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Article

Ginsenoside Rc Modulates SIRT6-NRF2 Interaction to Alleviate Alcoholic Liver Disease

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ABSTRACT: Alcoholic liver disease (ALD) is a serious worldwide health problem. Ginsenoside Rc is a major active ingredient isolated from *Panax ginseng*, whose pharmacological effects counteract oxidative stress, inflammation, and lipid accumulation. However, it is still unclear whether ginsenoside Rc might exert beneficial effects on alcohol-induced liver injury. To this aim, mice primary hepatocytes (MPHs) were challenged with alcohol to test ginsenoside Rc's effects on their intracellular alcohol metabolism. C57BL/6J mice or SIRT6$^{alb-/-}$ mice were chronically fed a diet with added alcohol or given a single gavage of alcohol with or without ginsenoside Rc. Analyses of alcohol metabolism, oxidative stress, inflammation, lipid metabolism, and RNaseq expression were conducted to explore potential targets exploited by ginsenoside Rc to protect against ALD. Our results showed that ginsenoside Rc attenuated alcohol-induced liver injury by regulating oxidative stress, inflammation, and lipid accumulation both *in vivo* and *in vitro*. Ginsenoside Rc did increase the deacetylase activity of SIRT6, thereby lowering acetylated NRF2 levels, which elevated NRF2's stability, and subsequently exerting an antioxidant effect. In keeping with this, the hepatic knockout of SIRT6 almost abolished the hepatoprotective effects of ginsenoside Rc against ALD. Therefore, our results suggest that ginsenoside Rc attenuated hepatocytes' damage and oxidative stress in ALD by up-regulating the SIRT6/NRF2 pathway. Hence, ginsenoside Rc may be a promising drug to treat or relieve ALD.

KEYWORDS: *Ginsenosides Rc, Alcoholic liver disease, Oxidative stress, SIRT6, NRF2*

INTRODUCTION

Alcoholic liver disease (ALD) induced by excessive alcohol consumption is a serious global health concern causing about 3 million deaths and 132.6 million disability-adjusted life years.[1] ALD includes a spectrum of hepatic pathological changes, such as steatosis, steatohepatitis, and cirrhosis.[2] Besides inflammation and lipid accumulation, oxidative stress too is a major pathogenetic factor induced by alcohol metabolism.[3−5] In addition, various reports showed that an overproduction of reactive oxygen species (ROS) and reduced antioxidant activity do aggravate ALD.[6,7]

Alcohol metabolism increases the reduction of nicotinamide adenine dinucleotide (NAD+) to nicotinamide adenine dinucleotide phosphate (NADH).[8] It has been reported that the NAD+/NADH ratio values decrease in alcohol-treated model mice.[9,10] The main production of endogenous ROS occurs when NADPH is reoxidized.[11,12] Moreover, acetaldehyde, a product of alcohol metabolism, triggers the immune response of Kupffer cells (KCs). Next, lipopolysaccharides (LPS) produced by alcohol-induced alterations of the gut microbiome activate NADPH oxidase in KCs, thereby increasing the generation of both ROS and tumor necrosis factor (TNFα), which advance liver inflammation and injury.[13] Oxidative stress too has been implicated in alcoholic hepatotoxicity as it dysregulates lipid metabolism, thereby inducing hepatic steatosis.[14−16] Therefore, attenuating the level of oxidative stress may be one of the potential targets for ALD treatment.

Sirtuin 6 (SIRT6), an important regulator of NAD$^+$-dependent histone deacetylase, has been widely involved in the regulation of longevity, metabolism, and inflammation.[17−19] Reportedly, SIRT6 reduced oxidative stress by regulating nuclear factor erythroid 2-related factor 2 (NRF2) in ischemic brains, mesenchymal stem cells, and nonalcoholic fatty livers.[20,21] Being a vital regulator of the antioxidant defense system, NRF2 modulates the expression of intracellular detoxifying enzymes and antioxidant proteins, such as heme oxygenase 1 (HO-1) and superoxide dismutase 2 (SOD2), which promote ROS elimination.[22−25] However, the specific mechanism by which SIRT6 activates NRF2 is still unclear. And there is a need for more experimental evidence to prove that the activation of the SIRT6/NRF2 signaling pathway may effectively protect the liver from acute or chronic alcohol injury.

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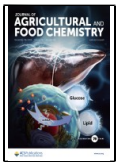

Ginsenosides, the major active ingredients isolated from *Panax ginseng*, exert multiple pharmacological activities on obesity, diabetes, hepatotoxicity, inflammation, cancer, cardiovascular diseases, and so on.[26−30] Among them, ginsenoside Rc showed a significant ability to prevent endogenous ROS production.[31,32] In addition, ginsenoside Rc could protect the liver from LPS-induced inflammation and lipid accumulation.[33,34] However, thus far it is still unknown whether ginsenoside Rc does protect the liver from ALD.

In this study, we evaluated ginsenoside Rc's hepatoprotective effects from chronic or acute ALD and investigated their underlying mechanisms. Our results clearly showed that ginsenoside Rc administration up-regulated SIRT6-NRF2 interaction, which reduced alcohol consumption-elicited oxidative stress, an effect that associated with the improvement of ALD. Thus, our data suggest that ginsenoside Rc is a drug potentially apt for treating ALD.

MATERIALS AND METHODS

Animals and Treatment. Male C57BL/6J mice aged 6−8 weeks were purchased from the Model Animal Research Center of Guangzhou University of Chinese Medicine. Hepatocyte SIRT6 deficient mice ($SIRT6^{alb-/-}$ mice) have been previously described.[35] All mice were maintained under standard humidity- and temperature-controlled conditions (temperature 22 °C, 12 h light/dark cycle) with free access to food and water and habituated to the laboratory environment for 1 week before being used. All animal care and experiments were conducted under protocols approved by the guidelines of the Animal Ethics Committee of Guangzhou University of Chinese Medicine (20210722014).

For chronic ALD models, C57BL/6J mice or $SIRT6^{alb-/-}$ mice were subjected to the NIAAA alcohol model based on methods previously described.[36] Briefly, mice were randomly divided into six groups (n = 10 for each group): (I) Mice were fed a control diet (a control liquid diet (Bio-Serv, F1259SP) for 1 day and then fed an isocaloric control diet for 14 days, followed with isocaloric dextrin maltose (45%, wt/vol) gavage) and intraperitoneal injection with saline daily for the last 7 days as the vehicle control (Ctr + Saline) group. (II) Mice were fed a control diet and intraperitoneal injections were given with an optimal therapeutic dose of ginsenoside Rc (purity > 98%, Yuanye Co, Shanghai China) described above daily for the last 7 days as the Ctr+Rc(H) group. (III) Mice were fed an ethanol diet (a control liquid diet for 1 day, a liquid diet containing 1%−5% (v/v) ethanol content for day 2 to day 5, a liquid diet containing 5% (v/v) ethanol for day 6 to day 15, and then gavaged with ethanol (5g/kg, 31.5%, vol/vol) at day 16) and given intraperitoneal injections with saline daily for the last 7 days as the EtOH+Saline group. (IV) Mice were fed an ethanol diet and given intraperitoneal injections with 5 mg/kg of ginsenoside Rc daily for the last 7 days as the EtOH+Rc(L) group. (V) Mice were fed an ethanol diet and given intraperitoneal injections with 10 mg/kg of ginsenoside Rc daily for the last 7 days as the EtOH+Rc(M) group. (VI) Mice were fed an ethanol diet and given intraperitoneal injections with 20 mg/kg of ginsenoside Rc daily for the last 7 days as the EtOH+Rc(H) group. Six hours after EtOH gavage, mice were sacrificed, and blood and liver tissues were collected and stored at −80 °C for further analyses.

For acute ALD models, mice were fed as previously reported.[37] Briefly, mice were randomly divided into three groups (n = 10 mice per group): (I) mice gavaged with isocaloric maltose dextrin (45%, wt/vol) and saline as a vehicle control (Ctr+saline) group; (II) mice intraperitoneally injected with saline once a day for 7 days prior to a binge of ethanol (5 g/kg, 31.5%, vol/vol) gavage as the EtOH+saline group; (III) mice intraperitoneally injected with ginsenoside Rc (20 mg/kg) once a day for 7 days prior to ethanol gavage as the EtOH+Rc group. Then, 6 h after EtOH gavage, mice were sacrificed, and blood and liver tissues were collected and stored at −80 °C for further analyses.

