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Research article

Korean Red Ginseng attenuates ethanol-induced steatosis and oxidative stress via AMPK/Sirt1 activation

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ABSTRACT

Background: Alcoholic steatosis is the earliest and most common liver disease, and may precede the onset of more severe forms of liver injury.
Methods: The effect of Korean Red Ginseng extract (RGE) was tested in two murine models of ethanol (EtOH)-feeding and EtOH-treated hepatocytes.
Results: Blood biochemistry analysis demonstrated that RGE treatment improved liver function. Histopathology and measurement of hepatic triglyceride content verified the ability of RGE to inhibit fat accumulation. Consistent with this, RGE administration downregulated hepatic lipogenic gene induction and restored hepatic lipolytic gene repression by EtOH. The role of oxidative stress in the pathogenesis of alcoholic liver diseases is well established. Treatment with RGE attenuated EtOH-induced cytochrome P450 2E1, 4-hydroxynonenal, and nitrotyrosine levels. Alcohol consumption also decreased phosphorylation of adenosine monophosphate-activated protein kinase, which was restored by RGE. Moreover, RGE markedly inhibited fat accumulation in EtOH-treated hepatocytes, which correlated with a decrease in sterol regulatory element-binding protein-1 and a commensurate increase in sirtuin 1 and peroxisome proliferator-activated receptor-α expression. Interestingly, the ginsenosides Rb2 and Rd, but not Rb1, significantly inhibited fat accumulation in hepatocytes.
Conclusion: These results demonstrate that RGE and its ginsenoside components inhibit alcoholic steatosis and liver injury by adenosine monophosphate-activated protein kinase/sirtuin 1 activation both *in vivo* and *in vitro*, suggesting that RGE may have a potential to treat alcoholic liver disease.

1. Introduction

Alcoholic liver diseases (ALD) remain the most common cause of liver-related morbidity and mortality worldwide [1]. Chronic alcohol consumption leads to hepatic steatosis, which is the benign form of ALD and most general response to heavy alcohol drinking. ALD has a known cause, but the mechanisms by which alcohol mediates ALD pathogenesis are incompletely defined. A number of factors play a key role in the pathogenesis and progression of ALD including gender and ethnic differences, nutrition, dysregulation of energy metabolism, immunologic mechanisms, and oxidative stress [2].

Experimental and clinical studies increasingly show that alcohol-induced oxidative stress is considered to be an early and indispensable step in the development of ALD [3]. Several pathways contribute to alcohol-induced oxidative stress. One of the central pathways is through the induction of cytochrome P450 2E1 (CYP2E1) by alcohol, leading to the induction of lipid peroxidation in hepatocytes [4]. Indeed, transgenic mice overexpressing CYP2E1 showed significantly increased liver damage following alcohol

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administration when compared with wild type mice [5]. By contrast, CYP2E1 knockout mice [6], and pharmacological inhibitors of CYP2E1 such as diallyl sulfide [7,8], phenethyl isothiocyanate [7,8], and chlormethiazole [9] decreased ethanol (EtOH)-induced lipid peroxidation and pathologic alterations.

Chronic alcohol ingestion has been shown to increase levels of sterol regulatory element-binding protein-1 (SREBP-1), a master transcription factor that regulates lipogenic enzyme expression, including fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase-1 [10,11]. Alcohol intake also lowered levels of peroxisome proliferator-activated receptor-α (PPARα), a key transcriptional regulator of lipolytic enzymes, such as carnitinepalmitoyl-transferase-1 and uncoupling proteins [12]. In addition to regulating transcription factors associated with fat metabolism, alcohol affects the activities of enzymes involved in energy metabolism, including adenosine monophosphate-activated protein kinase (AMPK) and sirtuin 1 (Sirt1). AMPK, a conserved cellular energy status sensor, is a serine–threonine kinase that can phosphorylate and subsequently inactivate SREBP-1 in hepatocytes, thereby attenuating steatosis [13]. Expression of the Sirt1, nicotinamide adenine dinucleotide-dependent class III histone deacetylase, is decreased in mice fed with alcohol, resulting in increased levels of SREBP-1 acetylation [14]. In addition, hepatocyte-specific knockout of Sirt1 impaired PPARα signaling and β-oxidation, whereas overexpression of Sirt1 elevated the PPARα target gene expression [15]. Hence, the AMPK/Sirt1 signaling axis is a promising therapeutic target to attenuate lipogenesis and increase lipolysis in ALD.

Korean ginseng (*Panax ginseng* Meyer) is one of the oldest and most commonly used botanicals in the history of traditional Oriental medicine. It has a variety of pharmacological activities, including anti-inflammatory, -tumor, and -aging [16]. The ginseng saponins, ginsenosides, play a key role in most physiological and pharmacological actions of ginseng [17]. Korean Red Ginseng (KRG) is heat- and steam-processed to enhance biological and pharmacological activities [18]. Red ginseng contains higher amounts of ginsenosides, and some ginsenosides are only found in red ginseng [19]. Studies have been shown that red ginseng has beneficial effects on liver function. KRG protects aflatoxin B1- [20] and acetaminophen-induced hepatotoxicity [21] and increases liver regeneration after partial hepatectomy [22] in animal models. We recently reported that KRG effectively protects against liver fibrosis induced by chronic CCl_4 treatment [23]. However, the effects of KRG on alcohol-induced liver damage and the expression of lipogenic genes have not yet been fully established.

In the present study, we examined the effect of KRG in mice after chronic EtOH treatment and in EtOH-treated hepatocytes. Histopathology and biochemical analysis verified the ability of KRG extract (RGE) to protect against EtOH-induced fat accumulation and oxidative stress, and to restore liver function. Moreover, RGE recovered the activity of AMPK and Sirt1 in alcohol-fed mice. In agreement with the *in vivo* data, RGE and its major ginsenosides possess the ability to recover homeostatic lipid metabolism in hepatocytes. These results demonstrate that KRG inhibits alcohol-induced steatosis through the AMPK/Sirt1 signaling pathway *in vivo* and *in vitro*, suggesting that KRG may have a potential to treat ALD.

2. Materials and methods

2.1. Materials

Lieber–DeCarli liquid diet was purchased from Dyets, Inc. (Bethlehem, PA, USA). Antibodies directed against CYP2E1, 4-hydroxynonenal (4-HNE), PPARα, and SREBP-1 were supplied by Abcam (Cambridge, UK). Antibodies that specifically recognize phosphorylated AMPK, AMPK, phosphorylated ACC, and Sirt1 were obtained from Cell Signaling (Beverly, MA, USA). The nitrotyrosine polyclonal antibody was purchased from Millipore Corporation (Billerica, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and goat anti-mouse immunoglobulin G were provided by Zymed Laboratories Inc. (San Francisco, CA, USA).

