Glycoprotein Isolated from *Acanthopanax senticosus* Protects against Hepatotoxicity Induced by Acute and Chronic Alcohol Treatment

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The protective effect of a 30 kDa glycoprotein (GF-AS) isolated from the stem bark of *Acanthopanax senticosus* against acute and chronic alcohol-induced hepatotoxicity were studied. N-terminal amino acid sequence of GF-AS showed NH_2-Val-Ala-Tyr-Pro-Trp-Ala-Gly-Phe-Ala-Leu-Ser-Leu-Glx-Pro-Pro-Ala-Gly-Tyr-. GF-AS significantly increases the activities of alcohol-metabolizing enzymes, including alcohol dehydrogenase, microsomal ethanol metabolizing system, and acetaldehyde dehydrogenase in rats acutely treated with alcohol, resulting in decreased plasma alcohol levels. GF-AS also increases the activities of antioxidant enzymes and glutathione level. Markers of liver injury induced by alcohol: elevated serum levels of aspartate aminotransferase, alanine aminotransferase, triglyceride and cholesterol, are reduced by GF-AS in both acutely and chronically treated rats. The activities of lipogenic enzymes including malic enzyme, glucose-6-phosphate dehydrogenase, and 6-phosphoglucuronic acid dehydrogenase in chronic alcohol-treated rats are significantly decreased by GF-AS. Furthemore, GF-AS improves histological change in fatty liver and hepatic lesions induced by alcohol. Collectively, GF-AS may alleviate alcohol-induced hepatotoxicity through increasing ethanol and lipid metabolism, as well as antioxidant defense systems in livers injured by acute- and chronic-alcohol treatment.

Keywords *Acanthopanax senticosus*; glycoprotein; alcohol metabolism; lipogenesis; antioxidation; liver damage

Acanthopanax senticosus (*A. senticosus*) is a common Asian herb known as "Siberian Ginseng" or "*Eleutherococcus senticosus*"[1] and used for rheumatism and prophylaxis of various diseases including chronic bronchitis, hypertension, and ischemia. The herb has also been known to effectively relieve stress or fatigue, and symptoms associated with diabetes, neuralgia, and cancer.[2,3] Today this oriental herb is called "adatogen" in the U.S.[4] The major active components of *A. senticosus* are acanthoside, eleutheroside, chiisanoside, senticoside, triterpenic saponin, syringin, flavone, vitamin, minerals, β-sitosterol, sesamine and savinine.[4,5] Each chemical compound is known to produce diverse biological activities. In Korea, the extract of the *A. senticosus* plant is used a component in traditional herbal Korean medicine, and is available as a functional beverage commercially marketed for reducing liver damage and accelerating alcohol detoxification. The efficacy of *A. senticosus* in animal modes and the mechanisms underlying the aforementioned physiological properties involved in alcohol metabolism is unclear, and is therefore the purpose of this investigation.

As much as 80—90% of ingested alcohol is metabolized in the liver, where alcohol is oxidized to acetadehyde.[6—8] The process is catalyzed by 3 different enzymes: alcohol dehydrogenase (ADH), microsomal ethanol metabolizing system (MEOS), and acetaldehyde dehydrogenase (ALDH). Since acetaldehyde is much more toxic than alcohol, it is associated with a larger number of the metabolic abnormalities in liver disease induced by alcohol.[9,10] Under normal conditions, acetaldehyde is rapidly converted to acetate by ADH, and therefore very low level of acetaldehyde should remain in the liver tissue or blood. ALDH also plays an important role in the elimination of acetaldehyde through oxidative reactions.[11] Therefore, the severity of liver diseases can be proportional to reductions in ADH or ALDH activities.[2,12]

Development of fatty liver and hyperlipidemia frequently occurs in chronic alcoholics; mainly because ethanol becomes a preferred fuel for the liver and displaces fat as a source of energy, which results in fat accumulation. Furthermore, the redox state secondary to ethanol oxidation is altered, promotings lipogenesis through increasing α-glycerophosphate and acylglycerols. The depressed oxidative capacity of mitochondria caused by chronic alcohol also contributes to fatty liver. Increasing fat accumulations in the liver can also stimulate secretion of lipoproteins into the bloodstream, facilitating the development of hyperlipidemia. Acetyl CoA carboxylase (ACC) is an enzyme that catalyzes the first step in fatty acids biosynthesis and is a rate-limiting enzyme in lipogenesis.[5,13] Moreover, malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PDH), and 6-phosphoglucuronic acid dehydrogenase (6-PGDH) are also involved in lipogenesis by supplying NADPH, an essential cofactor for fatty acids and cholesterol biosynthesis. Alcohol has also been suggested to cause fatty liver by altering the NAD^+/NADH redox potential, which inhibits fatty acid oxidation and the TCA cycle addition to stimulating lipogenesis.[5,13,14]

Many of alcohol's toxic effects in the liver have been ascribed to oxidative stress caused by ethanol metabolism. Ethanol, or its metabolites, causes auto oxidation in hepatic cells, which induces marked hepatotoxicity by acting as a pro-oxidative agent or by reducing antioxidant levels. Lipid peroxidation and related membrane damage are key features in alcoholic liver injury. Generally, increased oxidative stress occurs as a consequence of induced MEOS and NADPH oxidation,[3,15] and ethanol is converted to ethyl and 1-droxyethylradical.[16] Additionally, acetaldehyde binds hepatic glutathione (GSH), depleting the antioxidant reserve.[17] Therefore, the ineffective removal of free radicals can adversely alter the lipid composition of cell membranes *via* lipid peroxidation and induce depletion of cellular antioxidants, result-

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ing in damage of liver membranes and cells. Circulating antioxidant enzymes and non-enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and GSH play important roles in alleviating tissue damage induced by free radical formation.[18,19] Therefore, a compound with antioxidant properties can therapeutically ameliorate the progression of lipid peroxidation and hepatocellular injury induced by alcohol.

We isolated glycoprotein GF-AS as a new component from the stem bark of *A. senticosus*, which is different from the components of *A. senticosus* mentioned above. It was previously observed to significantly protect against CCl_4-induced liver injury in an antioxidative manner by scavenging DPPH and inhibiting lipid peroxidation both *in vitro* and *in vivo*. Hence, this investigation explored whether GF-AS is effective against liver disease and damage, in which ROS are involved as a potent causative factor.