Cell Culture and Treatment. Mouse primary hepatocytes (MPHs) were isolated and cultured based on methods previously described.[38] Then, freshly prepared MPHs were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum and plated in six-well culture plates at 0.5 × 106 cells/well. After attachment, MPHs were washed with PBS, and media were replaced with RPMI-1640 medium supplemented with 10% fetal bovine serumand penicillin-streptomycin. Then, MPHs were exposed to 100 mM ethanol with the indicated dose of ginsenoside Rc or DMSO cotreatment for 24 h of treatment.[9,39] For CYC-116 (HY-10558, MedChemExpress) treatment, MPHs were treated with 1 μM CYC-116 for indicated times. Cells were harvested for further experiments.

CCK-8 Assay. CCK-8 assays were performed according to the manufacturer's instructions.[40] Briefly, MPHs were cultured in 96-well plates at 5 × 104 cells/well. After attachment, cells were treated with the indicated concentration of ginsenoside Rc for 24 h. Then, cells were incubated with the premixed medium containing 10% CCK-8 (Glpbio Co, USA). Cell viability was measured using the OD value at 450 nm with a microplate reader.

Transfections and Luciferase Reporter Assays. HEK293T or RAW264.7 cells were cultured in 24-well plates using Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. Then, HEK293T cells were transfected with NRF2 (pARE-Luc) luciferase reporter plasmid and Ramlila luciferase expression plasmid pGMLR-CMV-TK (Yeasen Biotechnology Co. Shanghai, China), and RAW264.7 cells were transfected with NF-κB (pNF-κB-Luc) and pGMLR-CMV-TK by using lipofectamine 2000TM (Invitrogen, Carlsbad, CA, USA). Twelve hours after transfection, HEK293T cells were cotreated with DMSO (vehicle control) or ginsenoside Rc (50 μM) for 24 h. RAW264.7 cells were cotreated with DMSO (vehicle control) or ginsenoside Rc (50 μM) for 24 h before or without 24 h-induction of LPS (1.0 μg/mL). After incubation, cells were harvested and assessed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega). Relative luciferase activity was corrected for Renilla luciferase activity of pCMV-RL-TK and normalized to the activity of the control.

Biochemistry Analysis. Serum samples and cell medium were assayed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and triglycerides (TG) levels using the commercially available enzymatic assay kits (Jiancheng Co, Nanjing China) according to the manufacturer's instructions. For hepatic biochemistry analysis, 20 mg of liver tissues was homogenized in 100−200 μL of PBS. Then, the mixture or supernatant was collected to measure glutathione (GSH; Beyotime Co, Beijing, China), superoxide dismutase (SOD; Jiancheng Co, Nanjing China), H_2O_2 (Elabacience Co, Wuhan, China), alkaline phosphatase (AKP; Jiancheng Co, Nanjing China), and malonaldehyde (MDA; Jiancheng Co, Nanjing China) levels according to the manufacturer's instructions.

In addition, serum levels of inflammatory cytokines including IL-6 and IL-1β were measured using the indicated enzyme-linked immunosorbent assay (ELISA) kit according to the instructions from the manufacturer (Elabacience Co, Wuhan, China). Serum TNF-α levels were determined using an ELISA kit (RUIXIN Co. Fujian, China) following the manufacturer's instructions.

Histological and Immunofluorescence Analysis. For hematoxylin and eosin (H&E) staining, liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 10 μm sections. Then, liver sections were stained with H&E to detect liver damage. For Oil Red O staining, liver tissues were frozen at the optimal cutting temperature (OCT), sectioned into 10 μm pieces, and stained with Oil Red O (Sigma) to evaluate hepatic lipid content.

For immunofluorescent staining of livers, sections were blocked with BSA (Abclonal Co, Wuhan, China) for 30 min at room temperature and incubated with primary antibodies F4/80 and CD11b (Affinity Co, USA) at 4 °C overnight. Then, the sections were incubated with secondary antibody goat-antirabbit conjugated antibody (Abclonal Co, Wuhan, China) for 40 min at room temperature. Finally, the sections were counterstained with DAPI (Abclonal Co, Wuhan, China). For immunofluorescent staining of

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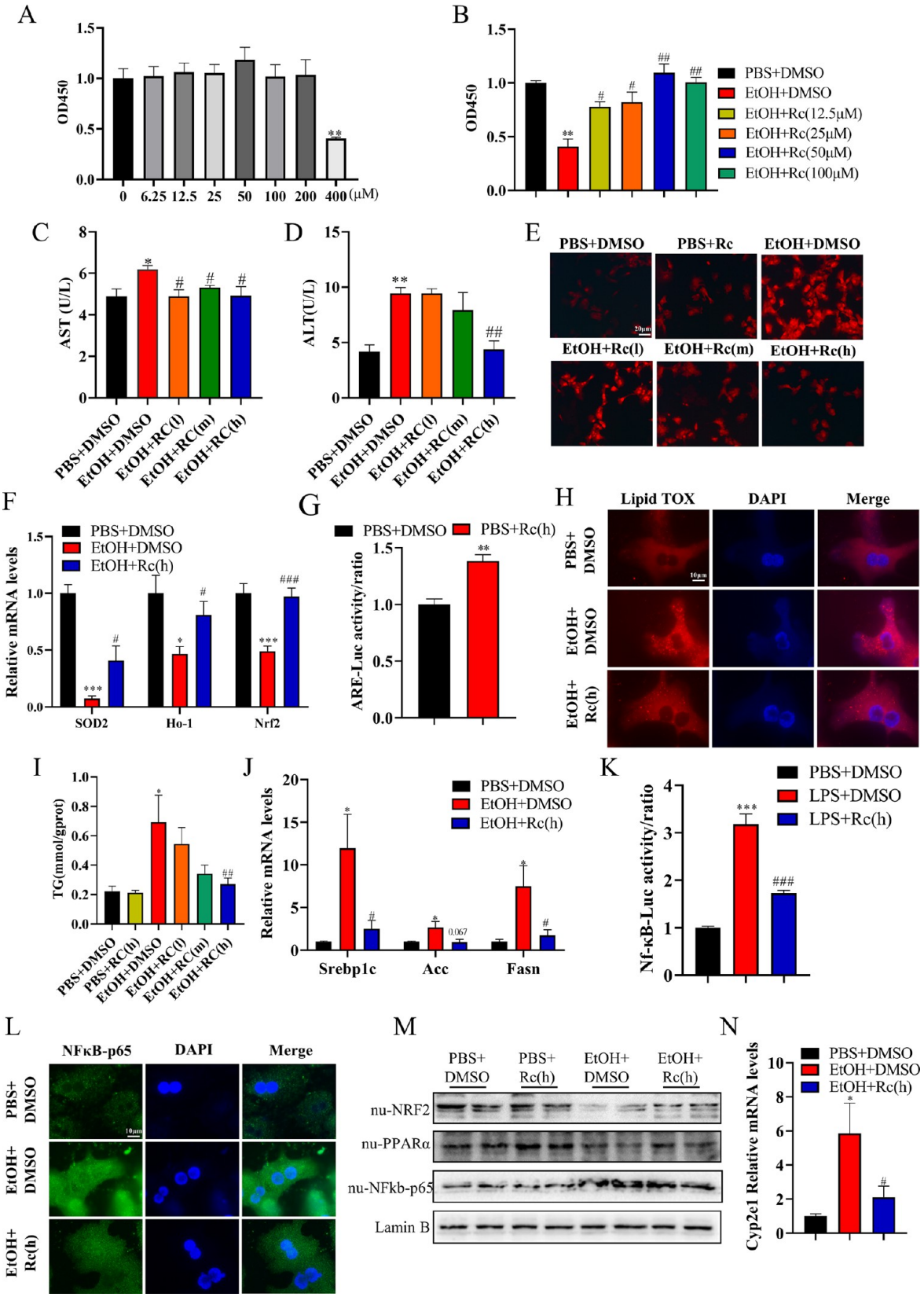

Figure 1. Ginsenoside Rc suppressed ethanol-induced hepatocellular damage by improving oxidative stress, inflammation, and lipid deposition in MPHs. (A, B) CCK8 assay; (C, D) hepatic ALT and AST levels; (E) ROS levels of MPHs (400×); (F) relative expression of oxidative stress mRNA; (G) dual luciferase assay of NRF2; (H) lipid TOX-stained MPHs (1000×); (I) hepatic TG levels; (J) relative expression of lipogenesis in MPHs; (K) dual luciferase assay of NF-κB; (L) immunofluorescence for NF-κB-p65 (1000×); (M) Western blotting of nuclear proteins; (N) expression of Cyp2e mRNA levels. Data are means ± SEM; n = 3−6/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the Ctr+DMSO group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the EtOH+DMSO group.