2.2. Preparation of RGE

RGE was kindly provided by KT&G Central Research Institute (Daejeon, Korea). Briefly, RGE was obtained from 6-year-old roots of *P. ginseng* Meyer. The ginseng was steamed at 90–100°C for 3 h and dried at 50–80°C. The red ginseng was extracted six times with water at 87°C for 12 h. The water content of the pooled extract was 36% of the total weight. Ginsenosides (Rb1, Rb2, and Rd) were obtained from Sigma-Aldrich Corporation (St Louis, MO, USA).

2.3. Animals and diets

Animal studies were conducted under the guidelines of the Institutional Animal Use and Care Committee at Chosun University, Gwangju, South Korea. C57BL6 mice were obtained from Oriental Bio (Sungnam, Korea) and acclimatized for 1 week. Mice ($n = 8$/group) were given free access to either the control diet or the Lieber–DeCarli liquid diet containing EtOH with or without RGE. The body weight and general condition of the animals were monitored at least once a week. The diet was kept refrigerated in the dark. EtOH was incorporated into the diet just before it was supplied to the animals. We used two animal models to evaluate the effect of RGE on alcohol-induced fatty liver and liver injury as previously reported [24–26]. First, a model of chronic EtOH intake was used. The mice were given free access to control diet or alcohol Lieber–DeCarli liquid diet for 4 weeks with or without RGE (250 mg/kg or 500 mg/kg, *per os*, $n = 8$) The mice were randomly assigned to the groups specified. The second was a mouse model of chronic–binge EtOH intake. The mice were fed with the control diet for 5 days, and then divided into four groups. The EtOH groups were fed with the Lieber–DeCarli liquid diet containing 5% EtOH for 10 days with or without RGE (250 mg/kg or 500 mg/kg, *per os*, $n = 8$). The control groups were pair-fed the control diet for 10 days. At Day 11, mice in EtOH groups were gavaged a single dose of EtOH (5 g/kg body weight, 20% EtOH), whereas mice in control groups were gavaged isocaloric dextrin maltose. The mice were sacrificed 9 hours after gavage.

2.4. Cell culture

AML12 cell lines were purchased from ATCC (Manassas, VA, USA). Cells were plated at a density of 3×10^5/well in 60 mm dishes and grown to 70–80% confluency. Cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 50 units/mL penicillin, 50 μg/mL streptomycin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 5 ng/mL selenium, and 40 ng/mL dexamethasone at 37°C in a humidified atmosphere with 5% CO_2. RGE or ginsenosides were dissolved in phosphate-buffered saline (PBS) and added to the cells. The cells were then incubated at 37°C for the indicated time period, and washed twice with ice-cold PBS prior to sample preparation.

2.5. Blood chemistry

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using Spectrum, an automatic blood chemistry analyzer (Abbott Laboratories, Abbott Park, IL, USA).

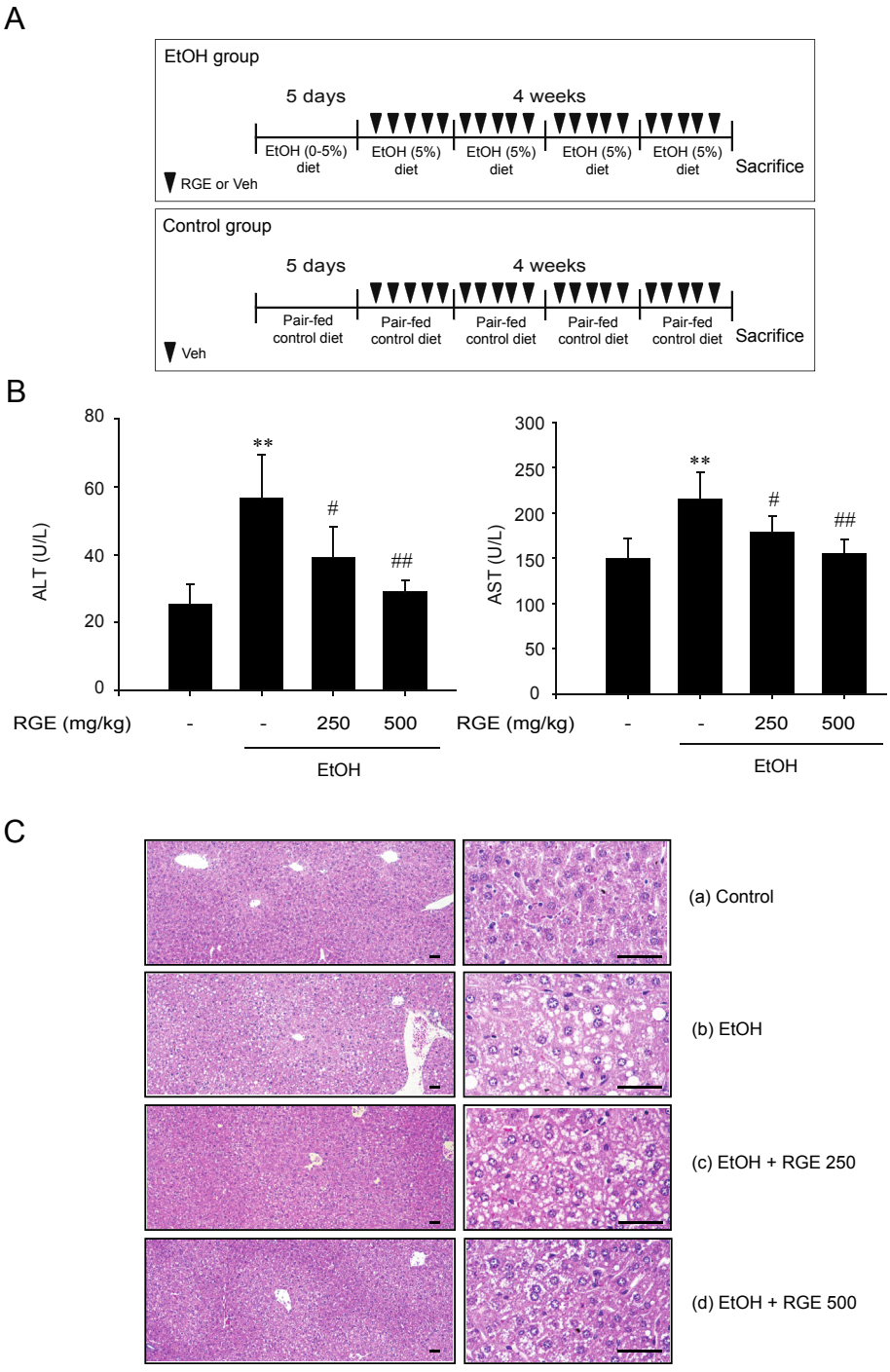

Fig. 1. Korean Red Ginseng extract (RGE) treatment prevents chronic ethanol-induced liver injury. (A) Feeding protocol. Mice were fed with pair-fed control or alcohol-containing diet (EtOH) for 4 weeks. During the feeding, the animals were orally administered with RGE five times/week. (B) Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were monitored in the serum of the mice. Values represent mean ± standard deviation from eight animals (significantly different from vehicle-treated control, $^{**}p < 0.01$, or EtOH alone, $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$). (C) Histopathology in mice. Hematoxylin and eosin-stained sections represent liver samples of mice fed control diet (control), alcohol-containing diet (EtOH), and alcohol-containing diet with RGE. EtOH = ethanol.