The present study was performed to evaluate GF-AS on alcohol detoxification, and the protective properties of GF-AS pretreatment in the acute and chronic alcohol-induced liver injury model. Elucidating the underlying mechanism involved was also observed. To this end, we measured serum alcohol concentrations, the activities of hepatic alcohol metabolizing enzymes, serum hepatotoxic indicators, serum and liver lipid levels, the activities of hepatic lipogenic enzymes, the status of antioxidant systems and lipid peroxidation, and histological analysis of liver damage in alcohol-injured livers pretreated with GF-AS.

MATERIALS AND METHODS

Preparation of Glycoprotein Fractions from *Acanthopanax senticosus* Extract The bark of *A. senticosus* originating from Korea was obtained from Oh Dae Mt, Kangwon-Do, Korea. Chopped *A. senticosus* stem bark placed in approximately 20 volumes of distilled water was homogenized for 1 min and then stirred overnight at 4 °C. After centrifugation for 30 min at 7000 rpm, the supernatant was transferred to a 70% saturated ammonium sulfate solution and slightly stirred at 4 °C overnight. Following a second round of centrifugation, the precipitate was collected and resuspended in an appropriate volume of PBS. For further purification, the extract was applied to sephadex-G50 gel filtration (2.5×90 cm, FPLC system Pharmacia Biotech, Sweden) and eluted at a speed of 1 ml/min with PBS. The eluent (GF-AS) was collected, and carried out SDS-PAGE electrophoresis along with the known molecular weight marker for the estimation of the molecular weight. We also reacted with anti-GF-AS antibody to confirm the presence of GF-AS (Fig. 1).[20] Anti-GF-AS antibody was produced by immunizing mice with GF-AS. BALB/c mice were immunized subcutaneously with Freund's adjuvant complete and 5 mg/ml of GF-AS. Mice were sacrificed 10 d after secondary immunization and then anti-GF-AS antibody was obtained from serum. After using anti-GF-AS antibody to confirm that the fraction contained GF-AS, protein content was measured (Bio-Rad Laboratories, Herculus, CA, U.S.A.).

Characterization of GF-AS The molecular size of GF-AS was measured on a SDS-PAGE electrophoresis along with the known molecular weight marker. The N-terminal sequence of GF-AS was achieved by Edman degradation using a Precise 491HT protein sequencer (Applied Biosystems, U.S.A.).

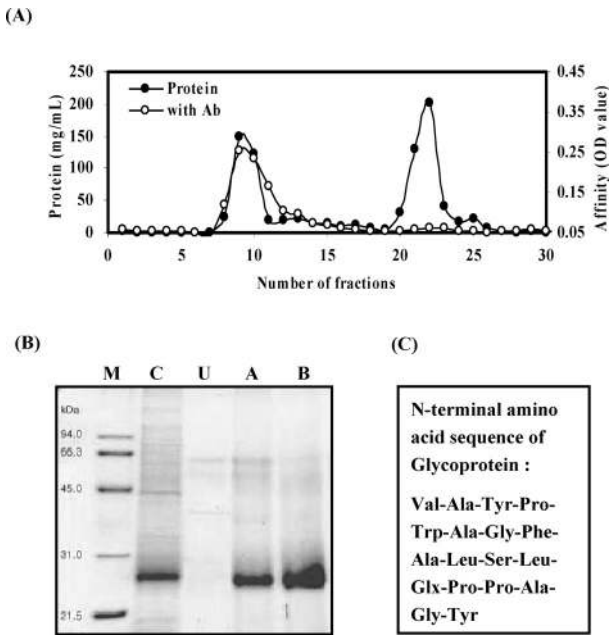

Fig. 1. Characterization of GF-AS

(A) Gel chromatography of GF-AS on a 2.5×90 cm Sephadex G-100 column. GF-AS (1 mg/ml) dissolved in PBS was applied to the column and eluted with at a flow rate of 1 ml/min. To determine the elution pattern, each fraction was measured for protein concentration, and GF-AS was confirmed by reaction with anti-GF-AS. Anti-GF-AS was made from BALB/c mice immunized with GF-AS. (B) SDS-PAGE gel electrophoresis of GF-AS using anti-GF-AS antibody. M; marker, C; crude GF-AS, U; unbound fraction, A and B; GF-AS fraction. (C) N-terminal amino acid sequence of GF-AS by Edman degradation method.

Animals The care and experimentation of animals in this investigation were conducted according to good welfare protocols in the Guide for Care and Use of Laboratory Animals at the NIH of Korea. Five-week-old, male mice (20—25 g) and SD rats (120—130 g) were obtained from SLC Inc. (Shizuoka, Japan) and were used after 1 week of adaptation. All animals were housed in polycarbonate cages in a temperature regulated (22 °C) and humidity (55%) controlled room with a 12-h light/12-h dark cycle. Water and a normal standard pellet diet were available *ad libitum* throughout the experimental period. BW was recorded twice a week throughout the course of the study.

Acute Alcohol-Induced Liver Injury in Mice Male mice were randomly assigned to 4 groups of 7 animals as shown in Table 1. The mice received a normal diet of standard pellets, and GF-AS in saline was administered i.p. at 0.5, 1.0, 2.5 mg/kg BW once daily for 3 consecutive days. One hour after the final GF-AS treatment, all mice received an acute ethanol dose of 5 g/kg BW diluted in water (50%, v/v). One hour after the administration of ethanol, blood samples were collected from mice by cardiac puncture to determine biochemical parameters. Subsequently, the animals were sacrificed. Livers collected were then weighed and a thin slice preserved in 10% buffered formalin solution for histological analysis. The remaining livers were frozen in liquid nitrogen and stored at −70 °C. Lethality after the single acute ethanol binge protocol was negligible (2.0%).