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cells, cells were seeded onto glasses and treated with ethanol and ginsenoside Rc for 24 h, washed with PBS, and fixed in 4% paraformaldehyde for 30 min at room temperature. Then, the fixed cells were blocked with BSA and incubated with primary antibody NF-κB-p65(Affinity Co, USA), NRF2 (Affinity Co, USA), H3K9ac, and H3K56ac (Abclonal Co, Wuhan, China) as described above. All images were taken using a microscope (Pannoramic MIDI 1000×) from Lingnan Medical Research Center of Guangzhou University of Chinese Medicine.

Reactive Oxygen Species (ROS) Analysis. For cellular ROS determination, cells were seeded in 12-well plates and treated as above, followed by incubation with DHE 5 μM (KeyGEN Co, Jiangsu, China) for 30 min at 37 °C in the dark. Then, cells were washed with PBS three times and visualized under a fluorescence microscope (Nikon 400×). For detecting ROS accumulation in the liver, mice were injected with DHE (10 mM, 10 μL via tail vein for 40 min before being sacrificed, and then the livers were harvested. ROS levels were measured using a Berthold Technologies LB983 NC100 from Science and Technology Innovation Center of Guangzhou University of Chinese Medicine.

Lipid TOX Staining. Cells were seeded in 12-well plates and treated as above, followed by fixing with 4% paraformaldehyde for 10 min. Then, cells were incubated with 1 × Lipis TOX neutral lipid stain (Invitrogen) at room temperature for 30 min. After nuclear staining with DAPI, the slips were visualized using a fluorescence microscope (Nikon 1000×).

Western Blotting. Total protein and nuclear protein were extracted from cultured cells according to the manufacturer's instructions (Beyotime Co, Beijing, China). The concentrations were determined by BCA assay kit (Beyotime Co, Beijing, China). In total, equal amounts of the protein (20−60 μg) were fractionated by 10% SDS-polyacrylamide gel, and separated proteins were transferred onto PVDF membranes. The membranes were incubated overnight at 4 °C with with various primary antibodies including anti-NRF2 (Affinity Co, USA), anti-PPARα (Proteintech Co, USA), anti-LAMINB1 (Proteintech Co, USA), and anti-β-ACTIN (Abclonal Co, Wuhan, China) and followed by an incubation with a secondary antibody. Finally, the blots were observed using BIO-RAD Gel Doc XR from Science and Technology Innovation Center of Guangzhou University of Chinese Medicine.

Co-Immunoprecipitation Assay. A co-immunoprecipitation assay was performed using a protein A/G PLUS-Agarose Kit (SANTA CRUZ, USA) according to the manufacturer's protocol. Briefly, cells treated with ethanol and ginsenoside Rc were lysed in RIPA lysis buffer containing a protease inhibitor (Beyotime Co, Beijing, China). Then, lysates were immunoprecipitated (IP) with anti-SIRT6 (Proteintch Co, USA) and anti-NRF2 (Proteintch Co, USA) antibodies or the mouse/rabbit lgG (Beyotime Co, Beijing, China). The total lysates or corresponding IP samples were immunoblotted with anti-SIRT6 and anti-NRF2.

For acetylation assays, ginsenoside Rc treated cells were exposed to anhydrous ethanol (100 mM) treatment for 24 h. Cells were lysed in lysis buffer containing a protease inhibitor and acetylatase inhibitor (Beyotime Co, Beijing, China). Anti-NRF2 antibody was used in IP endogenous NRF2 in protein samples. The acetylation levels of NRF2 were detected with acetyl-lysine antibodies (Abclonal Co, Wuhan, China) using Western blotting.

Quantitative PCR (qPCR). Total mRNA of liver tissues or MPHs was extracted with a TRIzol reagent. Revese transcription was performed using a high-capacity cDNA reverse-transcription kit (Applied Biological Materials Inc., Vancouver, Canada). cDNA was subjected to qPCR analysis with the PowerUp SYBRTM Green Master Mix (Abclonal Co, Wuhan, China). All gene expression was standardized with β-actin, and specific primer sequences are shown in Supplementary Table 1.

Statistical Analysis. The data were analyzed using GraphPad Prism (Version 8.0) and presented as means ± SEM. Statistical analysis was performed using the one-way analysis of variance followed by a post hoc Tukey test for comparisons. The value of $P < 0.05$ was considered statistical significance.

RESULTS

Ginsenoside Rc Suppressed Alcohol-Induced Hepatocellular Injury by Improving Oxidative Stress, Inflammation, and Lipid Deposition in MPHs. To start with, alcohol (100 mM)-incubated MPHs served to test the effects of ginsenoside Rc on alcohol-induced hepatic damage. Interestingly, ginsenoside Rc displayed a lower cytotoxicity (Figure 1A). Furthermore, ginsenoside Rc dose-dependently alleviated alcohol-induced hepatocytes' damage, with a maximum effect at 50 μM (h; Figure 1B): hence this concentration was used for the following *in vitro* study. Concurrently, ginsenoside Rc treatment significantly reversed alcohol-induced liver damage, as revealed by decreases in the levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the medium of MPHs Figure 1C,D). Consistently, the ginsenoside Rc treatment remarkably reduced the oxidative stress brought on by alcohol incubation in MPHs, as evidenced by a dose-dependent decrease in ROS generation (Figure 1E). At the same time, the treatment with ginsenoside Rc increased the expression of genes encoding for antioxidant proteins, including SOD2, NRF2, and HO-1 (Figure 1F). Moreover, ginsenoside Rc administration increased ARE-luc promoter luciferase activity and the nuclear level of NRF2—the latter result indicating the transcriptional activation of NRF2 (Figure 1G,M).

Alcohol metabolism generates ROS and consumes antioxidant substances, resulting in a large number of triglycerides (TG), whose fast accumulation causes hepatic steatosis.[41−43] Interestingly, ginsenoside Rc treatment dose-dependently reduced the intracellular lipid deposition levels in alcohol-incubated MPHs, as shown by decreases in TG levels and lipid TOX-staining (Figure 1H,I). Moreover, ginsenoside Rc treatment down-regulated the expression of genes related to lipogenesis, while the nuclear protein PPAR-α, which is connected to fatty acid oxidation, was up-regulated, implying that ginsenoside Rc could inhibit alcohol-driven lipid accumulation (Figure 1J,M).

In addition, by means of a dual luciferase assay, we also observed that ginsenoside Rc exerts a strong inhibitory effect on NF-κB-luc activity (Figure 1K). Ginsenoside Rc treatment also significantly decreased the expression of genes involved in inflammation, such as NF-κB, TNF-α, and IL-1β (Figure S1A). Furthermore, ginsenoside Rc administration significantly reduced total and nuclear NF-κB levels in alcohol-treated MPHs (Figure 1L,M).

Accumulating studies proved that alcohol metabolism produces a large amount of ROS, which are harmful to the liver. In addition, acetaldehyde, a product of alcohol metabolism under the action of Cyp2e1, induces intense toxic damage of liver tissue.[44−46] Therefore, we tested the effects of ginsenoside Rc on alcohol metabolism. Our results showed that ginsenoside Rc treatment significantly decreased the levels of Cyp2e1 in alcohol-exposed MPHs, while increasing the levels of ALDH1A1 and ALDH1B1 (Aldehyde Dehydrogenase 1 Family Members A1 and B1), both alcohol detoxifying enzymes that accelerate the catabolism of acetaldehyde to acetic acid (Figures 1N and S1B).

Overall, these data indicated that ginsenoside Rc alleviates alcohol-induced hepatocytes' injury by reducing oxidative stress, lipid deposition, and cytotoxicity in MPHs.

By Regulating Oxidative Stress Ginsenoside Rc Alleviated Hepatic Injury in ALD Mice. Given the

