Fulminant hepatic failure is a dramatic clinical syndrome that results from massive hepatocyte death. Although the nature of fulminant hepatic failure has been studied extensively, the mechanisms by which the organ damage occurs are not completely understood. D-Galactosamine (GalN) and lipopolysaccharide (LPS)-induced liver failure is a well-established experimental model (1). The model takes advantage of the ability of a transcriptional inhibitor, GalN, to potentiate the toxic effects of LPS, producing typical hepatic necrosis and apoptosis followed by fulminant hepatitis (2).

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Schisandra fructus, which is the fruit of *Schisandra chinensis* BAILL (Schizandraceae), is a well-known traditional Korean herbal medicine that is used as an antitussive, tonic, and sedative agent and to improve the liver function of patients with viral hepatitis (3). Schisandra fructus has potent antioxidative, antimicrobial, and nitrite scavenging effects (4). Its extract inhibits retinol-induced irritation and pro-inflammatory cytokine secretion (5). Recently, we have screened a number of liver protective agents from natural products, which are used traditionally in Korea for liver diseases (6, 7). Agents extracted from natural products used in folk medicine are expected to be therapeutically effective and to have a lower toxicity when used clinically.

Gomisin A is a lignan compound that is extracted and purified from dry fruits of *Schisandra chinensis* BAILL. Gomisin A improves histologically the hepatic cell

degeneration, necrosis, and resultant inflammatory reactions in experimental liver injury models induced by carbon tetrachloride, GalN, $\alpha$-naphthyl isothiocyanate, and orotonic acid (8). In addition, gomisin A has been reported to be effective in improving immunologically-induced acute hepatic failure (9) as well as in stimulating liver regeneration after a partial hepatectomy (10). However, the precise mechanism for its protective effects in liver failure is unclear.

Therefore, this study examined the hepatoprotective effects of gomisin A against fulminant hepatic failure, particularly on the extent of oxidative damage and apoptosis.

## Materials and Methods

*Extraction and isolation of gomisin A*

The dried fruits of *Schisandra chinensis* (20 kg) were refluxed with hot methanol two times. The methanol extract was concentrated in vacuum, yielding a residue (4.5 kg) that was suspended in water and successively partitioned with hexane, ethylacetate, and butanol and then evaporated to yield a hexane fraction (512 g), an ethylacetate fraction (554 g), and a butanol fraction (886 g). The hexane fraction was subjected to silica gel column chromatography (12 × 25 cm) eluted with gradient of hexane:acetone (100:1, 70:1, 50:1, 30:1, 10:1, 5:1, and 1:1, each 6 l) and separated into 7 fractions: H1 (0 – 6 l), H2 (6 – 12 l), H3 (12 – 18 l), H4 (18 – 27 l), H5 (27 – 35 l), H6 (35 – 40 l), and H7 (40 – 42 l). Fraction H5 (36 g) was chromatographed on a silica gel column (5 × 30 cm) eluted with hexane:acetone (10:1 and 6:1) to give ten subfractions (H5.1 – H5.10). Repeated chromatography of subfraction H5.8 on a silica gel column eluted with hexane:ethylacetate (5:1) yielded subfraction H5.8.5, from which gomisin A (2500 mg) was obtained by crystallization (Fig. 1).