Chronic Alcohol-Induced Liver Injury in SD Rats Male SD rats randomly assigned to 7 groups of 8 animals

Table 1. Experimental Groups

Experiment	Groups	(*n*)	Alcohol	GF-AS
Chronic alcohol treatment	NL	8	—	—
	NLAS-0.1	8	—	0.1 mg/kg BW
	NLAS-0.5	8	—	0.5 mg/kg BW
	CT	8	4 g/kg BW	—
	CTAS-0.1	8	4 g/kg BW	0.1 mg/kg BW
	CTAS-0.5	8	4 g/kg BW	0.5 mg/kg BW
	CTAS-1.0	8	4 g/kg BW	1.0 mg/kg BW
Acute alcohol treatment	CT	7	5 g/kg BW	—
	CTAS-0.5	7	5 g/kg BW	0.5 mg/kg BW
	CTAS-1.0	7	5 g/kg BW	1.0 mg/kg BW
	CTAS-2.5	7	5 g/kg BW	2.5 mg/kg BW

each as shown in Table 1 were administered alcohol or an isocaloric glucose substitute with or without GF-AS is as Table 1.

All rats received a normal diet of standard pellets throughout the experimental period. Animals not treated with alcohol received isocaloric glucose containing a 40% glucose solution. Alcohol-treated animals received an alcoholic dose of 4 g/kg BW diluted in water (30%, v/v). Alcohol and the isocaloric glucose solution were administered once a day orally for 30 d, 1 h after GF-AS treatment. GF-AS in saline or saline was administered intragastrically at concentrations of 0.1, 0.5, and 1.0 mg/kg BW once daily for 30 consecutive days. At the end of the experimental period, after overnight fasting, the rats were anesthetized and blood taken by heart puncture to determine biochemical parameters. The blood was centrifuged and the serum was harvested and stored at −20 °C. The rats were sacrificed, livers were weighed, and a thin slice was preserved in 10% buffered formalin solution for histological analysis. The remaining livers were frozen in liquid nitrogen and stored at −70 °C.

Preparation of Hepatic Subcellular Fractions Fresh livers were washed with 0.9% NaCl and homogenized with 9 vol (w/v) of 25 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA at 4 °C. After the crude homogenate was centrifuged at 4 °C for 10 min at 700×***g*** to remove the nuclear fraction and cell debris, the supernatant was transferred to another tube, and centrifuged at 4 °C for 30 min at 10900×***g***. The mitochondrial fraction was contained in the precipitate. To isolate the cytosolic and microsomal fractions, the supernatant was further ultracentrifuged at 4 °C for 60 min at 100000×***g***. The precipitate (microsomal fraction) and supernatant (cytosolic fraction) were separated and stored at −70 °C until the enzyme assay was conducted. These fractions were used to assay for alcohol-metabolizing, antioxidative, and lipogenic enzymatic activities.

Serum Measures of Hepatotoxicity Hepatotoxicity was assessed by quantifying the activities of serum aspartate aminotransferase (AST, AM 101-K), alanine aminotransferase (ALT, AM 101-K), lactate dehydrogenase (LDH, LDH-LQ), bilirubin (AM 301-K), γ-glutamyl transpeptidase (γ-GTP, AM 158-K), glucose (AM 201-K), cholesterol (CHOL, AM 202-K), HDL-cholesterol (HDL-CHOL, AM 203-K) and triglyceride (TG, AM 157S-K). Measurements were performed by spectrophotometric analysis using a diagnostic EIA kit obtained from the Asan Chemical Co. (Seoul, Korea) according to the manufacturer's instructions.

Hepatic Lipid Levels and Lipogenic Enzyme Activities The concentrations of CHOL and TG in whole livers were measured by the same method using blood specimens. The activities of lipogenic enzymes were measured using cytosolic fractions. ME was measured by the O'choa method[21] and G6PDH and 6-PGDH were measured by the Glock and McLean methods.[22] The activity was estimated by measuring the formation of NADPH at 340 nm. One unit of enzyme activity was defined as the enzyme activity resulting in the formation of 1 μmol of NADPH/min/mg protein.

Plasma Ethanol Level and Hepatic Ethanol-Metabolizing Enzyme Activities Plasma ethanol concentration was measured using the Sigma assay kit (N332-UV). ADH activities were determined using cytosolic fractions.[7] MEOS activities were determined using microsomal fractions.[7] ALDH activities were measured spectrophotometrically at 340 nm using acetaldehyde and NAD^+ as the substrates following NADH production.[9] Enzyme activities were expressed as nM of NADH formed/min/mg of protein in ADH, nM of NADH formed/min/mg of protein in MEOS and nM of NADH formed/min/mg of protein in ALDH.

Hepatic Antioxidant Enzyme Activities and Lipid Peroxidation Cytosolic fractions were used to assay for antioxidative enzyme activities including SOD (Bioxytech SOD-525), GSH-Px (Bioxytech GPx-340), GR (Bioxytech GR-340), GSH (Bioxytech GSH-400) and CAT (Bioxytech catalase-520). Activities of SOD, GSH-Px, GR, and CAT were measured by spectrophotometric analysis using a diagnostic EIA kit obtained from Oxiresearch (Portland, OR, U.S.A.) and carried out according to the manufacturer's instructions. Malondialdehyde (MDA) was measured using the method of Ohkawa *et al*.[23]

Hepatic Histological Examinations Fresh thin liver tissues were fixed in 10% neutral-buffered formalin for several days. Fixed tissues were processed routinely and then embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. The magnitude of chronic alcohol-induced liver injury was assessed by visual morphological changes in liver sections stained with H&E. Hepatic fibrosis and necrosis was evaluated by M&T staining.

Statistical Analysis All data were expressed as means± S.E. Significant differences among the groups were determined by one-way analysis of variance using the SAS statistical analysis program (SAS Institute, Cary, NC, U.S.A.). Statistical significance was considered at $p<0.05$. Additionally, Duncan's multiple range test was performed to determine differences among ethanol and GF-AS treatment or between paired groups.