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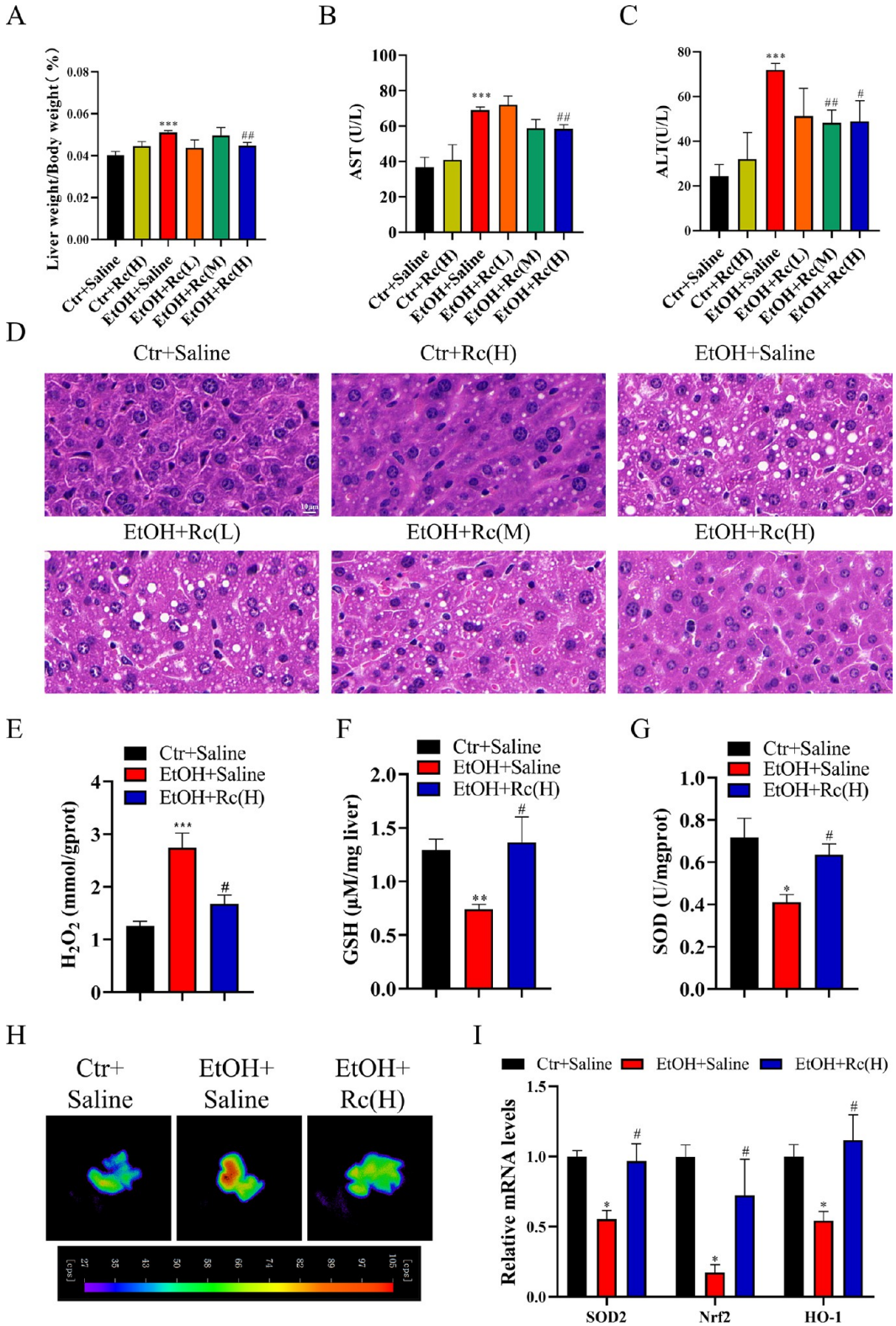

Figure 2. By regulating oxidative stress, ginsenoside Rc alleviated hepatic injury in ALD mice. (A) The liver weight/body weight ratio values; (B, C) serum ALT and AST levels; (D) H&E staining of liver tissue sections (1000×); (E) hepatic H_2O_2 measurements; (F) hepatic GSH measurements; (G) hepatic SOD measurements; (H) liver ROS levels; (I) expression of mRNA levels of genes related to oxidative stress. Data are means ± SEM; n = 5−8/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the Ctr+saline group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the EtOH+saline group.

effectiveness of ginsenoside Rc against oxidative stress in MPHs, we assumed that it might improve alcohol-feeding-induced ALD in mice. Thus, different daily doses of ginsenoside Rc were injected into mice for 7 days. The ginsenoside Rc treatment dose-dependently (5 mg/kg, 10 mg/kg, 20 mg/kg) reduced the liver weight/body weight ratio

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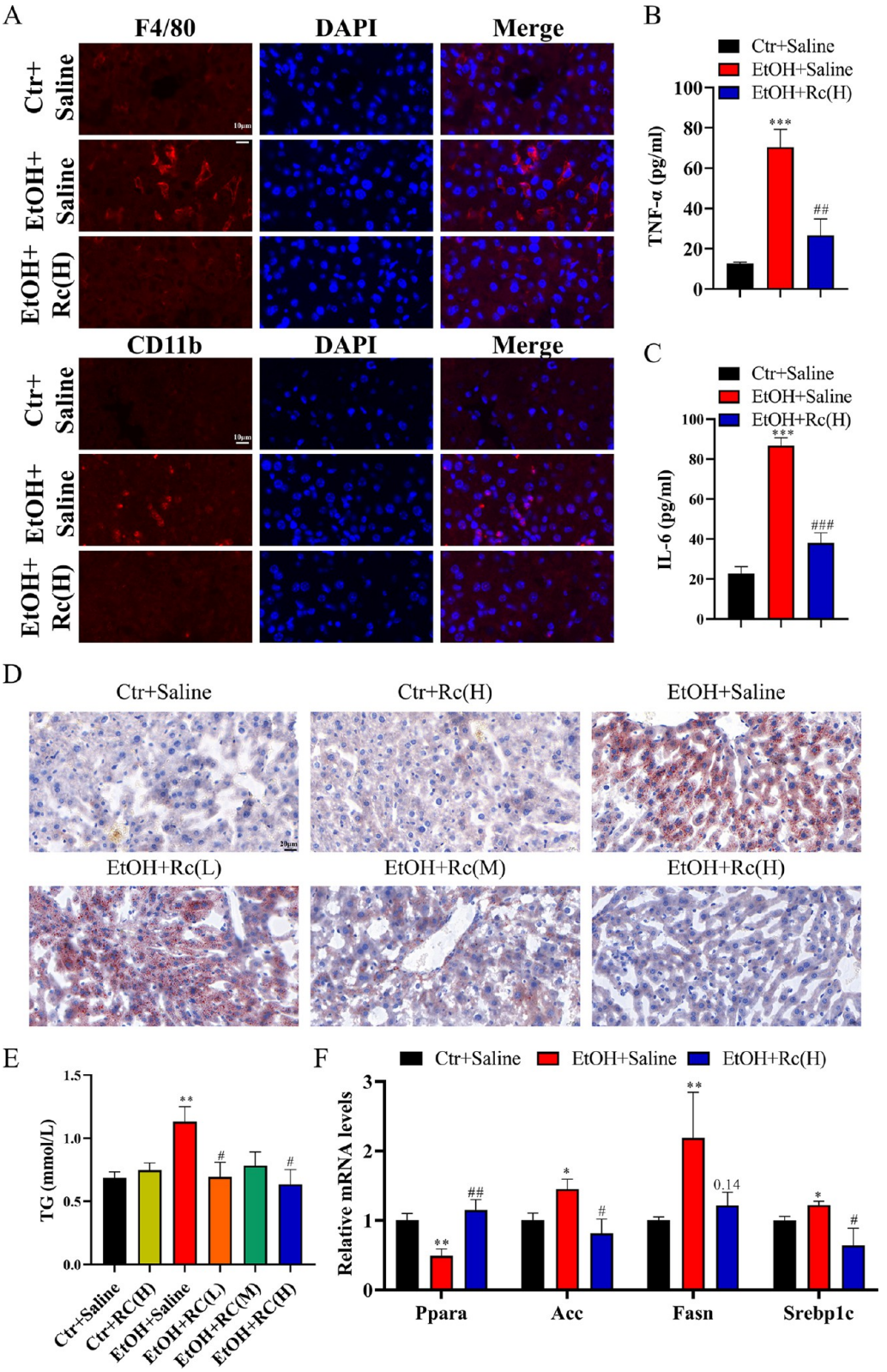

Figure 3. Ginsenoside Rc mitigated inflammation and hepatic steatosis in ALD mice. (A) Immunofluorescence analysis of F4/80 and CD11b (1000×); (B, C) serum ELISA levels of TNF-α and IL-6; (D) Oil Red O staining of liver tissue sections (400×); (E) serum TG levels; (F) expression of mRNA levels of hepatic genes involved in lipid metabolism. Data are means ± SEM; n = 5−8/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the Ctr+saline group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the EtOH+saline group.

values in ALD mice (Figure 2A). Moreover, biochemical analyses also showed a dose-dependent decrease in serum AST and ALT levels, and the levels of AKP in liver were also decreased with the ginsenoside Rc treatment (Figures 2B,C and S2A). Furthermore, as revealed by H&E staining, ginsenoside Rc (20 mg/kg) treatment markedly lessened the