2.6. Histopathology

Samples from the liver were separated and fixed in 10% neutral buffered formalin. The samples were then embedded in paraffin, sectioned (3–4 μm), and stained with hematoxylin and eosin (H&E) for general histopathological analysis. In addition, the effect of RGE treatment on the 4-HNE and nitrotyrosine immunoreactivity was also observed by immunohistochemical methods.

2.7. Oil Red O staining

For the analysis of fat accumulation in the liver, 10-μm sections were cut from frozen samples and stained with Oil Red O for 10 min. The slides were rinsed in water and counterstained with Mayer's hematoxylin, followed by analysis using light microscopy. Lipid droplet formation in hepatocytes was determined by Oil Red O staining. Cells were grown on a six-well plate. After treatment,

the cells were fixed 4% formaldehyde in PBS for 1 h and rinsed with 60% isopropanol. Cells were then stained with Oil Red O solution.

2.8. Hepatic lipid contents

Hepatic lipid content was measured as described previously [25]. Briefly, lipids from the total liver homogenate were extracted using chloroform/methanol (2:1), evaporated, and dissolved in 5% triton X-100. Triglyceride content was determined using Sigma Diagnostic Triglyceride Reagents (Sigma).

2.9. Immunoblot analysis

Samples were individually prepared from animals or AML12 cells treated as described above, and subjected to immunoblotting. Immunoblot analyses were performed according to a previously published procedure [24]. Proteins of interest in liver homogenates were resolved using a 9% or 12% gel and developed using an ECL chemiluminescence system (Amersham, Buckinghamshire, UK).

2.10. RNA isolation and real-time reverse transcription polymerase chain reaction analysis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA,USA) according to the manufacturer's instructions. To obtain cDNA, total RNA (1 μg) was reverse-transcribed using an oligo(dT)$_{16}$ primer. The cDNA was amplified using a high capacity cDNA synthesis kit (Bioneer, Daejon, Korea) with a thermal cycler (Bio-rad, Hercules, CA, USA). Real-time polymerase chain reaction (PCR) was performed with STEP ONE (Applied Biosystems, Foster City, CA, USA) using a SYBR green premix according to the manufacturer's instructions (Applied Biosystems). Primers were synthesized by Bioneer. The following primer sequences were used: mouse SREBP-1 5′- GAGGCCAAGCTTTGGACCTGG-3′ (sense) and 5′- CCTGCCTTCAGGCTTCTCAGG-3′ (antisense); mouse FAS 5′- ATTG CATCAAGCAAGTGCAG-3′ (sense) and 5′- GAGCCGTCAAACAGGAA GAG-3′ (antisense); mouse ACC 5′- TGAAGGGCTACCTCTAATG-3′ (sense) and 5′- TCACAACCCAAGAACCAC-3′ (antisense); mouse PPARα 5′- CTGCAGAGCAACCATCCAGAT-3′ (sense) and 5′- GCCGAAGGTCCACCATTTT -3′ (antisense); and mouse Sirt1 5′- ATCGGCTACCGAGACAAC-3′ (sense) and 5′- GTCACTA GAGCTGGCGTGT-3′ (antisense). The relative level of PCR products was determined on the basis of the threshold cycle value. Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene for normalization. Melting curve analysis was done after amplification to verify the accuracy of the amplicon.

2.11. Data analysis

One-way analysis of variance was used to assess significant differences among treatment groups. The Newman–Keuls test was used for comparisons of group means. Statistical analyses were carried out using IBM-SPSS Statistics ver. 21.0 (IBM Corporation, Armonk, NY, USA) for Windows software. Data represent the mean ± standard deviation. The criterion for statistical significance was set at $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Treatment with RGE improves chronic alcohol-induced histopathological changes

We first evaluated the effects of RGE on EtOH-induced steatosis. To induce alcoholic steatosis, we adopted the most commonly used voluntary feeding model with the Lieber–DeCali diet containing EtOH (Fig. 1A). After 4 weeks of alcohol feeding, serum ALT and AST levels were significantly increased. The EtOH-induced elevation in ALT and AST was notably decreased by concomitant treatment with 250 mg/kg or 500 mg/kg RGE (5 times/week, *per os*; Fig. 1B). To verify the effects of RGE on alcoholic steatosis, we performed histopathological analysis of changes in fat accumulation. Hepatic steatosis was observed in all of the EtOH-fed groups. However, alcohol-induced hepatic steatosis was markedly and dose-dependently inhibited by treatment of 250 mg/kg and 500 mg/kg RGE (Fig. 1C). Our data verified that RGE treatment improves alcohol-induced fatty liver.

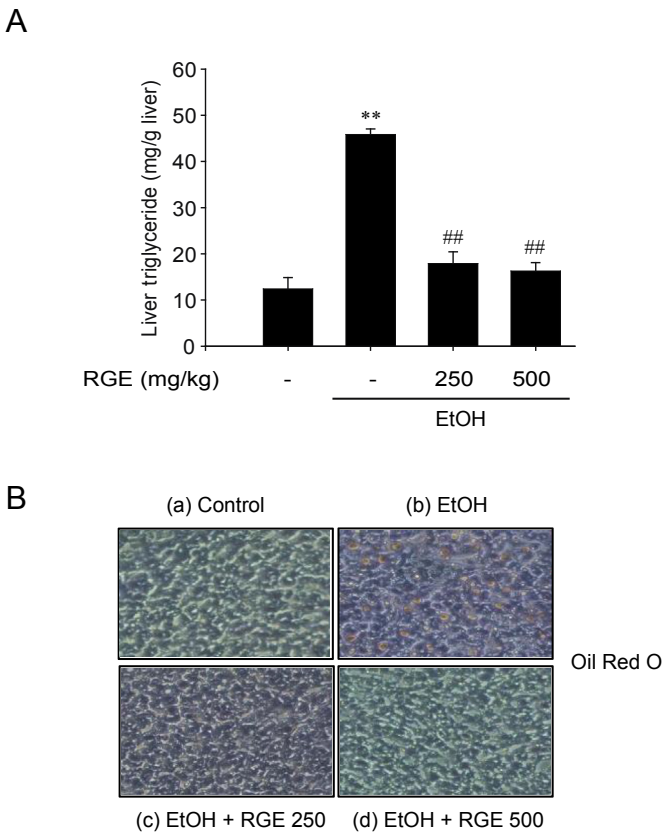

Fig. 2. Korean Red Ginseng extract (RGE) treatment inhibits chronic EtOH-induced fat accumulation in liver. (A) Hepatic triglyceride content was measured in lipid extracts. Data represent mean ± standard deviation from eight animals (significantly different from vehicle-treated control, $^{**}p < 0.01$, or EtOH alone, $^{\#\#}p < 0.01$). (B) Morphology in frozen liver sections as determined by Oil Red O staining. EtOH = ethanol.

