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Hepatoprotective Effects of Lactic Acid-fermented Garlic Extract against Acetaminophen-induced Acute Liver Injury in Rats

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Abstract The aim of the present study was to investigate the protective effect of fermented garlic extract by lactic acid bacteria (LAFGE) against acetaminophen (AAP)-induced acute liver injury in rats. Here we demonstrated that rats treated with LAFGE exhibit resistance to AAP-induced liver injury accompanied by lowered plasma alanine amino transferase levels and decreased proinflammatory responses. This function of LAFGE is linked to its capacity of suppressing AAP-induced apoptosis in the liver, partly via the inhibition of MAPK phosphorylation as well as down-regulation of p53. Our findings reveal that LAFGE modulates the signaling pathways involved in hepatic apoptosis through cellular redox control, as indicated by the inhibition of lipid peroxidation, glutathione and ATP depletion, and the elevation of antioxidant enzyme activities. Taken together, these findings indicate that LAFGE ameliorates AAP-induced liver injury by preventing oxidative stress-mediated apoptosis, thereby establishing LAFGE as a potential supplement in the treatment of AAP-induced liver injury.

Keywords: fermented garlic, acetaminophen, hepatoprotection, apoptosis

Introduction

Acetaminophen (AAP) is widely used as an ingredient in analgesic or antipyretic drugs such as tylenol, panadol, and pseudoephedrine hydrochloride. AAP is an over-the-counter (OTC) drug, the overdose of which often occurs and leads to acute liver failure (1). AAP is metabolized by cytochrome P450 2E1 (CYP2E1) in the liver, and CYP2E1 can produce electrophilic metabolites such as *N*-acetyl-*p*-benzoquinone imine (NAPQI), which covalently bind to the DNA and hepatocellular proteins (2). At therapeutic dosages, NAPQI is efficiently detoxified by glutathione (GSH) that forms an AAP-GSH complex, which is excreted through the kidney. Overdose of AAP, however, surpasses the elimination rate of NAPQI that leads to the accumulation of NAPQI in the liver and depletion of GSH (3). Owing to the depletion of GSH, residual NAPQI covalently binds to hepatocellular proteins and causes excessive reactive oxygen species (ROS) generation, mitochondrial dysfunction, lipid peroxidation, and DNA damage (4).

Garlic (*Allium sativum* L.) has antioxidant (5), anticancer (6), and immunomodulatory effects (7). A well-known garlic product is aged black garlic (ABG), which is produced by aging under high temperature and humidity; this process takes approximately a month (8). Although ABG is known as fermented garlic, it is not defined as an authentic fermented garlic product (9). The fermentation of garlic using lactic acid bacteria (LAB) can provide some advantages such as the enhancement of antioxidant effects, improvement of flavor, and enrichment with desirable metabolites produced by microorganisms (10). Allicin, a major antimicrobial agent in garlic, is converted from alliin by alliinase and interrupts the growth of microorganisms such as LAB (11-13). Thus, the inactivation of alliinase by heating could allow the fermentation of garlic using LAB (9). We previously prepared a fermented garlic extract using LAB to improve garlic-specific pungent taste and aroma, enhance functional properties of garlic, and overcome disadvantages during the manufacturing process of AGE. We previously found that lactic acid-fermented garlic extract (LAFGE) effectively protected alcohol-induced liver injury in an *in vivo* model by enhancing several antioxidant compounds in garlic during fermentation, including cycloalliin, *S*-allyl cysteine, *S*-methyl cysteine, and *S*-ethyl cysteine (14).

In the present study, we investigated the protective effects of LAFGE against AAP-induced liver injury in rats. AAP-induced liver injury was monitored by histological analysis and hepatic inflammatory response. To understand the protective mechanisms, we measured the activation of p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK), lipid peroxidation, DNA damage, and oxidative stress in the liver.

Materials and Methods

Chemicals and reagents Thiobarbituric acid (TBA), oxidized glutathione (GSSG), reduced glutathione (GSH), 1,1,3,3-tetraethoxypropane, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), NADPH, EDTA, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Detection kits comprising superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione *S*-transferase (GST) were supplied by BioVision (Milpitas, CA, USA). Antibody to cytochrome P450 2E1 (CYP2E1), Bcl-2, Bax, and caspase-3 were purchased from Cell Signaling (Danvers, MA, USA) and JNK, p-JNK, p38, and p-p38, were purchased from Santa Cruz Biotechnology (Santa Cruz, TX, USA). β-Actin was purchased from Abcam (Cambridge, UK).