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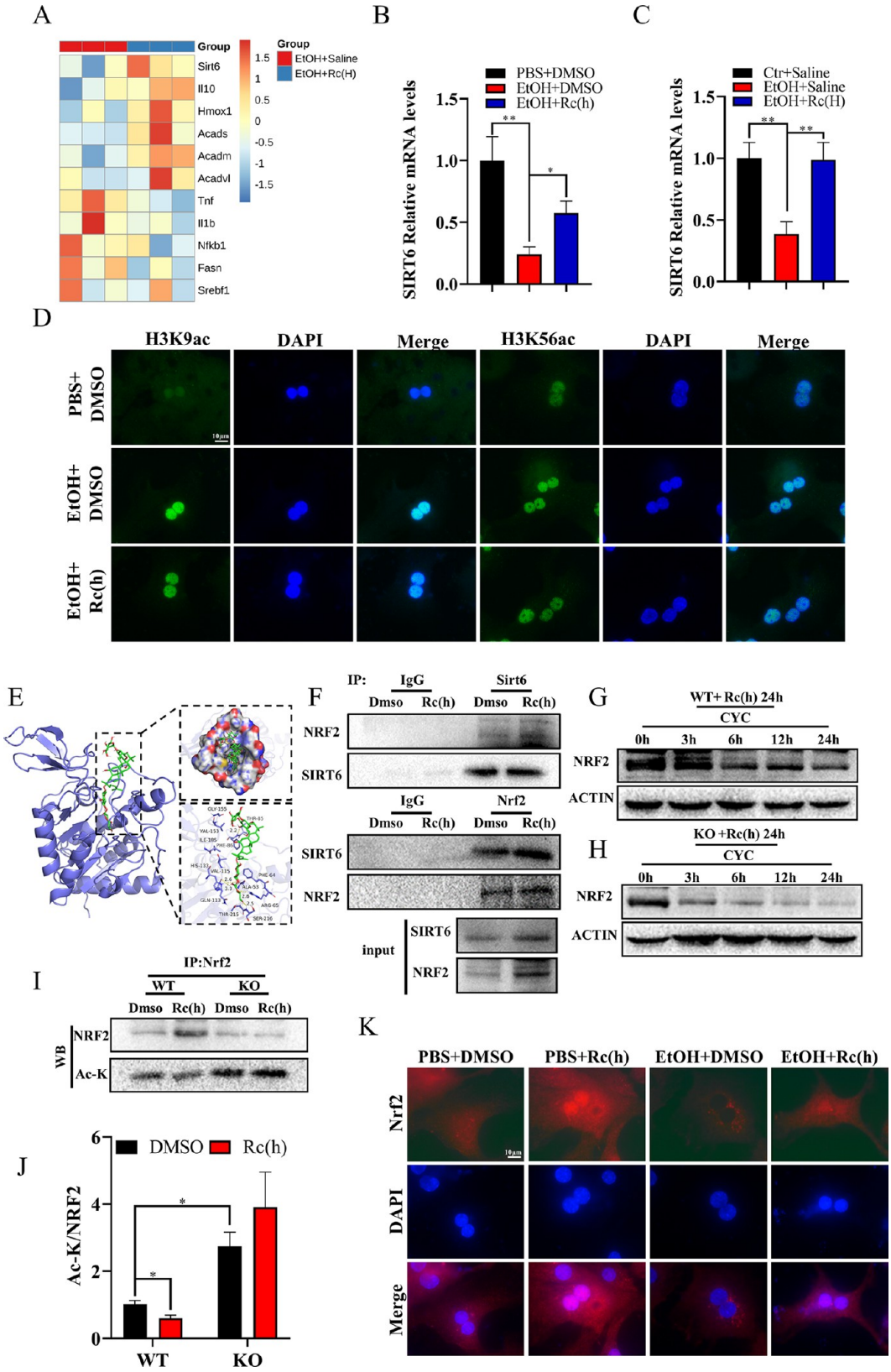

Figure 4. SIRT6 is the ginsenoside Rc's target as its beneficial antioxidant effects were related to NRF2 deacetylation by SIRT6. (A) Heatmap of SIRT6 and other genes involved in hepatic lipid metabolism from RNA-seq analysis; (B, C) SIRT6 expression *in vitro* (B) and *in vivo* (C); (D) immunofluorescent analysis of SIRT6 enzymatic activity (1000×); (E) the molecular docking model of ginsenoside Rc and SIRT6; (F) CO-IP assay of SIRT6 and NRF2; (G, H) NRF2 protein expression by MPHs isolated from livers of WT (G) and KO (H) mice; (I, J) the acetylation of NRF2; (K) immunofluorescent analysis of NRF2 (1000×). Data are means ± SEM; in J, $n = 3$; $n = 4–8$ in the other groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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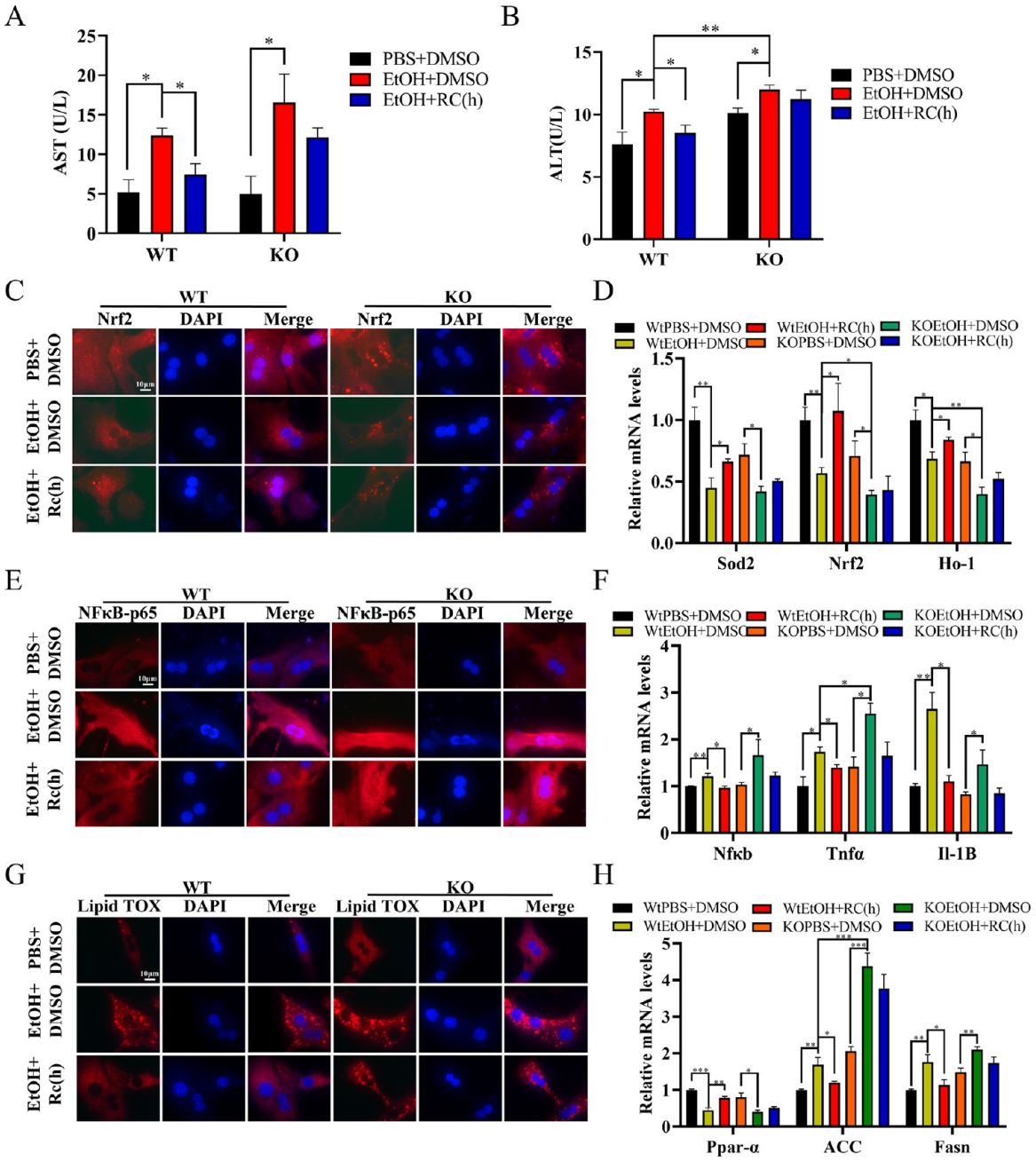

Figure 5. The protective effects of ginsenoside Rc were decreased in SIRT6-deficient MPHs. (A, B) Hepatic ALT and AST levels; (C) immunofluorescent analysis of NRF2 (1000×); (D) relative mRNA expression levels of genes related to oxidative stress; (E) immunofluorescent analysis of NF-κB-p65 (1000×); (F) relative mRNA expression levels of genes related to inflammation; (G) lipid TOX-stained MPHs (1000×); (H) relative mRNA expression levels of genes related to lipogenesis in MPHs. Data are means ± SEM; n = 4−6/group. *P < 0.05, **P < 0.01, ***P < 0.001.

alcohol-induced liver injury and lipid deposition (Figure 1D). Overall, ginsenoside Rc exerted a pronounced hepatoprotective effect against alcohol-induced hepatotoxicity.