RESULTS

Characterization of GF-AS Isolated *Acanthopanax senticosus* Extract Crude protein fraction obtained from *A. senticosus* (100 g) were precipitated with ammonium sulfate and obtained crude GF-AS fraction (10.24 g). Crude GF-AS fraction dialysed and obtained 6.7 ml of crude GF-AS with 768 μg/ml protein concentration. Crude GF-AS applied to gel filtration on sephadex G50 and eluted with PBS. As shown in Fig. 1, the eluent was found to be composed of 2 main protein peaks. Gel electrophoresis analysis revealed that the first peak (GF-AS) has an affinity for anti-GF-AS an-

tibody, but the second peak did not react with this antibody. The collected GF-AS fraction used for further study. We also measured the amount of GF-AS in herb using Elisa method. The 1000 μg/ml of the extract of *A. senticosus* was added to the well of anti-GF-AS antibody coated plate with GF-AS-HRP conjugate. Finally measured O.D. value was apply to the GF-AS standard curve and then calculated the concentrations. The GF-AS exist at the level of 59.85±2.93 μg/ml in the 1000 μg/ml of crude exract of *A. senticosus*. Based on previous results, GF-AS is a protein about 30.5 kDa, and the protein and neutral sugar concentration of GF-AS was 384.5 mg/ml and 222.4 mg/ml respectively, which suggests that GF-AS is a glycoprotein composed proteins and carbohydrates.[20] The amino acid composition analysis revealed that the GF-AS was composed of 18 amino acids. The N-terminal sequence of GF-AS was determined by Edman degradation as NH_2-Val-Ala-Tyr-Pro-Trp-Ala-Gly-Phe-Ala-Leu-Ser-Leu-Glx-Pro-Pro-Ala-Gly-Trp- (Fig. 1).

Acute Alcohol-Induced Liver Injury in Mice. Serum Alcohol Levels and Hepatic Alcohol Metabolizing Enzyme Activities The effects of GF-AS pretreatment on blood alcohol level and the activities of alcohol metabolizing enzymes were determined. As shown in Fig. 2, GF-AS treatment at a concentration of 2.5 mg/kg BW significantly decreased serum alcohol level at 1 h after acute alcohol treatment ($p<0.05$). GF-AS also significantly increased the activities of cytosolic ADH and ALDH in a dose dependent manner compared with the CT group, as well as the activity of MEOS in all CTAS groups fed both ethanol and GF-AS ($p<0.05$). Therefore, GF-AS appears to decrease the serum alcohol level by increasing the activities of hepatic alcohol metabolizing enzymes.

Serum AST, ALT, CHOL and TG Levels Serum levels of AST and ALT are cytosolic marker enzymes reflecting hepatocellular necrosis as they are released into the blood after cell membrane damage. Compared with the CT group, pretreatment with GF-AS reduced the activities of serum AST and ALT of alcohol-treated mice. Importantly, serum ALT was significantly decreased with GF-AS concentrations of 1.0 and 2.5 mg ($p<0.05$) (Fig. 3). Furthermore, as liver injury increases serum lipid levels, TG and CHOL levels were also measured. As shown in Fig. 3, GF-AS significantly decreased levels of serum TG and CHOL in a dose dependent manner ($p<0.05$), suggesting that GF-AS prevents hyperlipidemia induced by ethanol.

Antioxidation System The effects of GF-AS on the antioxidant systems were investigated to understand the mechanism involved in hepatoprotection by GF-AS. As shown in Fig. 4, GF-AS pretreatment increased SOD activity in a dose dependent manner, and significantly increased the activities of CAT and GSH-Px at GF-AS concentrations of 2.5 mg and 1.0 ($p<0.05$), respectively. Furthermore, pretreatment with 2.5 mg of GF-AS also significantly increased GSH level ($p<0.05$), non-enzymatic antioxidant levels, and decreased MDA levels ($p<0.05$). Taken together, antioxidative enzyme activities and GSH levels increased from GF-AS pretreatment causing MDA levels to decrease significantly.

Chronic Alcohol-Induced Liver Injury in Mice. **Body Weight** The final BW of alcohol-treated CT group were

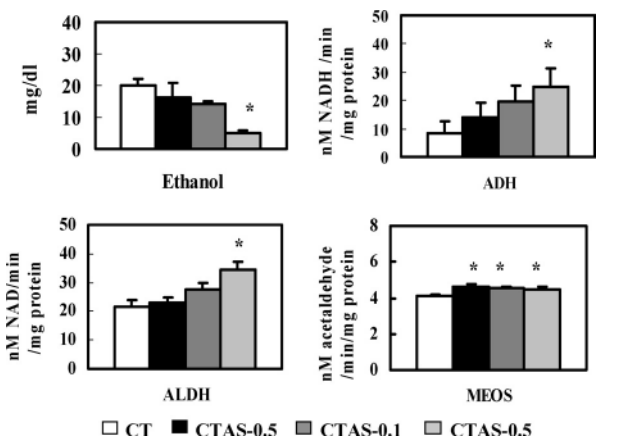

Fig. 2. Effect of GF-AS on Blood Alcohol Concentration and the Activities of Alcohol Metabolizing Enzymes in Acute Alcohol-Treated ICR Mice

Means±S.E. ∗ Significant different between GF-AS untreated CT group and GF-AS treated CTAS groups in all alcohol treated groups at $p<0.05$.

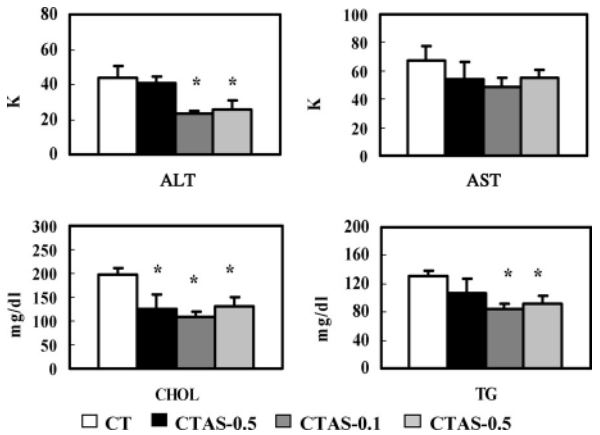

Fig. 3. Effect of GF-AS on the Activities of Serum AST and ALT, and the Level of Serum CHOL and TG in Acute Alcohol-Treated ICR Mice

Means±S.E. ∗ Significant different between GF-AS untreated CT group and GF-AS treated CTAS groups in all alcohol treated groups at $p<0.05$.