Preparation of lactic acid-fermented garlic extract (LIVERTECT^{TM}) Garlics were obtained from Gaweol Agricultural Association, Korea. Garlics (5 kg) were added to water (10 kg) and homogenized at 100°C for 3 h in a fermentor (MJS U3; Marubishi, Tokyo, Japan). *Lactobacillus plantarum* BL2 (International depositary authority: KCCM11019P) was inoculated at 2% (v/v) into homogenized garlic after cooling down to 37°C, and then additionally incubated for 36 h. After the extracts were sterilized for 1 h at 121°C, they were filtered, concentrated, and pulverized.

Animal experiment Wistar rats (weight, 180-200 g) were obtained from OrientBio (Sungnam, Korea). Animals were maintained in a controlled environment at 25±2°C under a 12-h dark/light cycle and acclimated for a week prior to the experiments. The animals were randomly divided into 5 groups (n=6): vehicle, control group, silymarin-treated group (100 mg/kg) as positive control, and LAFGE-treated groups (250 and 500 mg/kg). Silymarin and LAFGE suspended in saline were orally administered once a day for 7 consecutive days. Two hours after the final administration, rats were orally administered 2 g/kg of AAP suspended in 1% carboxymethylcellulose sodium (CMC-Na). They were anesthetized for collecting the blood and sacrificed to obtain the livers after the 24-h AAP administration followed by fasting. Serum was obtained by centrifugation at 5,000×g for 10 min and frozen at 80°C until further analysis. The livers were promptly removed, and a portion of the liver was fixed in 10% formalin; the remaining tissues were stored at 80°C.

Serum biochemical assays The levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were determined using an automatic biochemical analyzer (Cobas c-111; Roche, Basel, Switzerland), according to the instructions supplied with the commercial assay kits.

Histological analysis Pieces of fixed liver tissue were embedded in paraffin for histological analysis and observation of hepatic damage. The embedded tissues were sectioned at 5 μm thickness, stained using hematoxylin and eosin, and examined under light microscope (Olympus, Tokyo, Japan).

Measurement of GSH and ATP levels The hepatic total glutathione content was determined according to a method previously reported (15). In brief, 50 mg of liver tissues were homogenized in cold 5% metaphosphoric acid and centrifuged at 11,000×g for 10 min. Obtained supernatant was analyzed for the GSH levels in the reaction mixture containing 1 mM EDTA, 0.24 mM NADPH, 0.06 units glutathione reductase (GR), and 80 μM DTNB. 5-Thio-2-nitrobenzoic acid (TNB), a yellow product, was detected at 412 nm. The amounts of total GSH were calculated using the GSH standard curve. The hepatic ATP level was detected using the ATP detection kit (BioVision), according to the manufacturer's instructions.

Lipid peroxidation Lipid peroxidation was evaluated by the method reported by Fraga *et al*. (16). In brief, liver tissues were homogenized in 50 mM phosphate buffer containing 20 mM EDTA (pH 7.4), and 100 μL of tissue homogenate was mixed with 200 μL of 5% SDS, 2 mL of 0.1 N HCl, 300 μL of 10% phosphotungstic acid, and 1 mL of 0.5% TBA. The mixture was heated in boiling water bath for 45 min and cooled for 5 min; subsequently, 5 mL of *n*-butanol was added and mixed vigorously. After centrifugation, the absorbance of butanol layer was measured at 540 nm using a microplate reader (Model 680; Bio-Rad Inc., Tokyo, Japan). The TBA-reactive substance (TBARS) levels were expressed as malondialdehyde equivalents using 1,1,3,3-tetraethoxypropane as the standard.

Antioxidant enzyme activities The liver tissues weighing 50 mg were homogenized in 500 μL of cold Tris-HCl. The homogenate was centrifuged at 12,000×g for 10 min, and the supernatants were used for antioxidant enzyme assays. The activities of antioxidant enzymes, including SOD, CAT, GR, and GST, were detected according to the instructions in the kits.