Usually, the levels of ROS, H_2O_2, and MDA reflect the state of oxidative stress in the organism,[47,48] and antioxidant indicators, such as GSH and SOD, are often used to assess actual antioxidant levels.[48−50] Considering that a dose of 20 mg/kg of ginsenoside Rc (H) was effectively hepatoprotective against alcohol-induced liver injury in mice, we used this same dose for our *in vivo* study. As expected, after administering ginsenoside Rc, the levels of H_2O_2 and MDA significantly fell, whereas liver GSH and SOD levels increased (Figures 2 E−G and S2B). Concurrently, ginsenoside Rc also reduced the alcohol-induced hepatic ROS overproduction (Figure 2H). We also found that ginsenoside Rc also significantly up-regulated genes, such as SOD2, NRF2, and HO-1, involved in antioxidant activities (Figure 2I).

Ginsenoside Rc Mitigated Hepatic Inflammation and Steatosis in ALD Mice. Furthermore, we validated the ability of ginsenoside Rc to alleviate alcohol-induced hepatic inflammation and steatosis in mice. Immunofluorescence analyses showed that ginsenoside Rc reduced the markers F4/80 and CD11b proper of inflammatory monocytes in liver tissue. It also lessened the serum release of the pro-

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inflammatory cytokines IL-1β, IL-6, and TNF-α in ALD mice (Figures 3A−C and S2B). Moreover, ginsenoside Rc treatment down-regulated the inflammation-related genes NF-κB, IL-1β, IL-6, and TNF-α (Figure S2C,D).

Interestingly, ginsenoside Rc administration also dose-dependently and markedly decreased the alcohol-induced lipid accumulation in the liver, as revealed by Oil Red O staining and by the diminished hepatic TG contents (Figure 3D,E). We also found that ginsenoside Rc treatment effectively activated the genes related to lipid β-oxidation while repressing the fatty acid synthase (FAS) and acetyl CoA-carboxylase (ACC) genes. Moreover, ginsenoside Rc also down-regulated genes related to lipogenesis (Figure 3F). In addition, ginsenoside Rc regulated alcohol metabolism-related genes too in ALD mice (Figure S2F). Overall, these data suggested that under alcohol exposure ginsenoside Rc powerfully regulates oxidative stress and mitigates hepatic inflammation and steatosis.

Ginsenoside Rc Targeted SIRT6 As Its Beneficial Antioxidant Effects Required NRF2 Deacetylation by SIRT6. To further explore the mechanisms underlying ginsenoside Rc's therapeutic effects, we analyzed RNaseq data of liver samples from ALD mice treated with ginsenoside Rc or saline. Consistent with the above data, ginsenoside Rc administration to ALD mice significantly upregulated genes involved in antioxidation activity, such as HO-1, and genes related to β-oxidation, such as ACADV1 and ACADM, while downregulating genes involved in lipogenesis and inflammation. Along with these changes, a significant upregulation of SIRT6 occurred in ginsenoside Rc-treated mice (Figure 4 A). qPCR analyses further confirmed the increased expression of SIRT6 *in vivo* and *in vitro* (Figure 4B,C). SIRT6 normally functions as a transcriptional repressor by deacetylating the H3K9ac and H3K56ac on H3 histone bound to gene promoters.[51,52] Therefore, we tested the levels of H3K9ac and H3K56ac in alcohol-exposed cultured MPHs. Indeed, ginsenoside Rc increased the deacetylase activity of SIRT6, thereby reducing the levels of H3K9ac and H3K56ac (Figure 4D). Moreover, further docking analysis indicated a good binding affinity of ginsenoside Rc and Sirt6 via hydrophobic interaction (Figure 4E).

The NRF2/HO-1 pathway plays a critical role in counteracting oxidative stress. Under ROS influence, NRF2 is released from this complex and translocates from the cytoplasm into the nucleus, where it activates the HO-1 gene, thus playing an antioxidant role.[23,24] Previous studies also reported that the SIRT6/NRF2 complex plays critical antioxidant roles.[53] Thus, we tested the interaction between SIRT6 and NRF2 under ginsenoside Rc treatment. Indeed, NRF2 and SIRT6 exhibited a direct physical interaction under ginsenoside Rc administration (Figure 4 F). In the presence of a protein synthesis inhibitor (CYC-116), ginsenoside Rc treatment slowed NRF2's degradation in SIRT6$^{fl/fl}$ (WT) MPHs, an effect that could not be observed in SIRT6$^{alb-/-}$ (KO) MPHs (Figure 4G,H). Such results suggested a key role of ginsenoside Rc-activated SIRT6 in the regulation of NRF2. At the same time, we found that ginsenoside Rc led to the deacetylation of NRF2 in WT MPHs, but this did not happen in KO MPHs (Figure 4I,J). Moreover, immunofluorescence analysis results showed that ginsenoside Rc increased the translocation of NRF2 from the cytoplasm into the nucleus (Figure 4K). Altogether, we found that ginsenoside Rc could activate the SIRT6/NRF2 pathway, and this could be ginsenoside Rc's key mechanism of action in ALD.

Ginsenoside Rc's Hepatoprotective Effects Are Abolished in SIRT6-Deficient MPHs. To further confirm the role of SIRT6 as a modulator of ginsenoside Rc-induced therapeutic effects, MPHs isolated from SIRT6$^{alb/-}$ mice (KO) and SIRT6$^{fl/fl}$ mice (WT) were cultured in an alcohol-containing medium. Interestingly, as compared with WT MPHs, SIRT6 deficiency abrogated the ginsenoside Rc-induced beneficial effects on hepatocellular damage, as shown by the heightened ALT and AST levels in KO MPH medium (Figure 5A,B). Moreover, ginsenoside Rc failed to reduce ROS levels in SIRT6-deficient (KO) MPHs (Figure S3A). Immunofluorescence results and decreased nuclear NRF2 levels also showed that the increased translocation of NRF2 from cytoplasm into the nucleus driven by ginsenoside Rc was impaired by SIRT6 deficiency (Figures 5C and S3D). Concurrently, no changes took place in the genes related to antioxidant activities, even under the treatment with ginsenoside Rc, in KO MPHs (Figure 5D). In addition, SIRT6 deficiency abolished the ginsenoside Rc-induced mitigation of inflammation (Figure 5E,F). Moreover, ginsenoside Rc failed to reduce the alcohol-elicited hepatic lipid deposition in SIRT6-deficient MPHs (Figures 5G and S3B). Also, ginsenoside Rc treatment failed to alter the expression of the hepatic genes involved in β-oxidation and lipogenesis in KO MPHs (Figures 5H and S3C). Finally, the nuclear levels of PPAR-α did not increase under ginsenoside Rc treatment (Figure S3D). Collectively, these data suggested that a SIRT6-dependent mechanism meditates ginsenoside Rc's protective effects in alcohol-induced cellular toxicity in MPHs.

Ginsenoside Rc Failed to Alter the Alcohol-Induced ROS Burden in Sirt6-Deficient Mice. Consistent with ginsenoside Rc's abolished effects on alcohol-induced oxidative stress in KO MPHs, ginsenoside Rc treatment also failed to alter the liver weight/body weight ratio values in SIRT6-deficient mice (SIRT6$^{alb-/-}$ mice) fed an alcohol-added diet (Figure 6A). Also, in the presence of a hepatic SIRT6 deficiency, ginsenoside Rc did not reduce the increased liver AST, ALT, and AKP levels (Figures 6B,C and S4A). At the same time, no changes in alcohol-induced liver injury and lipid deposition occurred after ginsenoside Rc treatment in SIRT6$^{alb-/-}$ mice, as illustrated by H&E staining (Figure 6D). Moreover, hepatic antioxidants', such as SOD and GSH, levels were not changed by ginsenoside Rc in hepatic SIRT6 deficient mice (Figure 6E,F). We also found that H_2O_2, ROS, and MDA levels were unchanged in ginsenoside Rc-treated SIRT6$^{alb-/-}$ mice (Figures 6G,H and S4B), and there occurred also no change in the expression of genes, such as SOD2 and NRF2, associated with antioxidant activity (Figure 6I,J). Overall, these data supported the view that ginsenoside Rc's mitigation of alcohol-induced oxidative stress requires the activation of SIRT6 in mice.