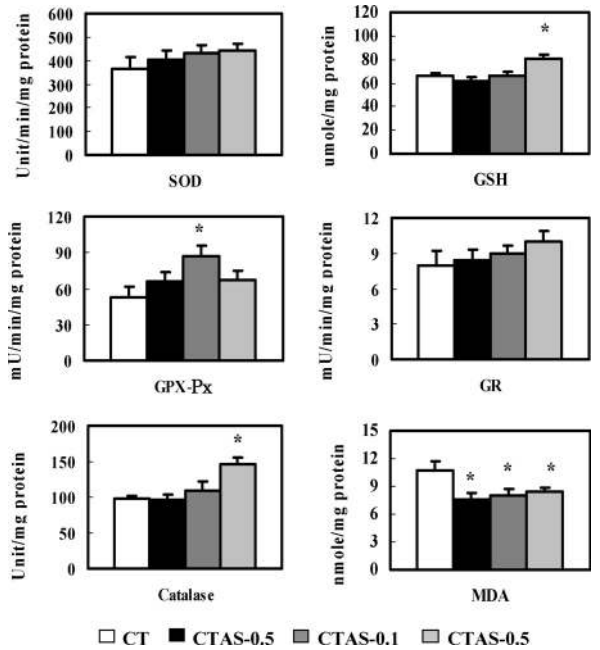

Fig. 4. Effect of GF-AS on the Hepatic Antioxidative System and Lipid Peroxidation in Acute Alcohol-Treated ICR Mice

Means±S.E. ∗ Significant different between GF-AS untreated CT group and GF-AS treated CTAS groups in all alcohol treated groups at $p<0.05$.

Table 2. Effect of GF-AS on the Activities of Alcohol Metabolizing Enzymes of Chronic Alcohol-Treated SD Rats

Groups	Alcohol dehydrogenase (nmol NADH/min/mg protein)	Acetaldehyde dehydrogenase (nmol NADH/min/mg protein)	Microsomal enzyme oxidizing system (nmol acetaldehyde/min/mg protein)
NL	38.76±6.54	41.67±3.0	22.07±3.44
NLAS-0.1	28.41±3.33	38.04±6.43	16.34±2.03
NLAS-0.5	29.80±4.40	36.06±3.59	17.43±2.07
CT	13.29±0.69$^{\#}$	23.77±4.42$^{\#}$	13.78±2.92$^{\#}$
CTAS-0.1	23.67±1.44$^{\#,*}$	38.35±3.04*	11.16±1.40$^{\#}$
CTAS-0.5	24.15±2.47$^{\#,*}$	32.92±2.34	12.90±2.47$^{\#}$
CTAS-1.0	21.35±2.35$^{\#,*}$	36.03±3.94*	11.16±11.40$^{\#}$

Means±S.E. Different superscript letters indicate significant difference at $p<0.05$ between groups. # Significant different between alcohol treated CT and CTAS groups and alcohol untreated NL group at $p<0.05$. * Significant different between GF-AS untreated CT groups and GF-AS treated CTAS groups in all alcohol treated groups at $p<0.05$.

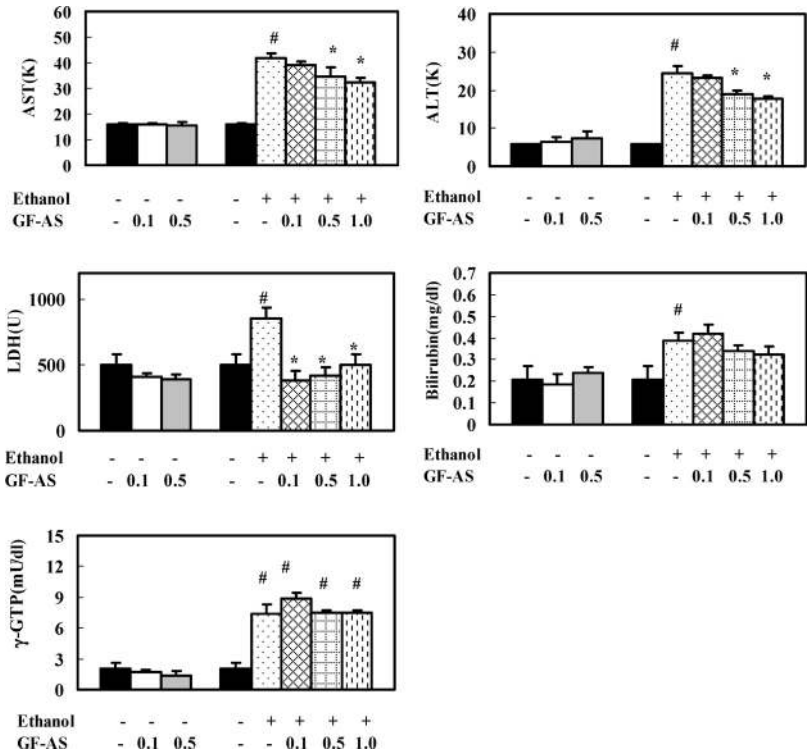

Fig. 5. Effect of GF-AS on the Activities of Serum AST, ALT, γ-GTP and LDH, and the Level of Serum Total Bilirubin of Chronic Alcohol-Treated SD Rats

Means±S.E. # Significant different between alcohol treated CT and CTAS groups and alcohol untreated NL group at $p<0.05$. * Significant different between GF-AS untreated CT groups and GF-AS treated CTAS groups in all alcohol treated groups at $p<0.05$.

significantly lower ($p<0.05$) than from alcohol untreated NL group. However, the BW of CT group animals were not significantly different from alcohol with GF-AS-treated groups. Furthermore, weight gain of alcohol untreated NL group did not show significant difference from alcohol untreated groups supplemented with GF-AS.

Hepatic Alcohol Metabolizing Enzyme Activities The activities of cytosolic ADH and ALDH were significantly decreased ($p<0.05$) in CT group administered alcohol compared to NL group untreated with alcohol. However, pretreatment with GF-AS (CTAS-0.1, CTAS-0.5 and CTAS-1.0 groups) restored enzymatic activities in rats treated with alcohol ($p<0.05$) (Table 2). Reduced MEOS activity from alcohol consumption was not restored by pretreatment with GF-AS (Table 2).