Real-time and semi-quantitative RT-PCR The total RNA was isolated using easy-BLUE^{TM} Total RNA extraction kit (Intron Biotechnology Inc., Seongnam, Korea), and reverse transcribed to cDNA using a cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), as described in the manufacturer's protocol. For the real-time qPCR analysis, cDNA was used as a template for the relative quantitation for the selected target genes with predesigned TaqMan gene expression assay kits. Each 20 μL reaction contained 100 ng cDNA, 2× Taqman Gene Expression Mastermix, forward and reverse primers, and TaqMan probe. All reactions were performed in duplicate with an ABI7500 system (Applied Byisystems, Foster, CA, USA) under the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were normalized to glyceraldehyde 3-phosphate dehydrogenase as an internal standard, and relative quantities of each gene were presented in terms of 2-ΔΔCt, calculated using the ΔCt and ΔΔCt values.

For semi-quantitative RT-PCR, PCR products were separated through electrophoresis on 1.8% agarose gel stained with ethidium bromide. PCR products were visualized under UV light and photographed using gel documentation system. The intensities of the bands were densitometrically quantified using Image J software (National Institute of Health, NIH Version v1.32j). Relative expression levels of target genes were normalized to β-actin and then data was represented as ratio compared to control.

Immunoblot analysis The liver tissues lysed using radioimmunoprecipitation assay buffer (Cell Signaling). Tissue lysates were centrifuged at 12,000× *g* for 20 min. Total protein concentration was determined by Bradford assay. Equal amounts of protein were separated on 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and the proteins were transferred to polyvinylidene difluoride membranes. The membranes were then blocked for 30 min in a PBS solution containing 0.5% BSA and 0.1% Tween-20. After washing, membranes were incubated for 1 h with horseradish peroxidase-linked secondary antibodies in PBS containing 0.5% BSA and 0.1% Tween-20 in PBS (PBST). Finally, after washing three times for 10 min with PBST, proteins were visualized by ImageQuant LAS 4000 (General Electric Fairfield, CT, USA). Band intensities were quantified with Image J software.

Statistical analysis Statistical significances were analyzed using one-way analysis of variance followed by Duncan's multiple range test at $p<0.05$ using Statistical Analysis System (SAS) program (SAS 8.2, SAS Institute Inc., Cary, NC, USA).

Results and Discussion

LAFGE attenuates AAP-induced acute liver injury To investigate the protective effects of LAFGE against AAP-induced liver injury, liver sections were histologically analyzed. As shown in Fig. 1A, large hemorrhagic area and massive necrosis were observed in AAP-treated rat livers. However, LAFGE pretreatment attenuated these pathological phenotypes in a manner similar to silymarin treatment as a positive control. Furthermore, the activities of serum ALP, AST, and ALT in AAP-treated rats were markedly elevated compared with those of serum ALP, AST, and ALT in the controls (Fig. 1B-1D), and pretreatment using LAFGE significantly decreased serum ALP, AST, and ALT levels ($p<0.05$). Serum ALT and AST levels increase on hepatocellular damage (17). The significantly elevated serum ALP level indicated liver dysfunction, particularly in biliary flow ($p<0.05$) (18). Pretreatment using LAFGE attenuated the increased activities of the aforementioned enzymes in serum and the hepatic damages observed in histological analysis. These findings suggested that LAFGE preserved hepatocellular membrane integrity and decreased enzyme leakage against AAP-induced liver injury.

Furthermore, a previous study demonstrated that inflammatory responses are closely related to AAP-induced liver injury (19). Several proinflammatory cytokines, including tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β), are potentially deteriorative to liver injury (20). In the present study, the mRNA expressions of hepatic inflammatory cytokines were analyzed using real-time qPCR. Futhermore, the levels of TNFα and IL-1β mRNA in the liver markedly increased in the AAP-treated group compared with the controls, and LAFGE pretreatment downregulated the increased mRNA levels of TNF-α and IL-1β by AAP administration in the liver (Fig. 1E-1F).