3.2. Treatment with RGE decreases in hepatic triglyceride content

To firmly establish the effects of RGE on alcoholic steatosis, hepatic triglyceride levels were measured. The increase in hepatic triglyceride accumulation after EtOH feeding was significantly inhibited by RGE treatment (Fig. 2A). Lipid accumulation was also assessed by Oil Red O staining. Control mice did not show steatosis, whereas EtOH-fed mice exhibited a substantial increase in lipid droplets, which was in line with the results of H&E microscopy (Fig. 2B). RGE completely inhibited lipid infiltration in the liver, confirming the ability of RGE to prevent hepatic fat accumulation. The expression of hepatic fat metabolism-related genes was also assessed by quantitative real-time PCR. As shown in Fig. 3A, hepatic expression of several lipogenic gene, including SREBP-1, FAS, and ACC was upregulated by EtOH feeding. This enhancement was completely reversed by RGE treatment. As previously reported,

chronic alcohol consumption decreased fat oxidation-related genes, such as Sirt1 and PPARα. However, RGE prevented EtOH-mediated decreases in lipogenic gene expression (Fig. 3A). Furthermore, RGE abolished the EtOH-induced enhancement SREBP-1 and depletion of PPARα protein in the liver (Fig. 3B). These results demonstrate that RGE inhibits EtOH-induced lipogenesis and restores alcohol-mediated decreases in fatty acid oxidation.

3.3. Treatment with RGE inhibits chronic alcohol-induced oxidative stress

Sustained exposure to EtOH leads to prolonged oxidative stress, which promotes lipid peroxidation and generation of reactive aldehydes, such as 4-HNE [27]. Previously, 4-HNE-positive cells were markedly increased in mice fed alcohol. However, RGE treatment led to a significant, dose-dependent reduction in 4-HNE positive cells (Fig. 4A). These data provide direct evidence that RGE effectively inhibits lipid peroxidation and the formation of 4-HNE to protect hepatocytes from necrotic changes caused by EtOH.

It is well known that prolonged reactive oxygen species (ROS) exposure leads to increased nitrotyrosine levels [28]. Nitrotyrosine immunoreactive cells were increased in the chronic EtOH-administration group as compared with the control. However, RGE treatment dramatically reduced the number of nitrotyrosine positive cells (Fig. 4B). We next assessed whether RGE treatment inhibited the induction of CYP2E1 caused by chronic alcohol intake. As anticipated, RGE significantly repressed the induction of CYP2E1 by EtOH (Fig. 4C). Our present data suggest that RGE protects against chronic alcohol-induced oxidative stress and hepatic injury.

3.4. RGE treatment prevents chronic alcohol-mediated AMPK phosphorylation

Next, we examined whether the effect of RGE on hepatic steatosis is associated with AMPK activation. Immunoblot analysis

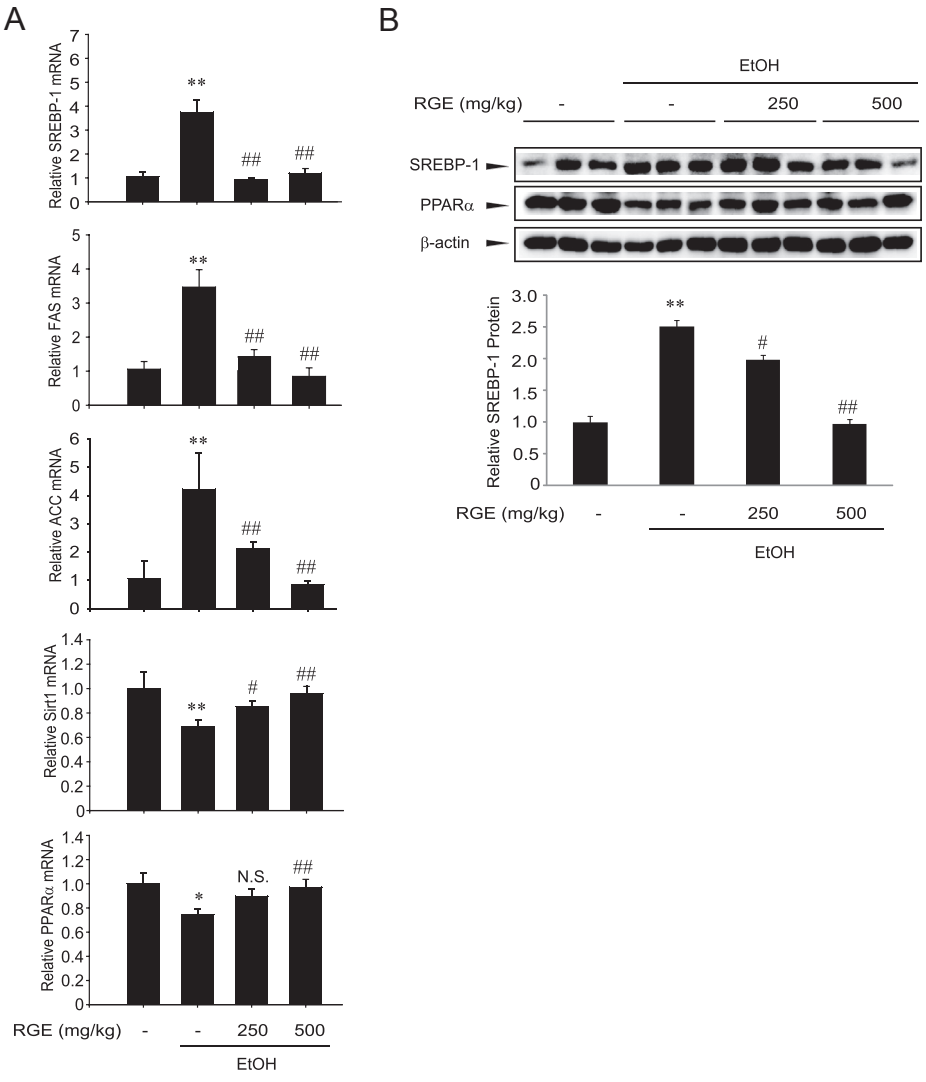

Fig. 3. Korean Red Ginseng extract (RGE) treatment improves fat metabolism gene profile. (A) Real-time reverse transcription polymerase chain reaction analysis. Mice were treated with vehicle or RGE, as described in the legend to Fig. 1. The transcripts of fat metabolism genes were analyzed by real-time reverse transcription polymerase chain reaction assays, with the mRNA level of glyceraldehyde-3-phosphate dehydrogenase used for normalization. (B) Immunoblotting. Sterol regulatory element-binding protein (SREBP-1) and peroxisome proliferator-activated receptor-α (PPARα) were immunoblotted in samples prepared from mice treated as described in the legend to Fig. 1. Equal loading of protein was verified by probing the replicate blots for β-actin. Data represent mean ± standard deviation from eight animals. Statistical significance of the differences between each treatment group and the vehicle-treated control ($^{*}p < 0.05$, $^{**}p < 0.01$) or ethanol alone ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, N.S.; not significant) was determined.