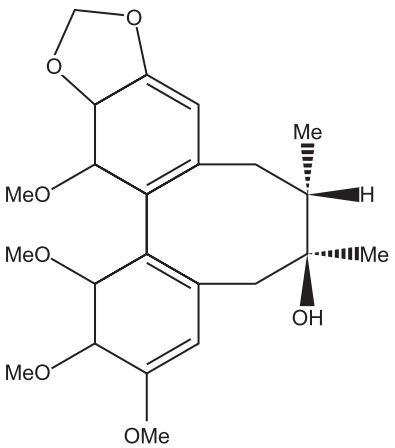


**Fig. 1.** Chemical structure of gomisin A.

The characteristics of gomisin A were as follows: mp: 80°C – 85°C; UV, $\lambda_{max}^{EtOH}$(log $\varepsilon$) 218, 235, 280 nm; IR, $\nu_{max}$ (KBr) 3470, 1618, 1590, 1462, 1402, 1271, 1113 $cm^{-1}$; FABMS [$M^+$] 416 *m/z*; $^1$H-NMR (300 MHz, $CDCl_3$) $\delta$: 0.81 (3H, d, $J$ = 7.5 Hz, 8-$CH_3$), 1.24 (3H, s, 7-$CH_3$), 1.85 (1H, m, H-8), 2.33 (1H, dd, $J$ = 8.1, 14.1 Hz, H-9), 2.34 (1H, d, $J$ = 13.5 Hz, H-6), 2.57 (1H, dd, $J$ = 1.5, 14.1 Hz, H-9'), 2.67 (1H, d, $J$ = 13.5 Hz, H-6'), 5.96, 5.97 (1H, d, $J$ = 1.5 Hz, $OCH_2O$), 6.47 (1H, s, H-11), 6.62 (1H, s, H-4), 3.51 – 3.90 ($OCH_3$). $^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$: 152.1 (C-1), 140.6 (C-2), 151.9 (C-3), 110.2 (C-4), 132.4 (C-5), 40.5 (C-6), 71.6 (C-7), 42.0 (C-8), 33.7 (C-9), 131.9 (C-10), 105.8 (C-11), 147.2 (C-12), 134.5 (C-13), 141.1 (C-14), 121.7 (C-15), 124.0 (C-16), 15.8 (C-17), 30.1 (C-18), 100.7 ($OCH_2O$), 55.9 – 61.0 ($OCH_3$).

*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. For preparation of the mice with GalN/LPS-induced fulminant hepatic failure, the mice (except for the normal control) were injected intraperitoneally with GalN (700 mg/kg; Sigma Chemical Co., St. Louis, MO, USA) and LPS (10 $\mu$g/kg *Escherichia coli* 026:B6, Sigma) dissolved in phosphate-buffered saline. Gomisin A was suspended in 10% Tween 80 – saline (vehicle) and administered at a dose of 25, 50, 100, and 200 mg/kg intraperitoneally 1 h before the GalN/LPS treatment. Six treatment groups were examined: a) vehicle-treated control; b) vehicle-treated GalN/LPS; c – f) gomisin A (25, 50, 100, and 200 mg/kg)-treated GalN/LPS groups. This gomisin A dose was selected because it has been evaluated previously (9, 10). The mice were sacrificed by decapitation at different time points, and blood and liver samples were collected for further examination.

*Determination of lethality and histopathological analysis*

The survival rate of the mice was monitored for 24 h after the GalN/LPS injection. The liver specimens for the histopathological analysis were obtained 8 h after administering the GalN/LPS. The sample was fixed in 10% neutral-buffered formalin. The sample was then embedded in paraffin, sliced into 5-$\mu$m sections, and stained with hematoxylin-eosin for a blind histological assessment. The morphologic criteria used to determine the degree of necrosis were portal inflammation, hepatocellular necrosis, inflammatory cell infiltration, and loss of cell architecture. The histological changes were evaluated in nonconsecutive, randomly chosen ×200

histological fields.

*Serum alanine aminotransferase activity and tumor necrosis factor-α (TNF-α) levels*

The serum alanine aminotransferase (ALT) activity at 8 h after the GalN/LPS injection was determined with a Hitachi 747 automatic analyzer (Hitachi, Tokyo). The serum TNF-$\alpha$ level was quantified at 1 h after the GalN/LPS injection using an enzyme-linked immunosorbent assay with a commercial mouse TNF-$\alpha$ ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

*Hepatic lipid peroxidation levels and glutathione contents*

The hepatic lipid peroxidation levels and glutathione content were measured 8 h after the GalN/LPS injection. The steady-state level of malondialdehyde, a lipid peroxidation end product, was analyzed 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 (11) using 1,1,3,3-tetraethoxypropane (Sigma) as the standard. The total glutathione in the liver homogenate was determined spectrophotometrically at a wavelength of 412 nm, with yeast glutathione reductase, 5,5'-dithio-bis(2-nitrobenzoic acid) and NADPH, according to the method reported by Tietze (12). The GSSG level was measured using the same method in the presence of 2-vinylpyridine (13), 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 GalN/LPS injection according to the method reported by Johnson and Lardy (14). 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 $600 \times g$ for 10 min and the supernatant was centrifuged for 5 min at $15{,}000 \times g$ to obtain the mitochondrial pellet. The mitochondrial pellet was then washed with a medium containing no added EDTA and centrifuged for 5 min at $15{,}000 \times g$. The resulting pellet contained approximately 50 mg protein/ml, which was determined using the Bradford method (15).