Activities of Serum AST, ALT, γ-GTP, LDH and Bilirubin The activities of serum AST, ALT, γ-GTP, LDH, and level of bilirubin were significantly increased ($p<0.05$) in the alcohol-fed CT group as compared with alcohol-untreated NL groups (Fig. 5). However, pretreatment with GF-AS in ethanol-treated rats (CTAS groups) significantly reduced elevation in serum AST, ALT, and LDH induced by ethanol ($p<0.05$) (Fig. 5), whereas treatment in normal rats (NLAS groups) did not significantly alter elevations (Fig. 5).

Serum and Liver Lipid Levels and Activities of Hepatic Lipogenic Enzymes Since alcohol reportedly causes fatty liver by stimulating lipogenesis as well as inhibiting fatty acid oxidation and the TCA cycle, the effect of GF-AS on hepatic lipogenesis induced by alcohol intoxification was observed in this study. Levels of serum TG and CHOL were significantly increased ($p<0.05$) by alcohol-treatment as compared with the alcohol-untreated group (Fig. 6). However, pretreatment with 1.0 mg of GF-AS in alcohol-treated rats significantly reduced serum levels of TG ($p<0.05$) but

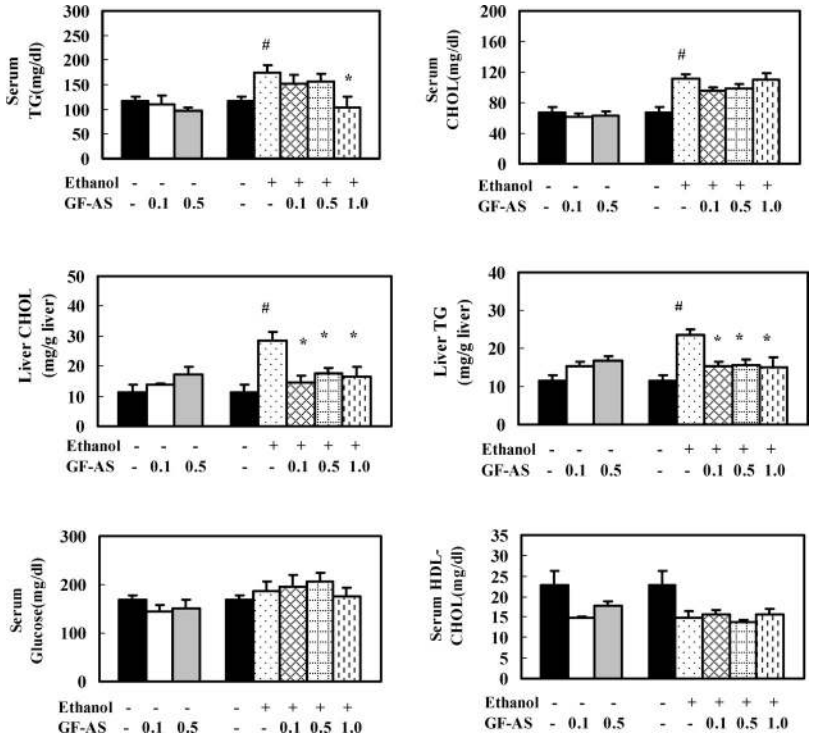

Fig. 6. Effect of GF-AS on the Level of Glucose and Lipid Composition in the Serum and Liver of Chronic Alcohol-Treated SD Rats

Means±S.E. # Significant different between alcohol treated CT and CTAS groups and alcohol untreated NL group at $p<0.05$. * Significant different between GF-AS untreated CT groups and GF-AS treated CTAS groups in all alcohol treated groups at $p<0.05$.

Table 3. Effect of GF-AS on the Activities of Hepatic Lipogenic Enzymes in Chronic Alcohol Treated SD Rats

Groups	Malic enzyme	6-Phosphoglucuronic acid dehydrogenase	Glucose-6-phosphate dehydrogenase
	(nmol NADH/min/mg protein)	(nmol NADH/min/mg protein)	(nmol NADH/min/mg protein)
NL	6.66±0.56	23.82±2.12	24.20±1.07
NLAS-0.1	9.67±0.99	30.58±3.19	28.90±1.63
NLAS-0.5	10.68±0.81^{+}	15.54±1.13	30.70±1.05
CT	15.05±1.83$^{\#}$	43.62±5.56$^{\#}$	36.48±3.42$^{\#}$
CTAS-0.1	8.20±0.72$^{\#,*}$	30.25±1.98*	25.40±0.99*
CTAS-0.5	10.03±0.58$^{\#,*}$	30.84±3.03*	22.48±2.29*
CTAS-1.0	7.94±1.31$^{\#,*}$	22.99±5.16*	17.76±3.55*

Means±S.E. Different superscript letters indicate significant difference at $p<0.05$ between groups. # Significant different between alcohol treated CT and CTAS groups and alcohol untreated NL group at $p<0.05$. * Significant different between GF-AS untreated CT groups and GF-AS treated CTAS groups in all alcohol treated groups at $p<0.05$. + Significant different between GF-AS untreated NL group and GF-AS treated NLAS groups in all alcohol untreated groups at $p<0.05$.

did not reduce CHOL levels (Fig. 6). The levels of serum glucose and HDL-CHOL were not affected by GF-AS pretreatment (Fig. 6). Levels of TG and CHOL in liver tissues were also measured. They were also significantly increased ($p<0.05$) by alcohol-treated CT group as compared with alcohol-untreated NL group (Fig. 6). However, pretreatment with GF-AS at a concentration of 0.1 mg/kg BW in alcohol-treated rats significantly reduced TG and CHOL levels ($p<0.05$) (Fig. 6), whereas levels were unaffected in animals in the alcohol-untreated control groups (NLAS-0.1 and NLAS-0.5 groups) (Fig. 6). To characterize the effects of GF-AS on the activities of lipogenic enzymes, hepatic lipogenic enzymes were measured. As compared with the alcohol-unfed NL group, GF-AS pretreatment in alcohol-fed rats significantly decreased the activities of NADPH-linked hepatic lipogenic enzymes ($p<0.05$), including G6PDH, 6-PGDH and ME induced in the alcohol-fed CT group (Table 3). These results suggest that the activity of lipogenic enzymes is positively correlated with hepatic and plasma TG levels.