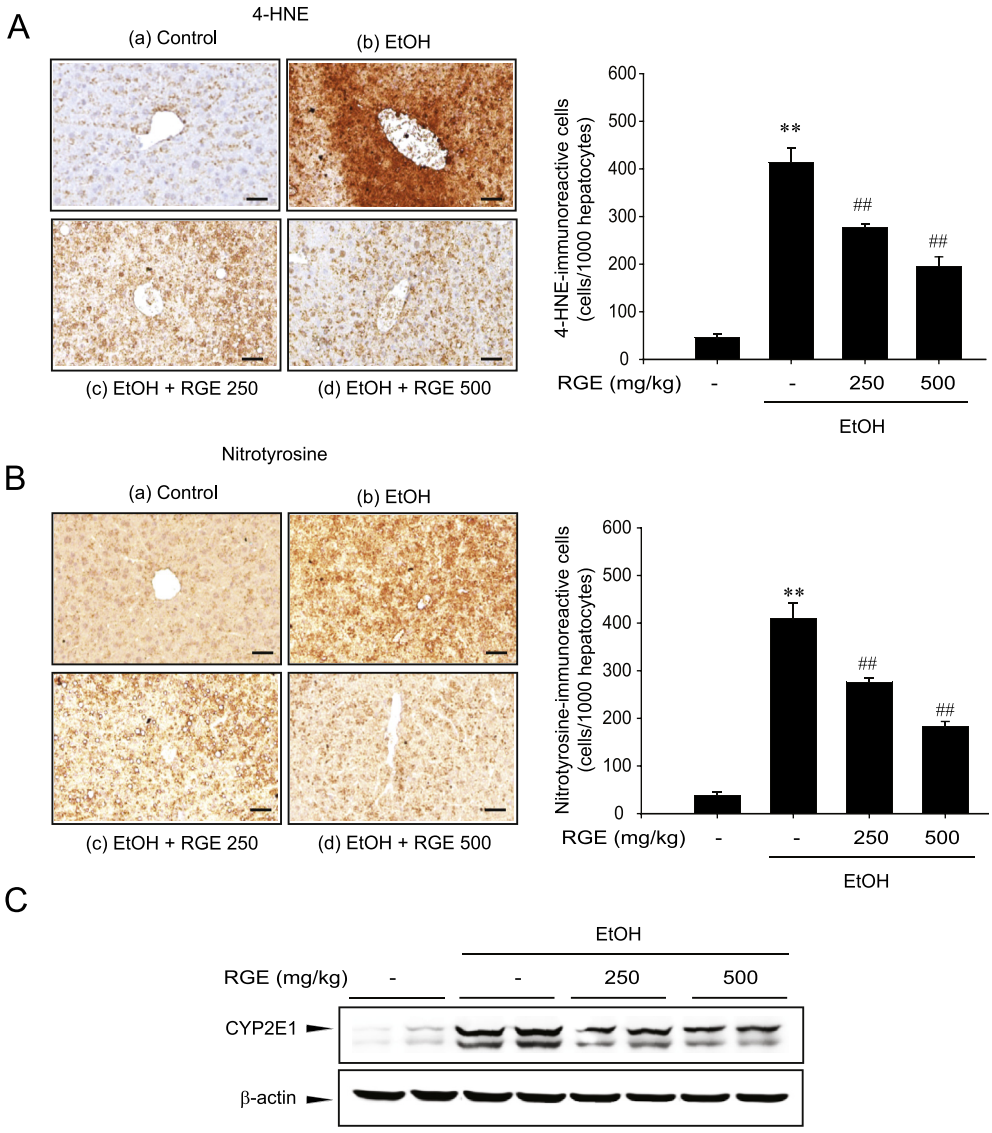

Fig. 4. Korean Red Ginseng extract (RGE) treatment abrogates chronic alcohol-induced oxidative stress. (A) 4-hydroxynonenal (4-HNE). Representative immunohistochemical staining of 4-HNE positive cells in liver section. (B) Nitrotyrosine. Representative immunohistochemical staining of nitrotyrosine positive cells in liver section. Statistical significance of the differences between each treatment group and the vehicle-treated control ($^{**}p < 0.01$) or ethanol (EtOH) alone ($^{\#\#}p < 0.01$) was determined. (C) Cytochrome P450 2E1 (CYP2E1) was immunoblotted in the samples prepared from mice treated as described in the legend to Fig. 1. Equal loading of proteins was verified by probing the replicate blots for β-actin.

showed that the level of phosphorylated AMPKα in liver homogenates notably decreased after 4 weeks of alcohol administration as previously reported (Fig. 5) [24]. Treatment of alcohol-fed mice with RGE resulted in a complete recovery of AMPKα phosphorylation levels. We further measured the levels of phosphorylated ACC, a direct downstream substrate of AMPK. Consistent with AMPKα phosphorylation, alcohol consumption inhibited ACC phosphorylation in the liver, and phosphorylation levels were recovered after RGE treatment. Our results demonstrate that chronic alcohol feeding results in a decrease in AMPK activity, which is recovered by RGE treatment.

3.5. Treatment with RGE prevents chronic-binge EtOH-induced liver injury

Previously, we reported that feeding mice with a Lieber–DeCarli diet containing 5% EtOH for 10 days, followed by a single dose of

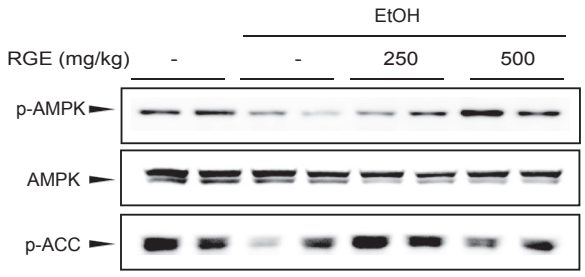

Fig. 5. Korean Red Ginseng extract (RGE) treatment increased adenosine monophosphate-activated protein kinase-α (AMPKα) phosphorylation. Phosphorylated AMPKα and acetyl-CoA carboxylase (ACC) was immunoblotted in the liver homogenates prepared from mice. Equal loading of proteins was verified by probing the replicate blots for AMPK.