LAFGE prevents AAP-induced liver injury through the regulation of apoptosis-related proteins We further examined the apoptosis-related factors including B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax), and caspase-3, in the liver. As shown in Fig. 2A decrease in the Bcl-2 mRNA and protein levels and an increase in the Bax mRNA and protein levels were observed in the liver of the AAP-treated group compared with those in the controls. The levels of these mRNAs and proteins were markedly reversed by LAFGE pretreatment. Moreover, the increased mRNA level of pro-caspase-3 in the controls was downregulated in the LAFGE-pretreated groups. The cleaved caspase-3/pro-caspase-3 ratio was significantly elevated in the control group compared with that in the vehicle group, while it was markedly reversed in the LAFGE-treated groups ($p<0.05$). AAP-induced liver injury has contradictory points pertaining to whether AAP-induced cell death is considered as necrosis or apoptosis (21). However, there are several evidences supporting apoptosis-related signals in AAP-induced liver injury. Abnormal increase of oxidative stress leads to mitochondria-mediated apoptosis (22-24). Bax leads to the release of cytochrome c from mitochondria to cytosol and triggers cytochrome c-dependent caspase cascade reaction (25). We found that LAFGE prevented AAP-induced hepatic apoptosis through upregulation of Bcl-2 expression and downregulation of Bax. Moreover, LAFGE effectively suppressed caspase-3 activation, the main effector in apoptotic cell death, suggesting that LAFGE attenuated AAP-induced liver injury through anti-apoptotic effects.

Effect of LAFGE on CYP2E1, p53 expression, and phosphorylation of JNK and p38 To investigate possible protective mechanisms of LAFGE against AAP-induced liver injury, changes in protein levels of CYP2E1, p53, JNK, and p38 were examined. AAP-induced hepatotoxicity is initiated by the CYP2E1-mediated bioconversion of AAP to NAPQI that covalently bind to the nucleus and cellular proteins, resulting in promoting the liver injury (26). In this regard, we observed the LAFGE-suppressed CYP2E1 expression in AAP-treated rat liver (Fig. 3), suggesting that it contributes to a reduction in NAPQI formation, consequently suppressing AAP-induced oxidative stress in liver injury. Moreover, p53, a tumor suppressor protein, is activated to trigger apoptosis in response to severe DNA damage by AAP-induced oxidative stress (27). p53 upregulates the Bax expression that plays a proapoptotic role and downregulates the Bcl-2 expression that plays an antiapoptotic role, consequently promoting apoptosis signaling