Antioxidation System The activities of SOD, CAT and GSH-Px decreased in alcohol-treated CT group as compared with those of the alcohol-unfed groups (NL, NLAS-0.1 and NLAS-0.5 groups) ($p<0.05$) (Table 4). GF-AS administration in alcohol-treated rats (CTAS-0.1, CTAS-0.5 and CTAS-1.0 groups) elevated the activities of SOD, CAT, GSH-Px and GR normally reduced by alcohol treatment (Table 4). However, alcohol-untreated control rats supplemented with GF-AS (NLAS-0.1 and NLAS-0.5 groups) did not show significant changes in the activities of SOD, CAT, GSH-Px and GR as compared to alcohol untreated NL group (Table 4). GSH level was not changed by alcohol treatment, but GF-AS pretreatment increased the GSH level (Table 4). Although the activities of antioxidative enzymes and GSH level were in-

Table 4. Effect of GF-AS on the Hepatic Antioxidative System and Lipid Peroxidation of Chronic Alcohol-Treated SD Rats

Groups	Superoxide dismutase (U/mg protein/min)	Catalase (U/mg protein/min)	Glutathione peroxidase (mU/mg protein/min)	Glutathione reductase (mU/mg protein/min)	Glutathione (μmol/mg protein)	Malondialdehyde (nmol/mg protein)
NL	200.56±31.83	213.74±11.08	41.99±3.28	9.71±1.11	30.41±1.31	41.38±2.71
NLAS-0.1	204.88±30.82	212.46±14.67	49.33±6.78	9.08±0.815	33.33±1.25	44.77±2.46
NLAS-0.5	217.24±5.52	241.58±12.04	48.00±3.86	8.76±0.58	35.29±0.70^{+}	40.87±3.37
CT	163.20±16.01	136.36±13.03$^{\#}$	35.08±3.08	9.60±0.45	29.35±2.07	50.33±5.59
CTAS-0.1	205.71±16.80	124.00±12.17$^{\#}$	49.11±3.16	10.39±0.53	34.20±0.81*	47.32±2.92
CTAS-0.5	244.69±21.40*	179.30±10.94*	44.70±3.98	11.34±0.49	35.60±1.36*	40.77±3.71
CTAS-1.0	219.21±15.84	165.93±9.82$^{\#,*}$	51.28±8.93	10.67±0.53	33.20±1.33	41.88±3.04

Means±S.E. Different superscript letters indicate significant difference at $p<0.05$ between groups. # Significant different between alcohol treated CT and CTAS groups and alcohol untreated NL group at $p<0.05$. * Significant different between GF-AS untreated CT groups and GF-AS treated CTAS groups in all alcohol treated groups at $p<0.05$. + Significant different between GF-AS untreated NL group and GF-AS treated NLAS groups in all alcohol untreated groups at $p<0.05$.

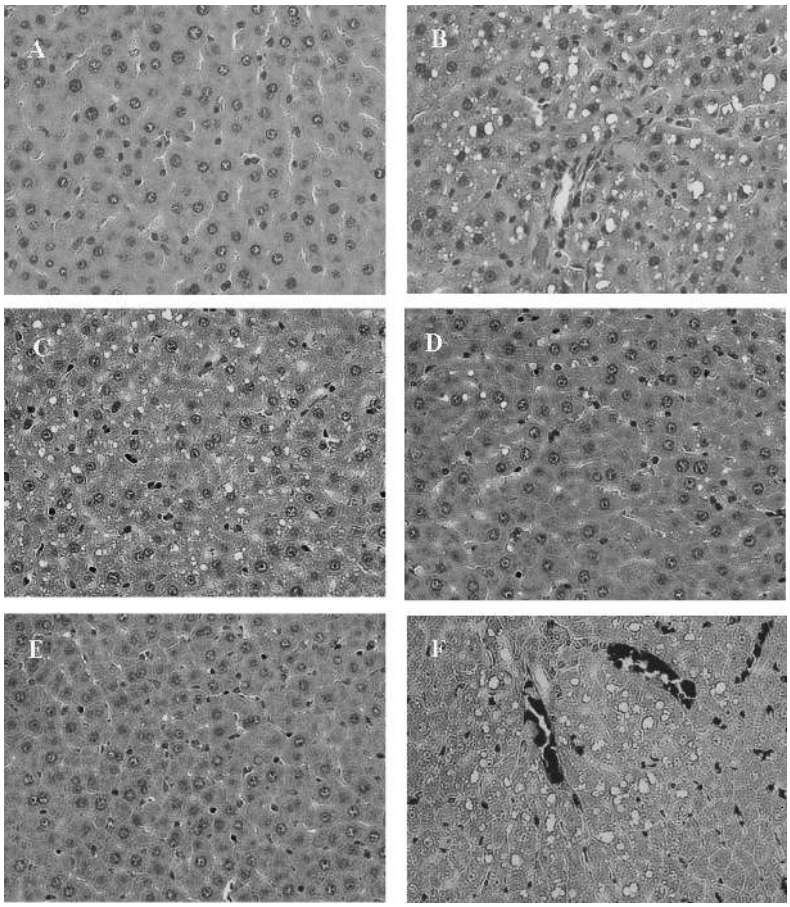

Fig. 7. Effect of GF-AS on Hepatic Morphological Changes of Chronic Alcohol-Treated SD Rats

(A) NL group; (B) CT group; (C) CTAS-0.1 group; (D) CTAS-0.5 group; (E) CTAS-1.0 group (H&E staining, X25). (F) M&T staining, X25 to investigate fibrosis and cirrhosis in liver tissues.

creased, MDA level was not significantly changed in CTAS groups pretreated with GF-AS (Table 4).

Histological Pathology of the Liver Histological effects were also examined in this study. In contrast to alcohol-untreated control rats (Fig. 7), alcohol-treated rats induced minimal multi-focal fatty changes, perivenular sinusoidal dilation, focal parenchymal hemorrhagic necrosis, and mild perivenular microvesicle formation in hepatocytes (Fig. 7). Pretreatment with GF-AS strongly prevented development of severe hepatic lesions induced by alcohol, with markedly reductions in necrosis (Fig. 7). These results were consistent with those of serum hepatotoxicity indices and the level of hepatic lipid peroxidation. In this study, fibrosis and cirrhosis were not observed in all groups, based on M&T staining (Fig. 7).