*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. (16). Briefly, the liver mitochondria (4 mg) were isolated from the animals in each experimental group and to each mitochondria sample, 4 ml of phosphate buffer containing 250 mM sucrose, 5 mM $KH_2PO_4$, and 1 $\mu$M rotenone (pH 7.2) was added at 25°C. The resulting mitochondria suspension (1.8 ml) was added to both the sample and reference cuvettes. Succinate (6 $\mu$M) was added to the sample cuvette only. The cuvettes were scanned at a wavelength of 520 nm.

*Caspase-3 activity*

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, Plynouth Meeting, PA, USA), according to the procedure reported by Morin et al. (17). Eight hours after the GalN/LPS treatment, a sample of liver tissue (1 g) was homogenized in 6 ml of a buffer containing 25 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA, and 50 $\mu$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 immediately to the samples before freezing. The caspase-3 activity was assayed in a total volume of 100 $\mu$l. A 30-$\mu$g sample of 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 $\mu$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.

*Detection of apoptotic cells and DNA fragmentation*

The liver tissues were obtained 8 h after the GalN/LPS injection. 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., Shiga). Under microscopy, the number of TUNEL-positive cells in ×200 histological fields was counted per liver section. The DNA fragmentation was assayed by agarose gel electrophoresis (18) with a slight modification. The frozen liver samples were minced, and 1 g of the sample was lysed with 10 ml of a lysis buffer (50 mM Tris, 10 mM EDTA, and 0.5% SDS). The lysates were treated with 10 mg/ml of proteinase K at 60°C for 90 min and then incubated with 10 mg/kg RNase for 30 min. After brief centrifugation of the lysates, the supernatants were

**Table 1.** Effect of gomisin A on lethality induced by GalN/LPS

| Groups | Dose (mg/kg, i.p.) | Dead/Total | | Lethality at 24 h (%) |
|---|---|---|---|---|
| | | 8 h | 24 h | |
| Control | — | 0/15 | 0/15 | 0 |
| GalN/LPS | — | 8/15 | 14/15 | 93.3 |
| Gomisin A + GalN/LPS | 25 | 4/15 | 7/15 | 46.7 |
| | 50 | 4/15 | 6/15 | 40.0 |
| | 100 | 1/15 | 3/15 | 20.0 |
| | 200 | 0/15 | 2/15 | 13.3 |