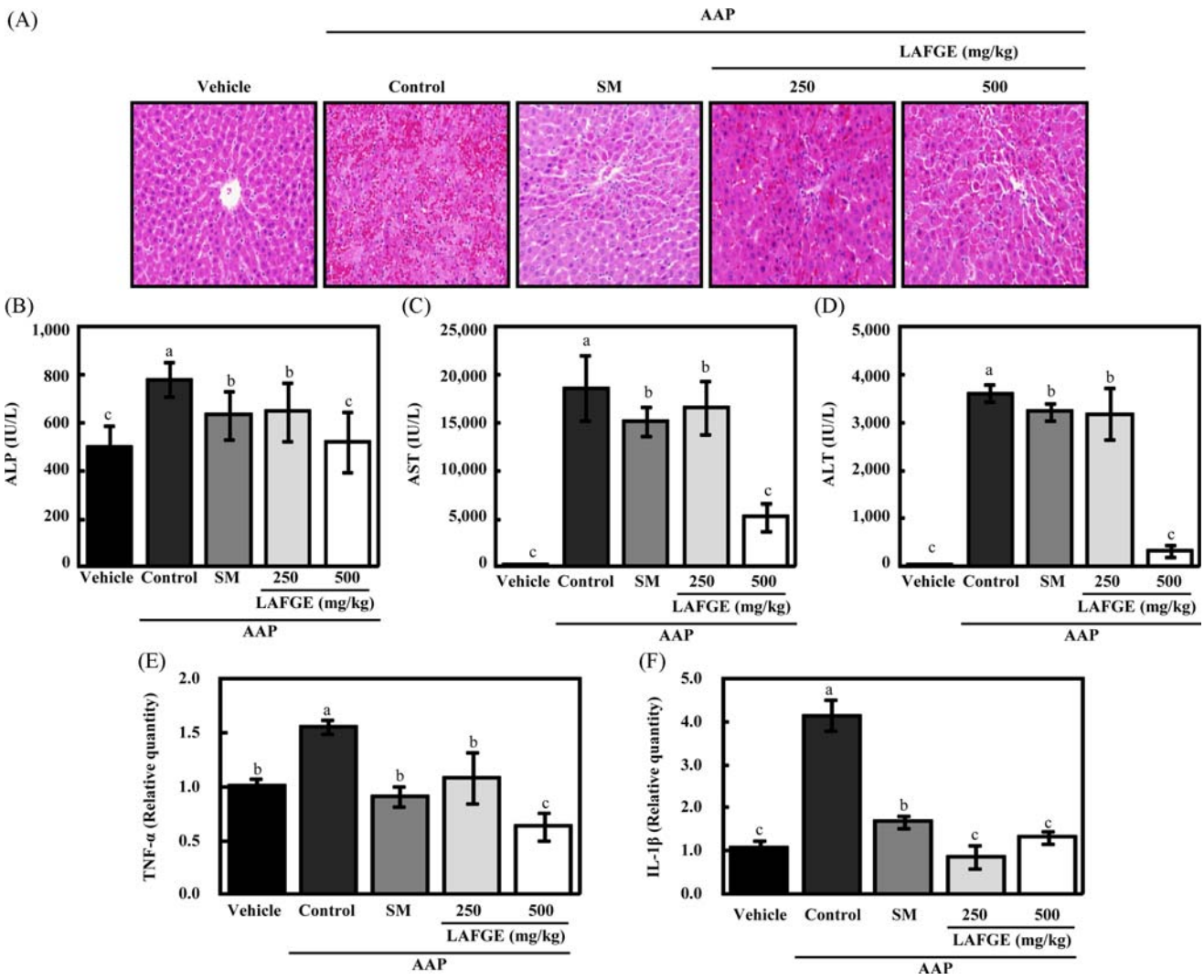

Fig. 1. Effects of the fermented garlic extract by lactic acid bacteria (LAFGE) on histopathological examination by hematoxylin and eosin staining (magnification×200) (A); serum alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine transaminase (ALT) levels (B-D); and hepatic inflammatory response against AAP-induced liver injury in rats (E, F). Data were expressed as means±SD. The different letters above the error bars mean significant differences at $p<0.05$.

pathway (28). We found that LAFGE downregulated p53 expression, suggesting that LAFGE prevents AAP-induced apoptotic liver injury.

Furthermore, MAPK plays critical roles in the regulation of survival, cell death, proliferation, migration, and inflammation (29). It was reported that the MAPK signaling pathway, including JNK and p38, is closely related to oxidative stress-mediated apoptosis (30,31). We found that AAP significantly increased hepatic p-JNK and p-p38 levels, which were reversed by LAFGE pretreatment ($p<0.05$). No significant changes were found in the total JNK and p38 expression ($p<0.05$). These results suggest that LAFGE prevents apoptotic liver injury through inhibition of AAP-induced MAPK activation.

LAFGE exhibits hepatoprotective effect against AAP-induced oxidative damages To evaluate the effects of LAFGE on AAP-induced oxidative damages in the liver, the GSH level, TBARS, ATP, and activities of antioxidant enzymes were analyzed. As shown in Fig. 4A-4D, AAP treatment resulted in lipid peroxidation and GSH and ATP depletion, whereas LAFGE pretreatment significantly reversed these changes ($p<0.05$). Moreover, hepatic SOD, CAT, GST, and GR activities were significantly reduced in AAP-administrated rat livers compared with the activities in the control rat livers ($p<0.05$). However, pretreatment using LAFGE significantly enhanced SOD, CAT, GST, and GR activities compared to control ($p<0.05$) (Fig. 4E-4H).

Oxidative stress is closely associated with the impairment of antioxidant defense system and AAP-induced hepatotoxicity (32). Lipid peroxidation by AAP-induced oxidative damage is also related to AAP-induced liver injury (33). In the present study, LAFGE pretreatment showed that AAP-induced GSH depletion and MDA formation were markedly improved. Furthermore, oxidative stress leads to mitochondrial dysfunction, including ATP depletion, membrane potential reduction, and induction of mitochondrial permeability transition (MPT) in AAP-induced hepatotoxicity (34).