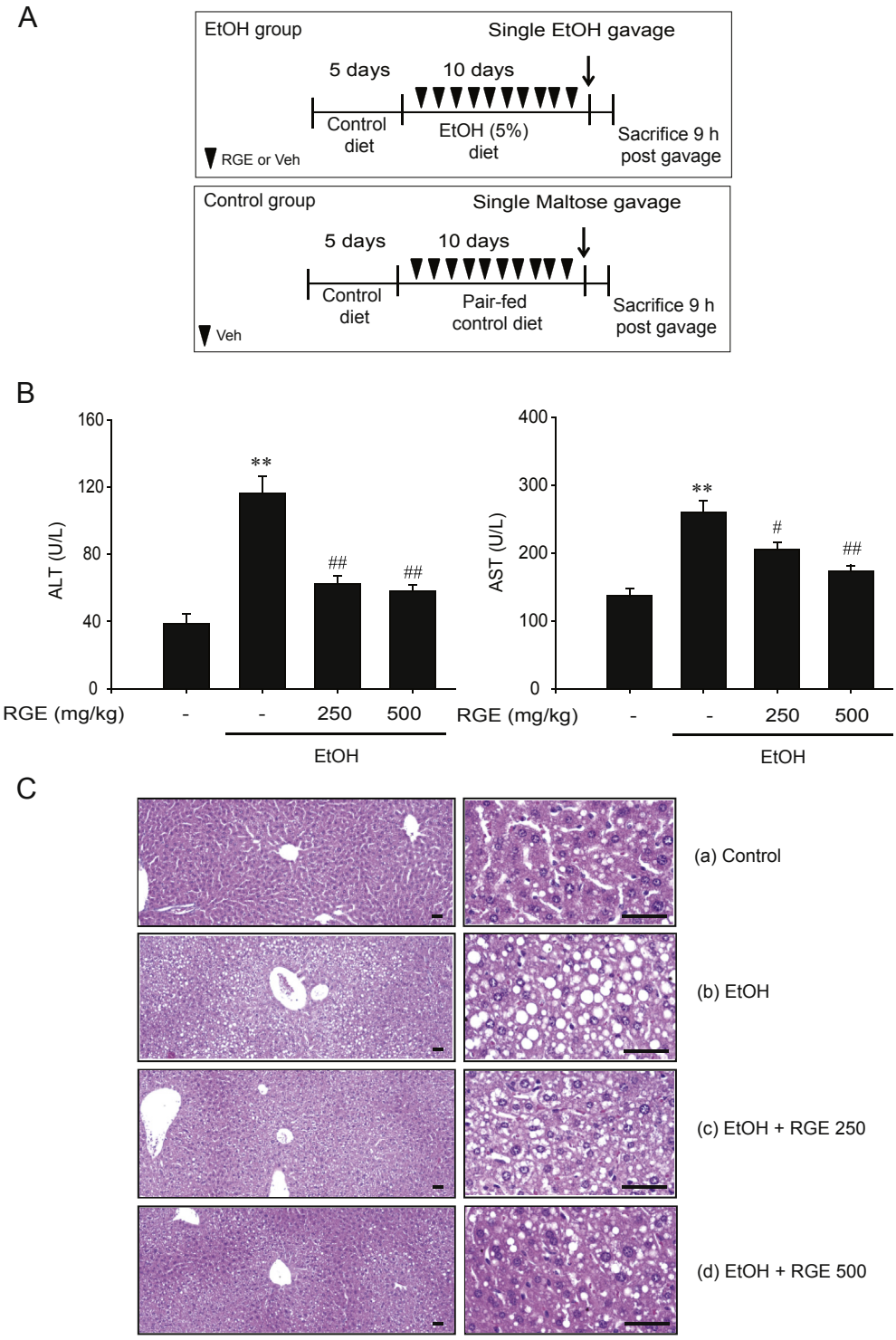

Fig. 6. Korean Red Ginseng extract (RGE) treatment prevents chronic–binge ethanol (EtOH)-induced liver injury. (A) Feeding protocol. Mice were fed with a liquid control diet for 5 days to allow for adjustment to the liquid diet, and then divided into four groups. The EtOH groups were fed with a liquid diet containing 5% EtOH for 10 days with or without RGE (250 mg/kg or 500 mg/kg, *per os*) and the control groups were pair-fed a control diet for 10 days. At Day 11, mice in EtOH groups were gavaged a single doses of EtOH (5 g/kg body weight), whereas control groups were gavaged isocaloric dextrin maltose. The mice were euthanized 9 hours after gavage. During the diet feeding, the animals were treated with RGE five times/week. (B) Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were monitored in the serum of the mice. Values represent mean ± standard deviation from eight animals (significantly different from vehicle-treated control, $^{**}p < 0.01$, or EtOH alone, $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$). (C) Histopathology in mice. Hematoxylin and eosin-stained sections represent liver samples of mice fed control diet (control), alcohol-containing diet (EtOH), and alcohol-containing diet with RGE. EtOH = ethanol.

EtOH gavage (5 g/kg body weight) (chronic–binge EtOH model) induces significant fatty liver and liver injury with oxidative stress (Fig. 6A) [25]. To investigate the effect of RGE for the treatment of ALD using the chronic–binge EtOH model, EtOH-fed mice were treated with RGE. Treatment with RGE decreased EtOH-induced serum ALT and AST levels (Fig. 6B). The protective effect of RGE on alcoholic steatosis was further confirmed by liver histology as shown by H&E staining. It was noted that treatment of alcohol-fed

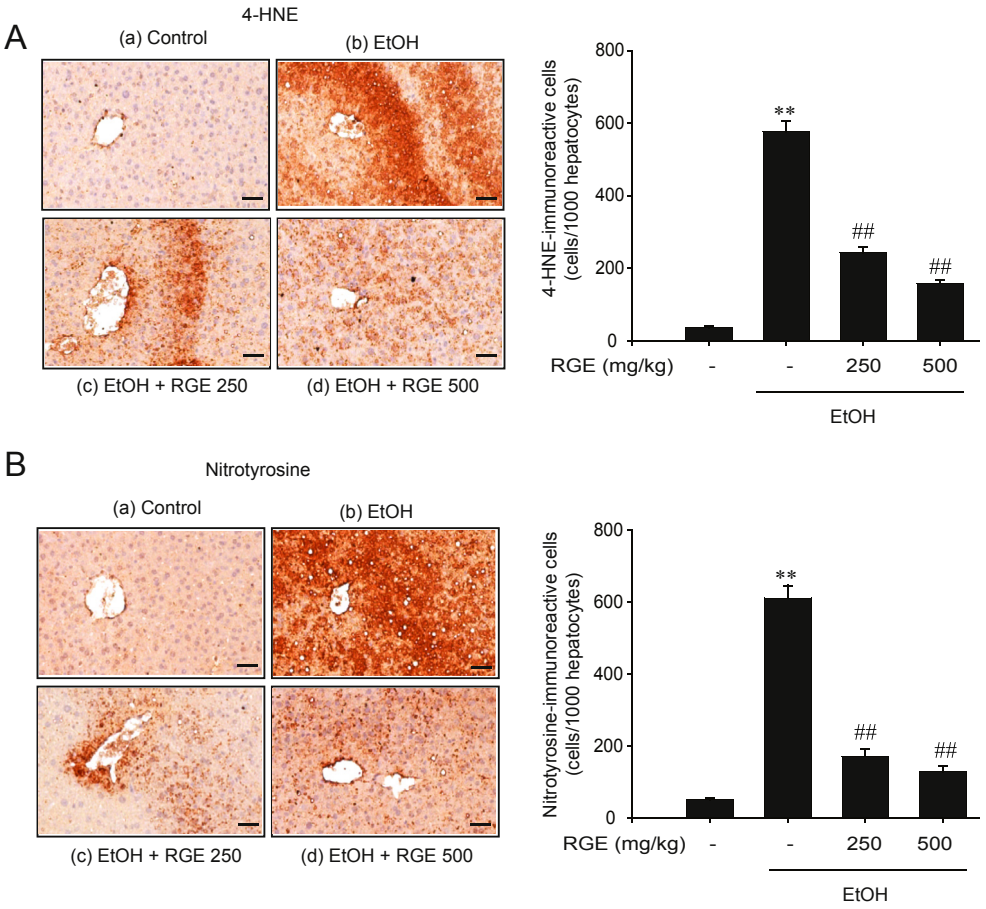

Fig. 7. Korean Red Ginseng extract (RGE) treatment abrogates chronic-binge EtOH-induced oxidative stress. Representative immunohistochemical staining of immunoreactive cells in liver section. (A) 4-hydroxynonenal (4-HNE). (B) Nitrotyrosine. Statistical significance of the differences between each treatment group and the vehicle-treated control ($^{**}p < 0.01$) or EtOH alone ($^{\#\#}p < 0.01$) was determined. EtOH = ethanol.