DISCUSSION AND CONCLUSION

Extract from the *A. senticosus* plant is used not only in traditional Korean medicine, but also as a functional beverage commercially available in Korea to reduce liver damage and accelerate alcohol detoxification. However, its mechanism of

action and efficacy remain unclear. The effects of GF-AS from the stem bark of *A. senticosus* were investigated for its capacity for alcohol detoxification, on activities of alcohol metabolizing enzymes, antioxidant defenses, and ultimately, hepatotoxicity in acute and chronic alcohol-treated rats. In the acute alcohol treatment model, results show that GF-AS significantly decreased the level of blood alcohol by increasing ADH, ALDH, and MEOS activities (Fig. 2). In the chronic alcohol treatment model, GF-AS also restored the activities of ADH and ALDH reduced by alcohol administration (Table 2). Therefore, GF-AS may improve the detoxification of alcohol and acetaldehyde by increasing the activities of alcohol-metabolizing enzymes, thereby preventing hepatic damage and abnormal liver function induced by alcohol.

Chronic consumption of alcohol causes injury to liver cells. Since the activities of AST, ALT, γ-GTP, LDH and levels of bilirubin in the circulation are indicators of hepatic damage, they were used as sensitive markers in the diagnosis of hepatic diseases. Chronic alcohol treatment for 30 d caused significant increases in levels of AST ($p<0.05$), ALT ($p<0.05$), γ-GTP ($p<0.05$), LDH ($p<0.05$) and bilirubin ($p<0.05$) in serum (Fig. 5), as well as increased the serum activity of AST from acute alcohol treatment (Fig. 3). However, pretreatment with GF-AS dose dependently prevented increases in serum AST, ALT, and LDH activity (Figs. 3, 5). Therefore, GF-AS may prevent hepatic injury and preserve the structural integrity of the liver by preventing the induction of enzymatic markers associated with acute and chronic alcohol consumption.

Chronic ethanol causes alterations in lipid metabolism and induces fatty liver, but its exact mechanism is complex.[24,25] Levels of TG in serum or liver tissue increased from alcohol consumption due to several processes, including increased availability of free fatty acids and L-glycerophosphate, decreased secretion of VLDL into the serum, and decreased removal of TG and CHOL from serum due to diminished lipoprotein activity.[25,26] Furthermore, ADH, which primarily proceeds hepatic oxidation of ethanol to acetaldehyde, reduces NAD^+ to NADH, and produces a striking redox change associated with metabolic disorders; reducing equivalents of NAD^+/NADH inhibit the TCA cycle and fatty acid oxidation, resulting in hepatic lipogenesis. Moreover, acetyl-CoA and NADPH are absolutely required for fat and cholesterol biosynthesis as a precursor and an essential cofactor, respectively.[13,27] G6PDH, 6-PGDH, and ME are related to hepatic lipogenesis since they are major enzymes producing cytosolic NADPH for lipid synthesis. Our results demonstrate that the activities of NADP-linked hepatic lipogenic enzymes, including G6PDH, 6-PGDH, and ME are significantly increased by alcohol feeding but can be significantly decreased by treatment with GF-AS (Table 3). Therefore, increased enzymatic activities induced by chronic alcohol consumption may in turn produce very high levels of NADPH/$NADP^+$, and stimulate fat accumulation in the liver. In our results, levels of TG and CHOL in the liver are significantly increased in alcohol treated rats compared with alcohol-untreated rats (Fig. 6). However, GF-AS treatment reduced TG and CHOL elevations in the liver induced by alcohol administration, which attributed to significant reductions in serum levels of TG and CHOL (Fig. 6). Collectively, these results demonstrate that GF-AS can prevent hyperlipidemia and fatty liver induced by chronic alcohol consumption through inhibition of lipogenic enzymes involved in hepatic lipogenesis. Morphological changes in fat accumulation were also observed. Chronic alcohol consumption exhibited typical signs of fatty liver, with accumulations in fat droplets throughout (Fig. 7), however, these morphological changes were improved by administration with GF-AS (Fig. 7).

Many studies[1,28—30] have demonstrated that liver injuries induced by ethanol are associated with free radicals and oxidative stress. Ethanol is converted to ethyl and 1-droxyethyl-radical. Free radicals adversely alter the lipid composition of all cell membranes *via* lipid peroxidation, resulting in membrane damage. Furthermore, ethanol and its metabolites can alter the redox balance towards a more oxidized state in the liver, which acts in a pro-oxidant manner and/or reduces antioxidant cell defenses. Oxidative stress is determined by increased ROS and enzymatic antioxidant systems including SOD, CAT, GSH-Px, GR, and non-enzymatic antioxidants systems such as reduced GSH and vitamin E, which act as protectors of oxidative stress. In the previous study, it was observed that GF-AS decreased lipid peroxidation by increasing antioxidative defense systems in the CCl_4-injured hepatotoxicity model *in vivo*, as well as scavenged DPPH radicals *in vitro* (paper submitted). In this study, lipid peroxidation also increased with alcohol treatment, whereas the activities of CAT ($p<0.05$), SOD, GSH-Px, and GR, and GSH level decreased when compared with alcohol non-treatment (Fig. 4, Table 4). However, treatment with GF-AS in alcohol-treated rats increases the activities of SOD ($p<0.05$), CAT ($p<0.05$), GSH-Px and GR, and GSH ($p<0.05$) levels, as well as decreases MDA levels (Fig. 4, Table 4), which alleviates deleterious effects induced by alcohol. It appears that the effect of GF-AS on the antioxidant system is weaker in the chronic alcohol model than in the acute alcohol model. Therefore, GF-AS appears to overcome ethanol toxicity caused by acute or chronic alcohol consumption by inducing the activities of antioxidant defense systems in livers greatly impaired by alcohol.

In conclusion, GF-AS appears to prevent hepatic injury by accelerating alcohol/acetaldehyde metabolism by increasing alcohol-metabolizing enzyme activities, increasing antioxidant system activities against oxidative stress, and by decreasing fat accumulation through inhibition of lipogenic enzyme activities, which is evidenced by decreased hepatotoxic indices in serum/liver tissue, and morphological observations in the liver.

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