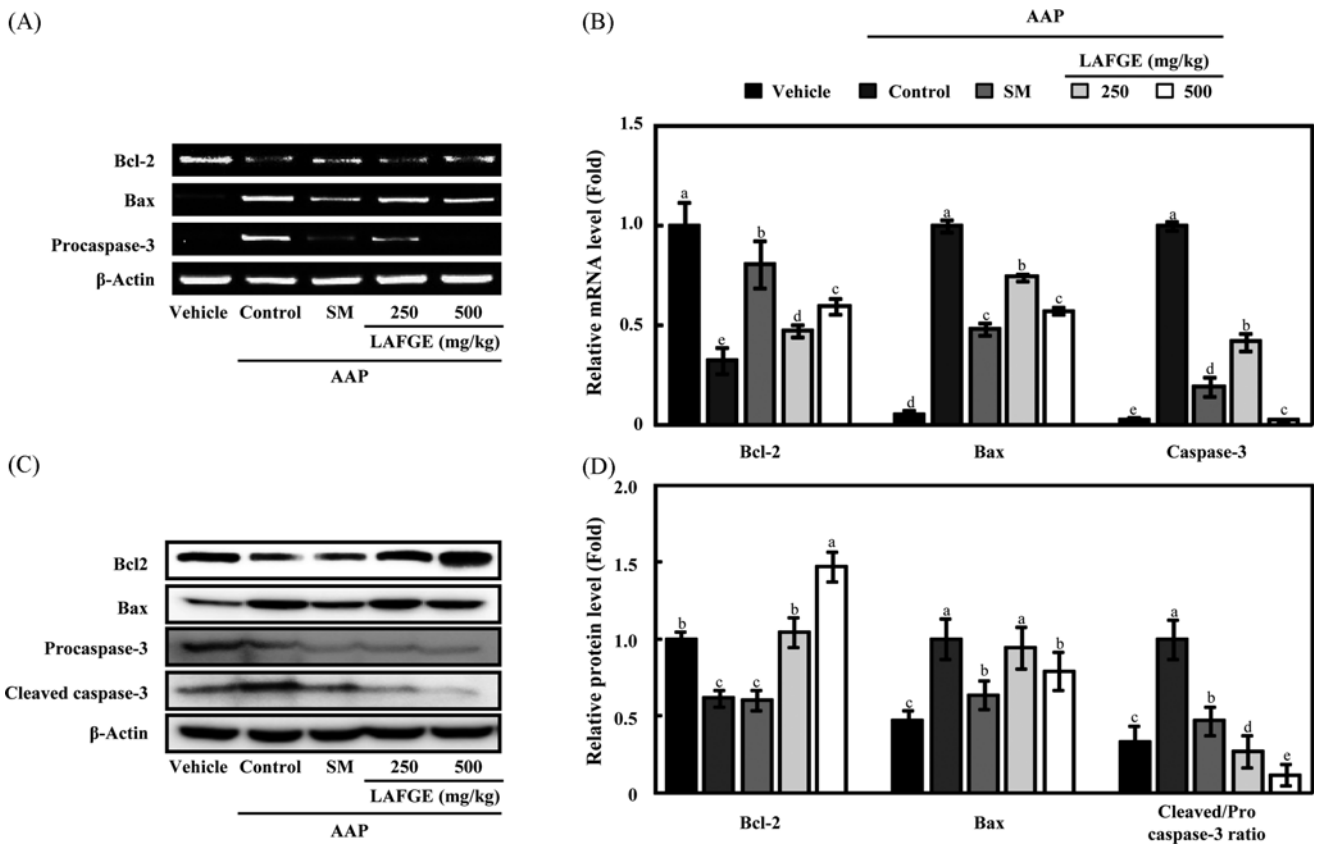

Fig. 2. Pretreatment of the fermented garlic extract by lactic acid bacteria (LAFGE) prevents AAP-induced hepatic apoptosis. (A) mRNA expressions of B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X protein (Bax), and procaspase-3 were detected by semi-quantitative RT-PCR. (B) The relative fold changes of mRNA intensity. Data were expressed as means±SD. The different letters above the error bars mean significant differences at $p<0.05$. (C) The protein expressions of Bcl-2, Bax, pro, and cleaved caspase-3 by Western blot analysis. (D) The relative fold changes of protein expression. Data were expressed as means±SD. The different letters above the error bars mean significant differences at $p<0.05$.