mice with RGE completely inhibited fat infiltration (Fig. 6C), confirming the ability of RGE to inhibit fat accumulation in liver.

Moreover, the chronic–binge EtOH model significantly increased 4-HNE positive cells, which is consistent with our previous report [25]. However, similar to the chronic EtOH model, the amount of 4-HNE positive cells was dose-dependently and significantly reduced by treatment with RGE (Fig. 7A). RGE also markedly attenuated nitrotyrosine positive cells, confirming that RGE is capable of inhibiting alcohol-induced oxidative stress in the chronic–binge EtOH animal model (Fig. 7B).

3.6. Treatment with RGE prevents EtOH-induced fat accumulation in AML12 cells

We next examined the effect of RGE on fat accumulation in a mouse hepatocyte cell line, AML12. EtOH treatment for 3 days increased fat accumulation in hepatocytes as shown by Oil red O staining. However, RGE (500 μg/mL or 1000 μg/mL) treatment reduced fat accumulation in a dose-dependent manner (Fig. 8A). To determine whether changes of fat accumulation in the hepatocyte were consistent with lipogenesis- or lipolytic-associated gene expression, the expression of SREBP-1, Sirt1, and PPARα was observed by Western blot analysis following concomitant treatment with 10–1000 μg/mL of RGE and EtOH for 3 days. In agreement with the *in vivo* data, RGE inhibited the ability of EtOH to induce SREBP-1 and repress Sirt1 and PPARα expression in AML12 cells (Fig. 8B).

3.7. Treatment with the major ginsenosides from red ginseng suppresses EtOH-induced fat accumulation in AML12 cells

The pharmacological properties of ginseng are primarily attributed to a group of active ingredients, the ginsenosides, which are a diverse group of steroidal saponins. Gum and Cho recently reported that total ginsenoside amount of RGE was 19.66 mg/g containing the major ginsenosides Rb1 (4.62 mg/g), Rb2 (1.83 mg/g), Rc (2.41 mg/g), Rd (0.89 mg/g), Re (0.93 mg/g), Rf (1.21 mg/g), Rg1 (0.71 mg/g), Rg2 (3.21 mg/g), Rg3 (3.05 mg/g), Rh1 (0.78 mg/g), and other minor ginsenosides [21]. Therefore, we next identified the major component of red ginseng required for the inhibition of hepatic steatosis. We determined the effects of the major ginsenosides Rb1, Rb2, and Rd on the EtOH-induced fat accumulation in AML12 cells. Interestingly, the ginsenosides Rb2 and Rd, but not Rb1, inhibited EtOH-induced fat accumulation, as shown by Oil red O staining (Fig. 9A). Consistent with this, Rb2 and Rd significantly reversed EtOH-mediated Sirt1 and PPARα suppression (Fig. 9B). The results suggest that RGE and its major ginsenosides inhibit alcohol-induced fatty liver and liver injury through the recovery of homeostatic lipid metabolism in the liver.

4. Discussion

ALD, which ranges from simple fatty liver to cirrhosis and hepatocellular carcinoma, remains a major cause of liver-associated mortality worldwide [29]. Early research on the pathogenesis of the

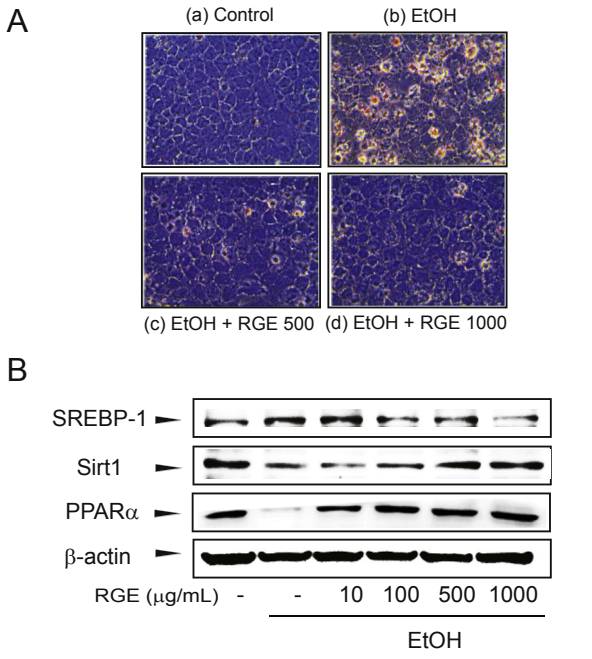

Fig. 8. Korean Red Ginseng extract (RGE) treatment inhibits alcohol-induced fat accumulation in AML12 cells. (A) Oil Red O staining. The cells were treated with the indicated concentrations of RGE for 3 days with EtOH in AML12 cells. Cells were fixed formaldehyde, stained with Oil red O, and observed under a Leica microscope (Wetzlar, Germany). (B) Sterol regulatory element-binding protein (SREBP-1), sirtuin (Sirt1), or peroxisome proliferator-activated receptor-α (PPARα) expression was assessed in the lysates by immunoblotting. The results were confirmed by three independent experiments. Equal loading of proteins was verified by probing the replicate blots for β-actin. EtOH = ethanol.

ALD primarily focused on alcohol metabolism-related oxidative stress, malnutrition, and activation of Kupffer cells by endotoxins [30,31]. Recently, the characterization of intra- and intercellular signaling pathways, innate and adaptive immune responses, epigenetic features, microRNAs, and stem cells has improved our knowledge of the pathobiology of ALD [31]. Despite improved understanding of the pathophysiology of ALD, there is no Food and Drug Administration-approved drug for the specific treatment of ALD. Therefore, the development of effective therapeutic strategies for ALD is pivotal.