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

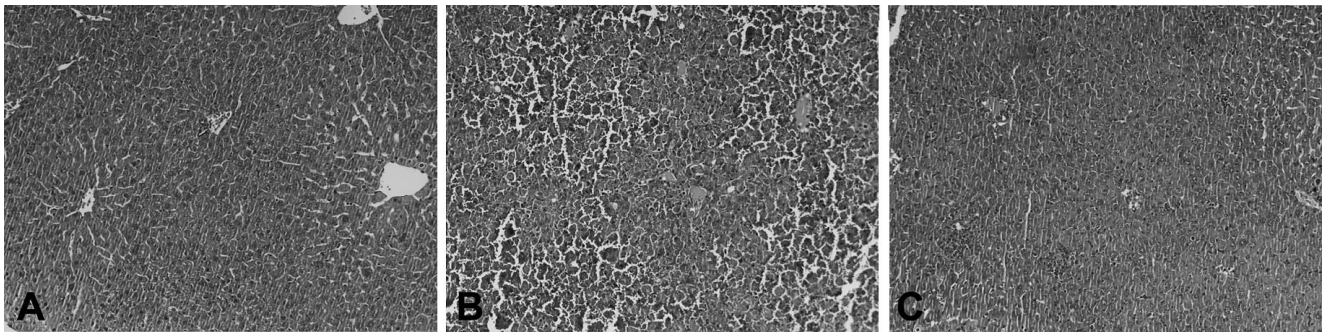


**Fig. 2.** H&E staining of the livers after GalN/LPS administration. Typical images were chosen from the different experimental groups (original magnification ×200). A: Control group: normal lobular architecture and cell structure, B: GalN/LPS group: multiple and extensive areas of portal inflammation and hepatocellular necrosis and a moderate increase in inflammatory cell infiltration, C: gomisin A (100 mg/kg)-treated GalN/LPS group: minimal hepatocellular necrosis and inflammatory cell infiltration and mild portal inflammation.

separated by electrophoresis on a 1.5% agarose gel containing 0.5 $\mu$g/ml ethidium bromide. The DNA fragmentation pattern was examined on the photographs taken under ultraviolet illumination.

*Statistical analyses*

The results are each presented as a mean ± S.E.M. The overall significance of the results was examined by using one-way analysis of variance (ANOVA). The differences between the groups were considered statistically significant at a $P$ value <0.05 with the appropriate Bonferroni correction made for multiple comparisons.

## Results

*Lethality and histological change*

The administration of GalN (700 mg/kg) / LPS (10 $\mu$g/kg) induced fulminant hepatic failure. The mice pretreated with vehicle instead of gomisin A began to die within 6 h of the GalN/LPS injection (data not shown), and the death rate was 93.3% at 24 h. However, pretreatment with gomisin A markedly reduced the death rate, as shown in Table 1. The histological features shown in Fig. 2 indicate a normal liver lobular architecture and cell structure of the livers from the control animals. However, the livers exposed to GalN/LPS showed multiple and extensive areas of portal inflammation, hepatocellular necrosis, which was randomly distributed throughout the parenchyma, and a moderate increase in inflammatory cell infiltration. These pathological changes were ameliorated by the gomisin A treatment.

*Serum alanine aminotransferase activity, lipid peroxidation, and reduced glutathione levels*

The serum ALT levels, which are the serum marker of hepatocyte necrosis, in the control animals were 33.4 ± 3.8 U/l. In the GalN/LPS-treated group, the serum ALT levels increased approximately 160-fold of the control at 8 h after the GalN/LPS injection. This increase was suppressed by the administration of both 100 and 200 mg/kg gomisin A. The administration of

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

| Groups | Dose (mg/kg, i.p.) | ALT (U/l) | Malondialdehyde (nmol/mg protein) | GSH ($\mu$mol/g liver) |
|---|---|---|---|---|
| Control | — | 33.4 ± 3.8 | 0.34 ± 0.02 | 6.7 ± 0.4 |
| GalN/LPS | — | 5353.1 ± 2757.6** | 0.67 ± 0.04** | 3.2 ± 0.5** |
| Gomisin A + GalN/LPS | 25 | 4670.5 ± 1499.3** | 0.57 ± 0.06** | 3.0 ± 0.7** |
| | 50 | 4250.9 ± 816.3** | 0.46 ± 0.07$^{++}$ | 4.4 ± 0.6* |
| | 100 | 764.3 ± 312.7**,$^{++}$ | 0.38 ± 0.03$^{++}$ | 5.0 ± 0.5*,$^{++}$ |
| | 200 | 611.9 ± 323.8**,$^{++}$ | 0.42 ± 0.04$^{++}$ | 4.7 ± 0.3*,$^{+}$ |

Each value is the mean ± S.E.M. of 8 – 10 animals per group. Significantly different (*$P$<0.05, **$P$<0.01) from controls. Significantly different ($^{+}P$<0.05, $^{++}P$<0.01) from GalN/LPS.