Liver SIRT6 Deficiency Lessens Ginsenoside Rc's Protective Effects against Alcohol-Induced Hepatic Inflammation and Steatosis. As ginsenoside Rc did reduce via SIRT6 lipid accumulation and inflammation in alcohol-exposed MPHs, we further studied its effects *in vivo*. The immunofluorescence analysis of F4/80 and CD11b markers showed that the ginsenoside Rc-driven improvement of hepatic inflammation was abolished in SIRT6$^{alb-/-}$ mice (Figures 7A and S4E). Ginsenoside Rc administration did not alter the serum release of the pro-inflammatory cytokines IL-1β, IL-6,

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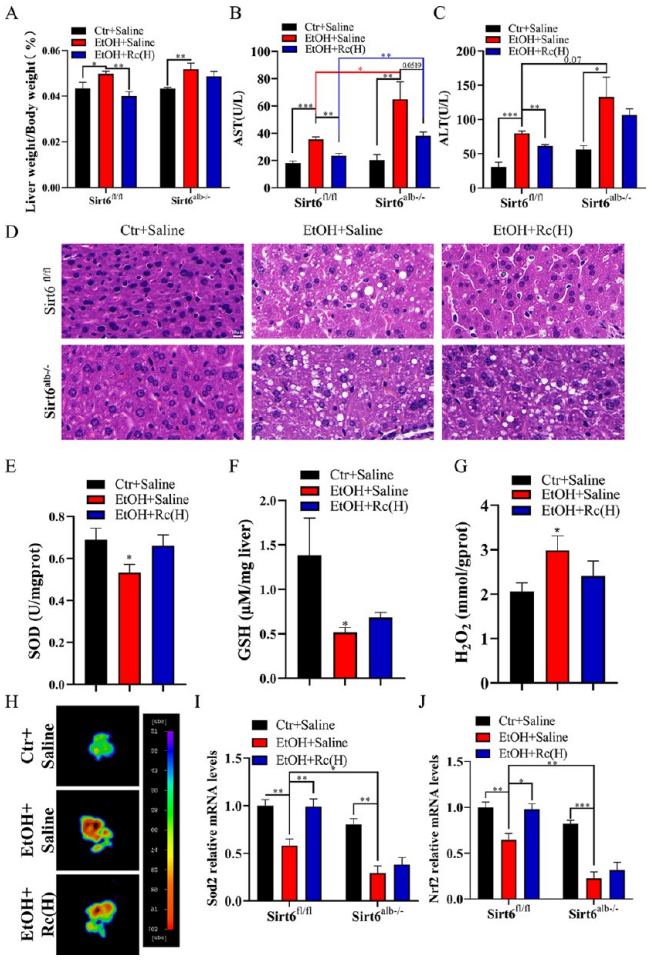

Figure 6. Ginsenoside Rc failed to alter the ethanol-induced ROS burden in SIRT6-deficient mice. (A) Liver weight/body weight ratio values; (B, C) serum ALT and AST levels; (D) H&E staining of liver tissue sections (1000×); (E) hepatic SOD measurements; (F) hepatic GSH measurements; (G) hepatic H_2O_2 measurements; (H) ROS levels in livers; (I, J) mRNA expression levels of oxidative stress-related genes. Data are means ± SEM; n = 5−8/group. *P < 0.05, **P < 0.01, ***P < 0.001.

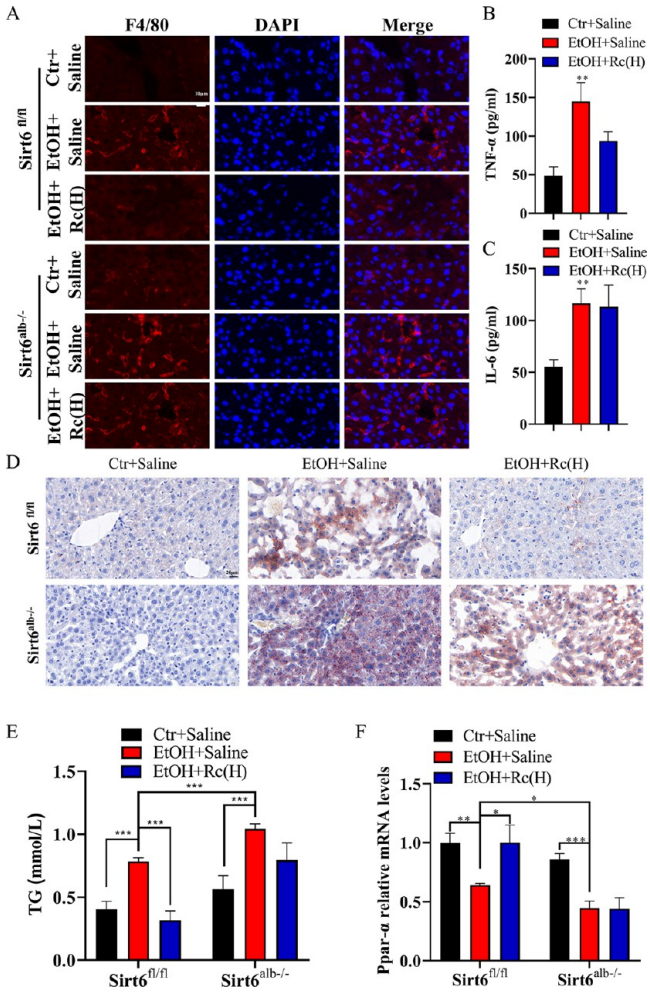

Figure 7. Hepatic SIRT6 deficiency diminished the protective effects of ginsenoside Rc against ethanol-induced hepatic inflammation and steatosis. (A) Immunofluorescence analysis of F4/80 (1000×); (B, C) serum ELISA levels of TNF-α, and IL-6; (D) Oil Red O staining of liver tissue sections (400×); (E) serum TG levels; (F) mRNA expression levels of hepatic gene involved in lipid metabolism. Data are means ± SEM; n = 5−8/group. *P < 0.05, **P < 0.01, ***P < 0.001.

and TNF-α, and the expression of NF-κB in SIRT6$^{alb-/-}$ mice (Figures 7B,C and S4C,D). Moreover, as Oil Red O staining showed, ginsenoside Rc treatment did not prevent lipid deposition in the livers of SIRT6$^{alb-/-}$ mice (Figure 7D). Concurrently, ginsenoside Rc did not change serum TG contents in hepatic SIRT6-deficient mice (Figure 7E). In the same animals, ginsenoside Rc also failed to regulate the genes involved in fatty acid oxidation (Figure 7F). Overall, these data suggested that ginsenoside Rc improved alcohol-induced hepatic inflammation and steatosis via SIRT6 activation in mice.

Via SIRT6 Activation Ginsenoside Rc Alleviated Acute Alcohol-Induced Liver Damage. Next, we examined the therapeutic effects of ginsenoside Rc on the liver injury induced by an acute alcohol intake. Ginsenoside Rc treatment significantly reversed the ethanol-induced increases in AST and ALT levels in SIRT6$^{fl/fl}$ mice, but exerted no beneficial effect in SIRT6$^{alb-/-}$ mice (Figure 8A,B). Concurrently, ginsenoside Rc altered serum TG contents but only in the presence of SIRT6 activity (Figure 8C). However, no change in Oil Red O staining occurred (Figure S5A). Because acute alcohol intake induced a small amount of lipid deposition in SIRT6$^{alb-/-}$ mice, ginsenoside Rc treatment marginally decreased lipid deposition under this condition (Figure 8D). Concurrently, we also found that ginsenoside Rc's alleviation of inflammation also depended upon SIRT6, as shown by the decreases in F4/80 and CD11b (Figures 8E and S5B). Furthermore, the treatment with ginsenoside Rc also reduced the hepatic ROS overproduction induced by an acute alcohol intake—an effect abolished in SIRT6$^{alb-/-}$ mice (Figure 8F). Taken together, these data suggested that ginsenoside Rc attenuated acute alcohol-induced inflammation and oxidative stress in a SIRT6-dependent fashion.