KRG has been shown to exhibit several beneficial effects in the treatment of liver diseases through the regulation of immune function and antioxidant activity [16]. However, the effects of KRG on alcohol-induced hepatic steatosis and oxidative stress have not been fully established. Here, we established the effects of RGE on alcohol-induced liver injury *in vivo* and *in vitro* and identified the major component of KRG with beneficial effects in ALD. Ginseng saponins, referred to as ginsenosides, play a major role in most pharmacological actions of ginseng; however, until now, the role of ginsenosides on EtOH-induced fat accumulation has remained observed. Interestingly, the ginsenosides Rb2 and Rd, but not Rb1, significantly restored EtOH-induced Sirt1 and PPARα suppression (Fig. 9B), consistent with RGE treatment to the mice. Moreover, the ginsenosides Rb2 and Rd inhibited EtOH-induced fat accumulation in AML12 cells (Fig. 9A). The increased lipolytic gene expression and inhibition of fat accumulation resulting from treating by RGE and its major ginsenosides indicates that RGE may be a promising hepatoprotective candidate against liver injury.

During the last 5 decades, several animal models of ALD have been studied, which has helped us understand the molecular basis of ALD. The most widely used model for ALD is the Lieber–DeCarli EtOH-containing diet, which is a liquid diet-based voluntary feeding model. Recently, we have developed and reported a more

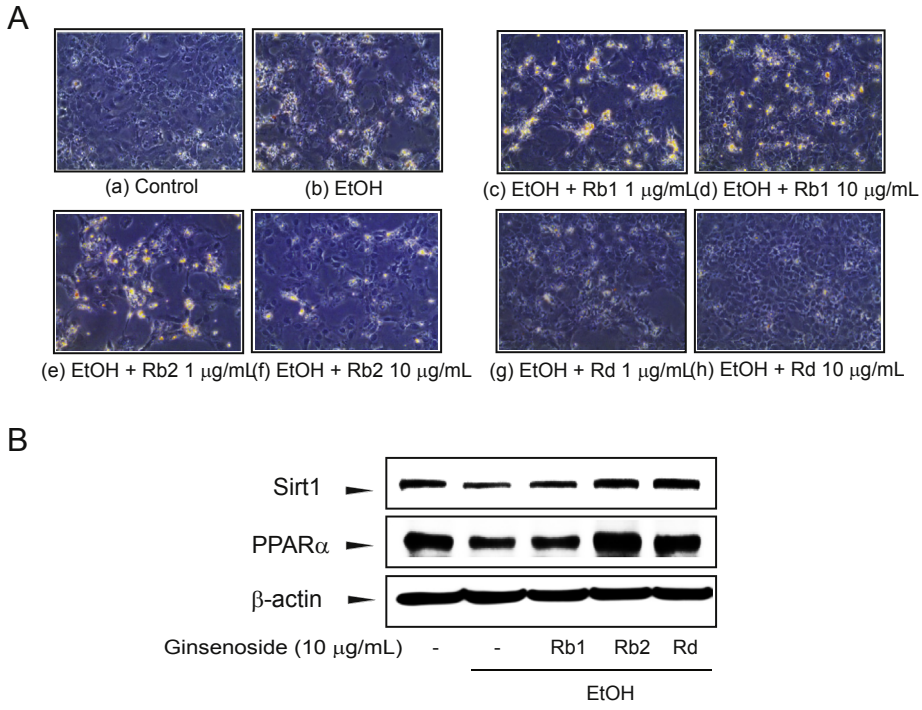

Fig. 9. Ginsenosides from red ginseng inhibit alcohol-induced fat accumulation in AML12 cells. (A) Oil Red O staining. Cells were treated with the indicated concentrations of ginsenosides for 3 days with ethanol (EtOH) in AML12 cells. Cells were fixed in formaldehyde, stained with Oil red O, and observed under microscope. (B) Expression of sirtuin 1 (Sirt1) or peroxisome proliferator-activated receptor-α (PPARα) was assessed in the lysates by immunoblotting. The results were confirmed by three independent experiments. Equal loading of proteins was verified by probing the replicate blots for β-actin. EtOH = ethanol.

severe alcohol-induced liver injury model (a chronic–binge EtOH model in mice), which is similar to drinking patterns in ALD patients who have a background of long-term drinking (chronic) and a history of recent heavy alcohol use (binge) [25,26]. Such chronic–binge EtOH feeding induced severe hepatic steatosis, oxidative stress, and inflammation in mice. Other laboratories have also confirmed the effect of the chronic–binge EtOH model in mice and rats [32,33]. Here we used two animal models, the chronic EtOH model and chronic-binge EtOH model to investigate the effect of RGE for the treatment of ALD. Treatment with RGE improved alcoholic fatty liver and liver injury in both models.

Alcohol is primarily metabolized in the liver by oxidative enzymatic breakdown by alcohol dehydrogenase. In addition, the microsomal electron transport system also regulates alcohol metabolism via catalysis by CYP2E1. CYP2E1 expression is induced during chronic alcohol consumption, and results in the formation of ROS and free radicals [3,4]. CYP2E1 also promotes the formation of highly reactive aldehydes, including acetaldehyde, 4-HNE, and MDA, which can form protein adducts. In the current study, we measured the CYP2E1 protein level through western blot (Fig. 4C) and 4-HNE and nitrotyrosine protein adducts, two major products of ROS and reactive nitrogen species, respectively, by immunohistochemistry (Figs. 4A and B, 7A and B). Treatment of mice with RGE was capable of inhibiting CYP2E1 induction caused by chronic alcohol consumption. In addition, 4-HNE-positive cells and nitrotyrosine-immunoreactive cells were significantly reduced after treatment with RGE. Thus, the beneficial effect of RGE against alcohol-induced fat accumulation and liver injury may be mediated, at least in part, through the inhibition of oxidative stress.

In recent years, several novel mechanisms regulating the pathogenesis of ALD have been described. Chronic alcohol ingestion in animal models is associated with impairment of the hepatic AMPK/Sirt1 axis, a central signaling pathway regulating energy metabolism [14,34]. The activation of AMPK/Sirt1 signaling in liver has been found to increase fatty acid oxidation and repress lipogenesis, primarily by modulating activity of SREBP-1 or PPARγ coactivator-α/PPARα [35,36]. Here, we confirmed that AMPK phosphorylation was significantly decreased after alcohol administration. Treatment of alcohol-fed mice with RGE restored AMPKα and ACC phophorylation levels (Fig. 5). Moreover, treatment of AML12 cells with RGE and ginsenosides resulted in a complete recovery of the Sirt1 and PPARα suppression induced by EtOH (Figs. 8 and 9). Consistent with this, RGE and ginsenosides inhibited EtOH-induced SREBP-1 expression and fat accumulation as evidenced by Oil red O staining in AML12 cells. These results indicate that the effect of RGE on alcoholic fatty liver and liver injury may be due to improvement of homeostatic lipid metabolism in the liver.

In summary, our present study demonstrated for the first time that RGE and major ginsenosides efficaciously ameliorated alcohol-induced fatty liver and liver injury through improving hepatic energy metabolism and prevention of oxidative stress. The effect of RGE and ginsenosides may be due to the activation of AMPK/Sirt1 signaling pathway. Therefore, our study provides crucial information about the possible use of KRG as a clinical candidate for the prevention and treatment of ALD.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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