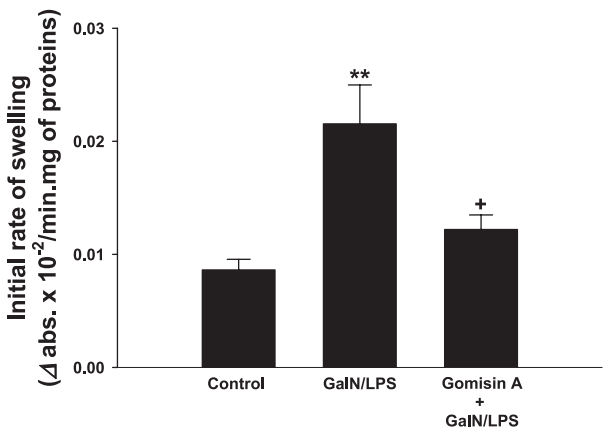


**Fig. 3.** Effect of gomisin A (100 mg/kg) on the rate of mitochondrial swelling in the liver of mice after GalN/LPS administration. Each value is the mean ± S.E.M. of 8 – 10 animals per group. Significantly different (**$P$<0.01) from controls. Significantly different ($^{+}P$<0.05) from GalN/LPS.

GalN/LPS increased the hepatic malondialdehyde level to approximately double that of the control animals. This elevation was attenuated by 50, 100, and 200 mg/kg of gomisin A. In contrast, the GSH content decreased significantly 8 h after the GalN/LPS injection, which was attenuated by 100 or 200 mg/kg gomisin A (Table 2).

*Mitochondrial swelling*

Figure 3 shows the succinate-driven increases in the initial swelling rates of the isolated liver mitochondria. The basal rate of succinate-driven swelling of the liver mitochondria isolated from the controls was low. However, the rate of mitochondrial swelling increased markedly 6 h after the GalN/LPS treatment, which was attenuated by the pretreatment with 100 mg/kg gomisin A.

*Serum TNF-α levels and caspase-3 activity*

As shown in Table 3, the serum TNF-$\alpha$ level in the control animals was quite low. In contrast, the serum TNF-$\alpha$ level increased 24.8-fold 1 h after the GalN/LPS treatment but was reduced by gomisin A. The caspase-3 activity in the cytosol fraction of the liver isolated from 8 h after the GalN/LPS treatment was significantly higher than the control animals but was attenuated by a pretreatment with gomisin A.

*Hepatocyte apoptosis and DNA fragmentation*

Apoptotic hepatocytes were detected by TUNEL staining. A large number of TUNEL-positive hepatocytes were observed in the mouse liver tissues obtained from 8 h after the GalN/LPS treatment. However, a few TUNEL-positive hepatocytes were observed in the livers from the animals pretreated with gomisin A (Fig. 4). The genomic DNA fragmentation was assayed to

**Table 3.** Effect of gomisin A on serum TNF-$\alpha$ levels and caspase-3 activities in mice after GalN/LPS-treatment

| Groups | TNF-$\alpha$ (pg/ml) | Caspase-3 (% of control) |
|---|---|---|
| Control | 45.1 ± 4.3 | 100.0 ± 17.3 |
| GalN/LPS | 1119.3 ± 145.8** | 753.7 ± 83.1** |
| Gomisin A + GalN/LPS | 551.2 ± 84.1**,$^{++}$ | 321.5 ± 79.2**,$^{++}$ |

Mice were pretreated with gomisin A (100 mg/kg) 1 h before GalN (700 mg/kg) / LPS (10 $\mu$g/kg). Serum TNF-$\alpha$ levels were determined at 1 h after and caspase-3 activities were determined at 8 h after GalN/LPS-treatment. Each value is the mean ± S.E.M. of 8 – 10 animals per group. Significantly different (**$P$<0.01) from controls. Significantly different ($^{++}P$<0.01) from GalN/LPS.