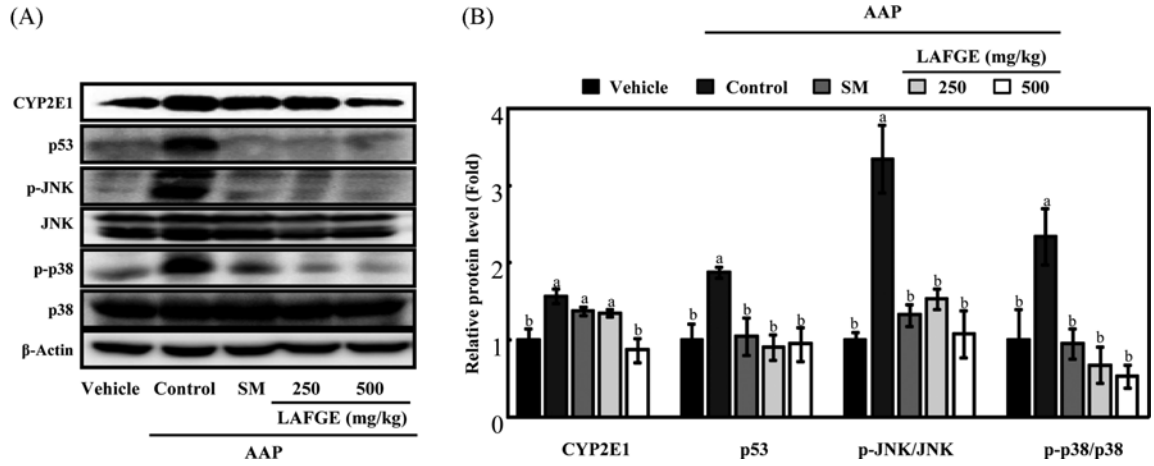

Fig. 3. Effects of fermented garlic extract by lactic acid bacteria (LAFGE) pretreatment on protein level of cytochrome P450 2E1 (CYP2E1), p53, and phosphorylation of c-Jun N-terminal kinase (JNK) and p38 in AAP-intoxicated rat livers. (A) LAFGE inhibited AAP-induced CYP2E1 and p53 expression and phosphorylation of JNK and p38. (B) Relative protein level of CYP2E1, p53, p-JNK/JNK, and p-p38/p38. Data were expressed as means±SD. The different letters above the error bars mean significant differences at $p<0.05$.

LAFGE supplementation markedly reversed AAP-induced ATP depletion and may influence the inhibition of mitochondria-dependent apoptosis signaling pathway.

LAFGE markedly inhibited lipid peroxidation and restored antioxidant enzyme activities in the liver of AAP-treated rats. These observations may result from increased organosulfur compounds, including *S*-allyl