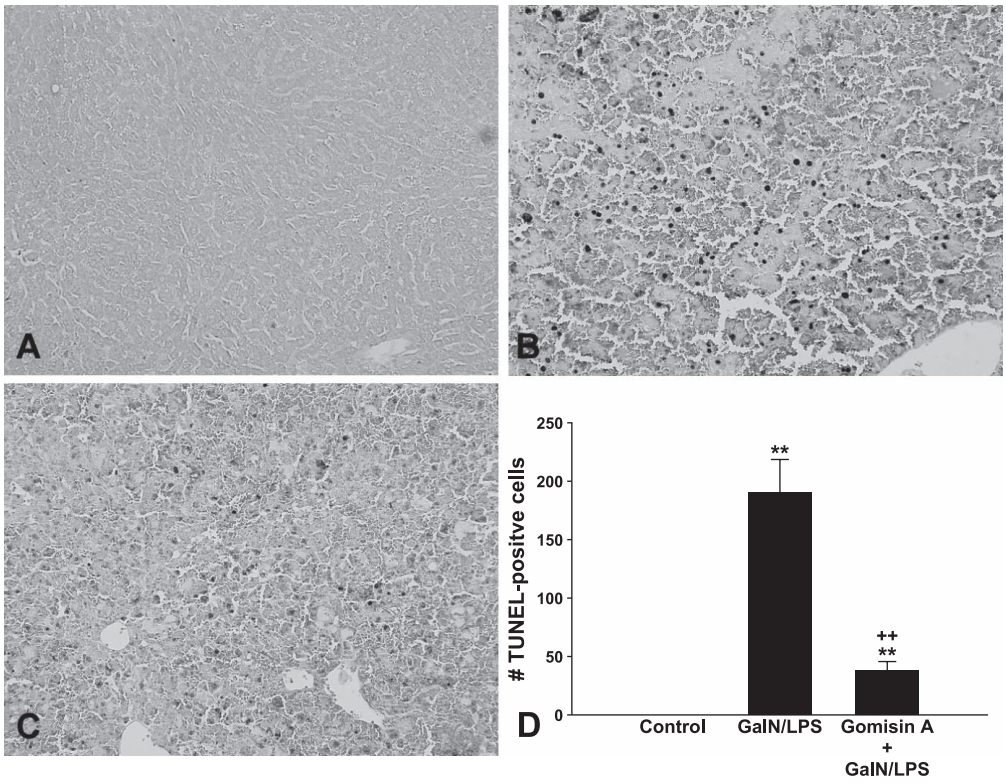


**Fig. 4.** Detection of apoptotic hepatocytes in the liver of mice after GalN/LPS injection (original magnification ×200). A: Control group, B: GalN/LPS group, C: gomisin A (100 mg /kg)-treated GalN/LPS group, D: TUNEL-positive hepatocytes. Numbers are apoptotic cells in randomly chosen ×200 histological fields. Significantly different (**$P$<0.01) from controls. Significantly different ($^{++}P$<0.01) from GalN/LPS.

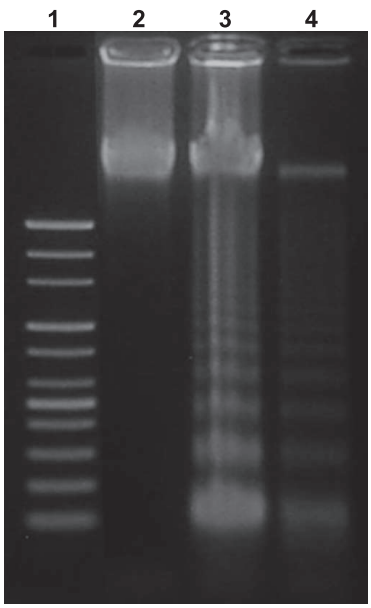


**Fig. 5.** DNA fragmentation in the liver of mice after GalN/LPS injection. Lane 1: molecular weight standard, lane 2: control group, lane 3: GalN/LPS group, lane 4: gomisin A (100 mg/kg)-treated GalN/LPS group.

confirm the suppressive effect of gomisin A on hepatocyte apoptosis. DNA fragmentation was detected in the liver tissues 8 h after the GalN/LPS injection, while very little DNA fragmentation was observed in the livers from the mice pretreated with gomisin A (Fig. 5).

## Discussion

Considerable efforts have been made to clarify the mechanism for the development of fulminant hepatitis and various measures have been taken to treat the disease. However, the prognosis of fulminant hepatitis is quite poor, and there is no effective therapy for the disease. Schisandra fructus has been used as a traditional medicine in Korea and East Asia to treat an increased serum aminotransferase activity in acute hepatitis. Schisandra fructus contains several lignans, including gomisin A, B, C, F, and G (19). Gomisin A is the most active component among these lignans (20). It was reported that gomisin A prevents some experimental acute liver injuries (8). However, the nature of the protective mechanisms involved remains unclear. This study clearly demonstrated that gomisin A prevented the lethality in mice with GalN/LPS-induced fulminant hepatic failure through the inhibition of necrosis and apoptosis of hepatocytes.

In this study, the death of the mice in the GalN/LPS-treated group occurred at 6 h, and the mortality rate reached an extremely high level of 93.3% (14 of 15) at 24 h. At 8 h after the GalN/LPS injection, severe

necrosis occurred as a result of GalN/LPS-induced fulminant hepatic failure. This was indicated by the significant increase in the serum ALT levels. These results are similar to those reported by Nakama et al. (1) in that the serum ALT levels began to increase at 6 h and reached a maximum at 8 h after the GalN/LPS injection. Moreover, the gomisin A treatment (100 and 200 mg/kg) markedly attenuated the lethality and the release of ALT Indeed, histological hematoxylin-eosin staining clearly showed that the GalN/LPS-induced hemorrhaged necrosis and hepatocyte degeneration were dramatically lower in the gomisin A-treated mice. Therefore, the decrease in mortality and serum ALT levels by gomisin A in this study is probably due to its protection against GalN/LPS-induced liver injury.

Although the underlying mechanisms for the hepatocyte injury after the GalN/LPS injection are unclear, it has been assumed that the overproduction of reactive oxygen species caused by GalN/LPS induces the cellular injury leading to necrosis (2). Accumulating evidence suggests that massive oxidative stress plays a major role in the pathogenesis of GalN/LPS-induced fulminant hepatic failure, and antioxidants such as allopurinol, superoxide dismutase, and catalase suppress remarkably the serum aminotransferase levels after a GalN/LPS treatment (21). Reactive oxygen species attack the biological membrane, which can lead to the oxidative destruction of the membrane polyunsaturated fatty acids through lipid peroxidation. The loss of membrane integrity caused by lipid peroxidation leads to the release of the cytosolic contents from ruptured hepatocytes such as ALT. GSH plays an important role as an antioxidant system to counteract the deleterious effects of reactive oxygen species. In the GalN/LPS-treated mice, the hepatic GSH level was significantly lower than the controls. In contrast, the level of hepatic lipid peroxidation was significantly higher at 8 h after the GalN/LPS treatment than the control. This suggests that the reactive oxygen species produced in the GalN /LPS-treated livers cause cell damage directly through GSH depletion and the subsequent lipid peroxidation. The treatment with gomisin A (100 and 200 mg/kg) attenuated the decrease in hepatic GSH content and lipid peroxidation, which indicate antioxidative action plays an important part in the hepatoprotective activity of gomisin A. These results are similar to those of Kiso et al. (20); gomisin A inhibited ascorbate/$Fe^{2+}$- and ADP/$Fe^{3+}$-induced lipid peroxidation by rat liver microsomes, suggesting that it mediates its inhibitory actions at the non-enzymatic step in the process of lipid peroxidation.

The current biochemical and pathological studies suggest that hepatocyte apoptosis plays an important role in the development of fulminant hepatic failure and that the regulation of undesired hepatocyte apoptosis is one approach for treating fulminant hepatic failure. Apoptosis has been observed in animal models of GalN/LPS injury (22). In addition to necrosis, it is possible that the cells sublethally damaged by reactive oxygen species could be triggered to undergo programmed cell death. Therefore, TUNEL staining and genomic DNA fragmentation (DNA-laddering) assay were performed to confirm the suppressive effect of gomisin A on hepatocyte apoptosis. The results using the GalN/LPS-induced fulminant hepatic failure model demonstrated striking hepatocyte apoptosis. Moreover, the gomisin A pretreatment inhibited the apoptotic process occurring in GalN/LPS injury, which translates to a protective effect.