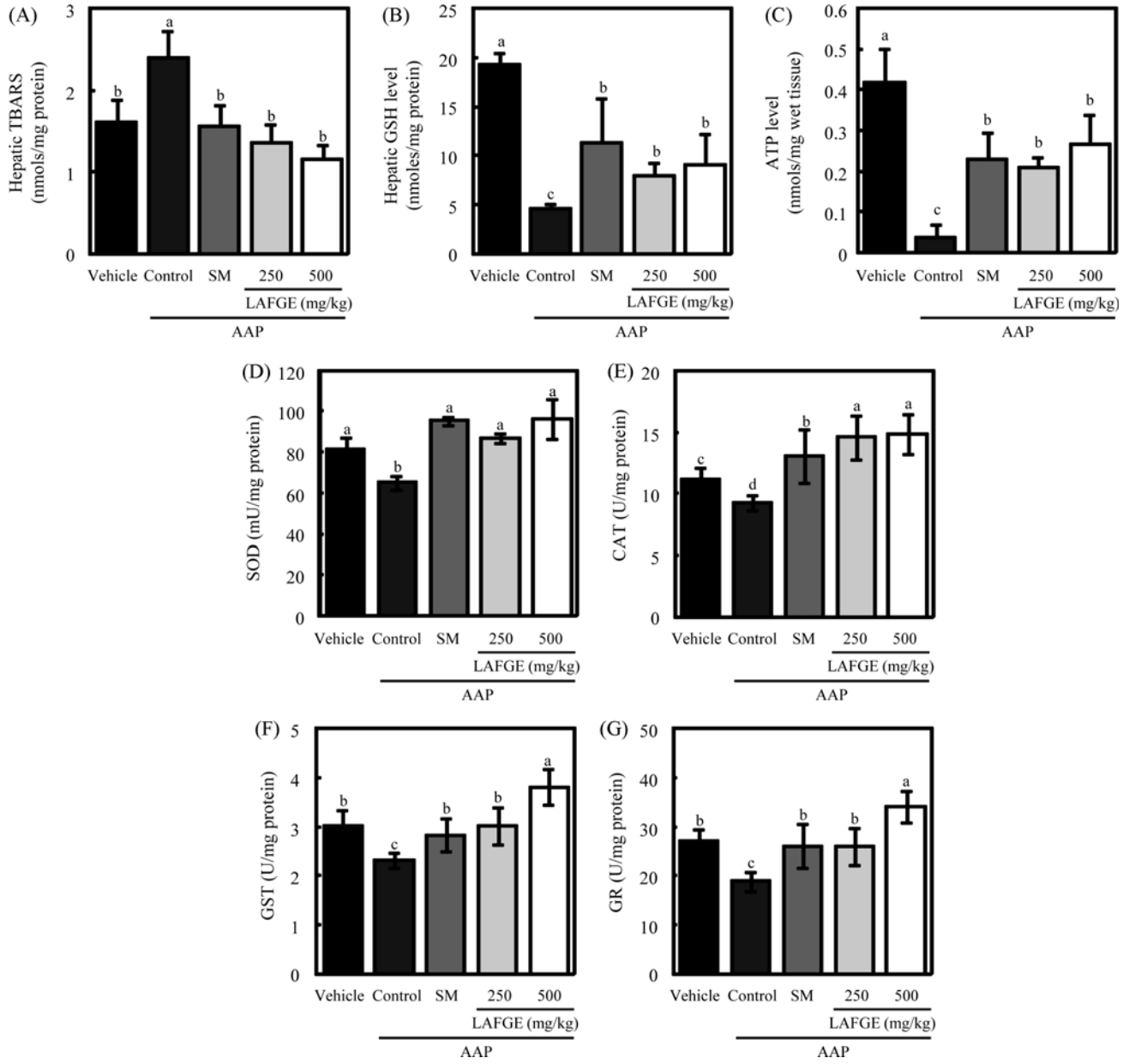

Fig. 4. Effects of fermented garlic extract by lactic acid bacteria (LAFGE) on lipid peroxidation (A), total glutathione (GSH) level (B), adenosine triphosphate (ATP) level (C), and antioxidant enzymes activities (D-G) in AAP-administered rat livers. Data were expressed as means±SD. The different letters above the error bars mean significant differences at $p<0.05$.

cysteine (SAC), *S*-ethyl cysteine, *S*-methyl cysteine, and cycloalliin in fermented garlic with *L. plantarum* (14). In addition, Hsu *et al.* (35) reported that SAC and *S*-propyl cysteine (SPC) attenuated GSH depletion, lipid peroxidation, as well as activities of antioxidant enzymes, including GPX and CAT, in AAP-intoxicated rat livers. These results indicate that hepatoprotective effects of LAFGE against AAP-induced oxidative liver injury may be because of enhanced antioxidant capacity followed by the increased organosulfur compounds after lactic acid fermentation.

In summary, the present study demonstrates that LAFGE protects against oxidative liver injury by AAP through inhibiting apoptosis, maintaining cellular GSH, and protecting from oxidative damage to mitochondria as well as suppressing liver MAPK activation. Our results suggest that antioxidant capacity of LAFGE protects against AAP-induced liver injury, suppressing oxidative stress-mediated hepatic apoptosis (Fig. 5). These results support that LAFGE is a feasible supplement for functional food to prevent AAP-induced liver injury.

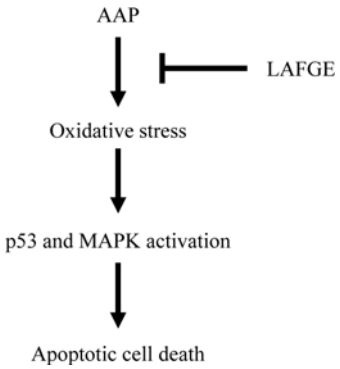

Fig. 5. Schematic representation of acetaminophen (AAP)-induced hepatotoxicity and its possible protective mechanism of LAFGE.

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Disclosure The authors declare no conflict of interest.

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