The mitochondria play an important role in energy production, $Ca^{2+}$ homeostasis, and cell death. In recent years, the role of the mitochondria in apoptotic and necrotic cell death has attracted considerable attention (23). In apoptosis and necrosis, the mitochondrial permeability transition, which leads to a disruption of the mitochondrial membranes and mitochondrial dysfunction, is considered 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, and oxidative stress triggers permeability transition pore opening, where increased intracellular $Ca^{2+}$ and reactive oxygen species are present (24). The opening of this pore leads to destruction of the mitochondrial membrane potential and mitochondrial swelling, resulting in cell death (25). Recent reports suggest that permeability transition pore opening is responsible for the release of cytochrome *c* and other apoptogenic factors, leading to the activation of the caspase activity to initiate apoptotic cell death (26). A significant increase in the swelling rate occurred at 6 h after the GalN/LPS treatment, which then declined at 8 h (data not shown). This suggests that GalN/LPS cause an obvious disruption of the mitochondrial structure that is characterized by swelling and opening of the pore, which is prevented by gomisin A. In accordance with these results, Soriano et al. (27) 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 with the GalN/LPS, offers protection from hepatotoxicity. Cytochrome *c* plays an important role in apoptotic cascade. Release of cytochrome *c* can also be caused by opening of the mitochondrial permeability transition. We tried to measure the release of cytochrome *c* into

cytosol from mitochondria at both 6 h and 8 h after GalN/LPS injection, the typical time-points of hepatocyte apoptosis, by Western blot assay. Unfortunately, we could not quantify the cytochrome *c* levels due to massive congestion in the liver at these time-points. The increases in the caspase-3 activity were measured in order to confirm the possible involvement of the mitochondria in the apoptotic pathway. There is increasing evidence suggesting that an injection of GalN/LPS increases the activity of caspase-3 as well as the number of apoptotic hepatocytes, leading to the death of the animal (1). These results show that the gomisin A pretreatment attenuates the increase in caspase-3 activity after the GalN/LPS injection.

The plasma TNF-$\alpha$ levels are elevated in patients with acute alcoholic hepatitis (28) as well as in the chronic hepatitis caused by a hepatitis B virus and C virus infection (29). After a GalN/LPS injection, the TNF-$\alpha$ level in the serum increases rapidly and reaches a maximum within 2 h (1). Some authors have indicated that the anti-TNF-$\alpha$ antibody prevents GalN/LPS-induced fulminant hepatic failure, resulting in a decrease in mortality (30). Therefore, TNF-$\alpha$ plays a role in the pathogenesis of not only an endotoxin-induced experimental liver injury, but also in human liver diseases (31). TNF-$\alpha$, a pleiotropic proinflammatory cytokine, is rapidly produced by macrophages in response to tissue damage (32). An increase in the TNF-$\alpha$ level has been directly correlated with the histological evidence of hepatic necrosis and the increase in the serum aminotransferase levels (33). According to the results of our histological studies, showing massive hepatic necrosis and portal inflammation, it seems likely that it is due to the increased level of TNF-$\alpha$. TNF-$\alpha$ is essential for GalN/LPS-induced fulminant hepatic failure (34) because it not only causes the production of reactive oxygen species but also directly 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, which is a downstream cysteine proteinase, through multiple apoptosis signal pathways (35). In vitro studies of hepatocytes have implicated over-activation of c-Jun $NH_2$-terminal kinase (JNK) signaling as a mechanism of TNF-$\alpha$–induced apoptosis. Recently, several studies, based on the gene-knock out approach, have convincingly demonstrated that the crucial role of JNK in hepatocytes apoptosis induced by GalN/LPS (34). Our study confirmed the dramatic increase in the serum TNF-$\alpha$ level at 1 h after the administration of GalN/LPS. These alterations were attenuated by the gomisin A pretreatment. Therefore, the protective effects of gomisin A against GalN/LPS-induced fulminant hepatic failure may be due to the inhibition of TNF-$\alpha$ production.

Overall, it appears that gomisin A prevents the GalN/LPS-induced fulminant hepatic failure by suppressing oxidative stress and apoptosis of hepatocytes. This study provides evidence that gomisin A may offer an alternative for the prevention of hepatic failure.

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