DISCUSSION

In the present study, we showed that ginsenoside Rc attenuated alcohol-induced hepatic oxidative stress, inflammation, and lipid accumulation by up-regulating the SIRT6/NRF2 pathway. Our study started when we found that ginsenoside Rc exerts protective effects against liver function damage in MPH models treated with alcohol. By using as

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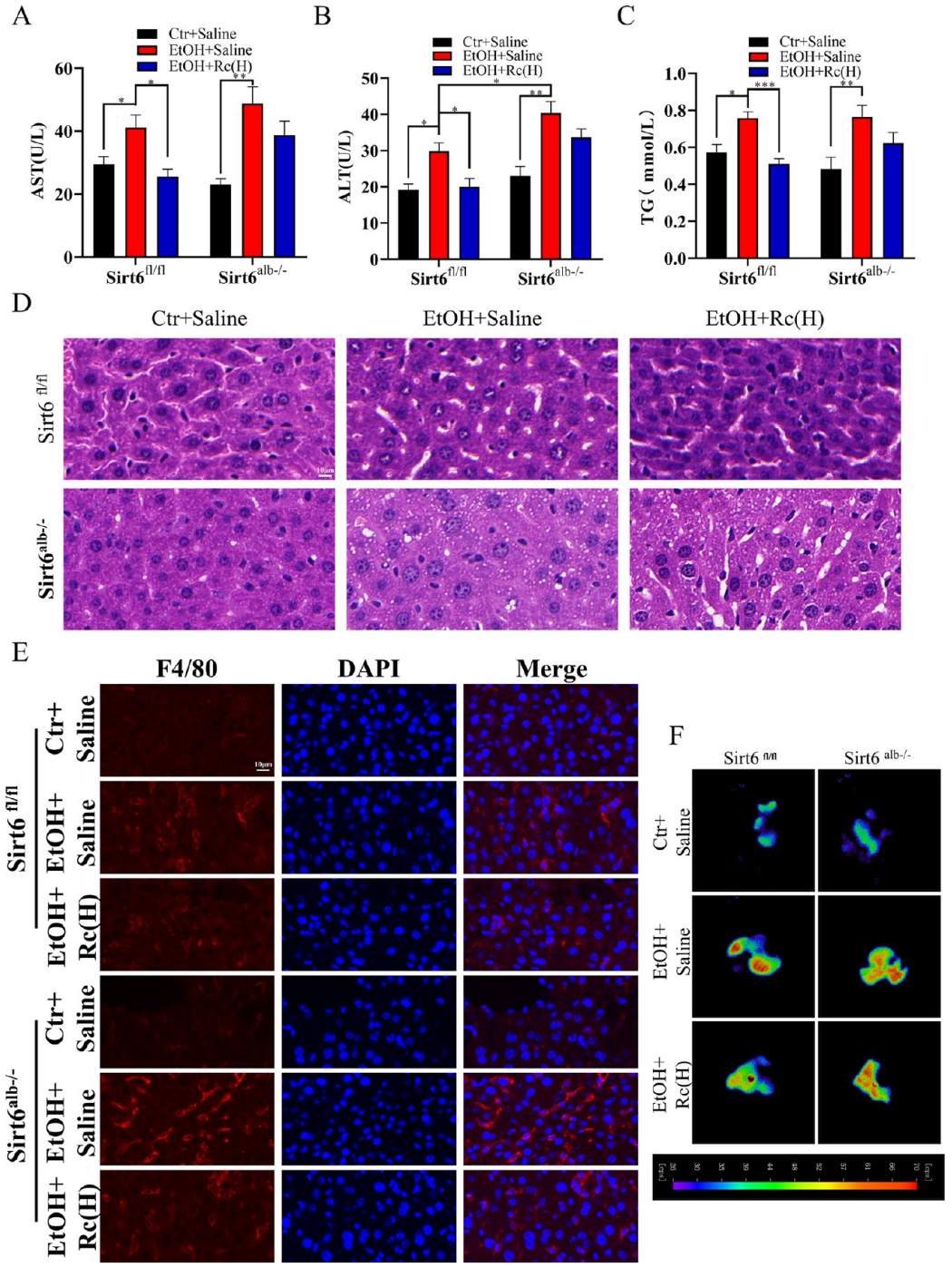

Figure 8. Ginsenoside Rc alleviation of acute alcohol-induced hepatic damage depends on SIRT6. (A−C) Serum AST, ALT, and TG levels; (D) H&E staining of liver tissue sections (1000×); (E) immunofluorescence analysis of F4/80 (1000×); (F) ROS levels in livers. Data are means ± SEM; n = 5−8/group. *P < 0.05, **P < 0.01, ***P < 0.001.

models C57BL/6J mice and hepatocyte-specific SIRT6 deletion we confirmed that ginsenoside Rc improved the alcohol-induced liver damage. We also showed that this protective function of ginsenoside Rc is absent in the mice with hepatocyte-specific SIRT6 deficiency. Mechanistically, ginsenoside Rc increases the physical interaction between NRF2 and SIRT6 and promotes NRF2 deacetylation and stabilization by SIRT6, thus boosting NRF2 transcriptional activity. Ultimately, by these effects, ginsenoside Rc heightens through the SIRT6/NRF2 pathway the liver tissue antioxidative capabilities. Therefore, these results are the proof of concept that ginsenoside Rc may be a promising therapeutic drug for ALD.

Chronic and excessive alcohol consumption can lead to ALD, which has become a global health concern.[54,55] Currently, behavioral interventions and nutritional support are ALD's most fashionable treatments. In addition, many drugs with antioxidant properties, such as liposoluble vitamins, and synthetic antioxidants are used to treat ALD.[24] Consequently, many scientists agree that inhibiting the oxidative stress is particularly important to managing ALD. Therefore, the search for suitable natural antioxidants reducing

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the levels of oxidative stress in hepatocytes and the liver damage brought about by alcohol's metabolism is an important endeavor to prevent and treat ALD. As ample evidence suggests, ginsenoside Rc is a therapeutic agent since it protects against liver function damage through its relevant antioxidant, anti-inflammatory, and fat accumulation inhibitory effects.[32,40] However, we must realize that our knowledge of the mechanisms that ginsenoside Rc operates in ALD is still inadequate.

It is well-known that alcohol-induced oxidative stress plays a key role in the pathogenesis of ALD. The liver damage due to oxidative stress is mainly brought about by the ROS-mediated oxidative damage, immune response, hepatocyte steatosis, mitochondrial structural dysfunction, and liver tissue inflammation.[12,41,42,50,56,57] After drinking alcohol, the alcohol dehydrogenase (ADH) and microsomal ethanol oxidizing system (MEOS; made by the cytochrome P450 enzymes) first oxidize ethanol to acetaldehyde, which is next further oxidized to acetate by ALDH.[44,58] Along this process, ROS are produced by the metabolism of alcohol to acetaldehyde and acetate as well as by the associated processes involving the reduction of $NAD^+/NADP^+$ to NADPH/NADPH.[24,59] At the same time, antioxidant agents, such as SOD and GSH, hinder the accumulation of ROS.[49] In this work, the treatment with ginsenoside Rc notably reduced hepatic MDA and H_2O_2 levels, while it increased SOD and GSH levels, thus lessening hepatic ROS generation in ALD mice. Our results are consistent with reports from Kim et al. and Yang et al. showing that ginsenoside Rc exerted powerful antioxidant, anti-inflammatory, and lipid-lowering effects.[32,40] However, we maintain that the knowledge about the operative mechanisms of ginsenoside Rc in ALD is still inadequate.

While the body metabolizes alcohol, some antioxidant pathways are activated to interfere with the alcohol-induced oxidative stress. The NRF2-mediated pathway is a key one of them.[60] After NRF2's transcription, the downstream antioxidant enzyme NADPH-quinone oxidoreductase 1 (NQO1) and the HO-1 gene are activated to suppress hepatic oxidative stress.[61,62] In the present study, we found that ginsenoside Rc treatment increased the mRNA expression of NRF2 and of its target genes HO-1 and SOD2. Moreover, ginsenoside Rc also promoted the translocation of NRF2 from the cytoplasm into the nucleus to exert its antioxidant effects. However, to our knowledge, the mechanisms through which ginsenoside Rc activates NRF2 signaling and plays an important antioxidant role in ALD had remained unknown. Intriguingly, a recent paper showed that the protein−protein interaction between NRF2 and SIRT6 conferred antioxidant activity in APAP-induced hepatotoxicity.[53] From this observation it might be inferred that ginsenoside Rc's protection against ALD might at least in part depend on its regulation of the SIRT6/NRF2 pathway. As we expected, Co-IP assays showed a direct physical interaction between NRF2 and SIRT6 in the presence of ginsenoside Rc. This interaction enhanced NRF2's stability and reinforced ginsenoside Rc's antioxidant activity. In contrast, a missing interaction between NRF2 and SIRT6 due to the deletion of SIRT6 in the hepatocytes reduced the induction of NRF2 expression to negligible levels even in the presence of ginsenoside Rc and diminished the latter's ability to inhibit or alleviate the alcohol-induced oxidant, pro-inflammatory, and lipid accumulating effects. These results suggested that through SIRT6's regulation ginsenoside Rc could inhibit ALD progression. Various studies also pointed out that SIRT6 is a histone deacetylase that normally functions as a transcriptional regulator by deacetylating H3K9ac and H3K56ac.[35,51] In fact, it has been proved that NRF2's deacetylation level is important for its transcriptional activity.[63] To address this topic, we found that ginsenoside Rc directly deacetylates NRF2 by activating SIRT6. Subsequently, it elevates NRF2 expression, thus reinforcing the latter's transcriptional activity, and finally exerts antioxidant effects. Conversely, in SIRT6-deficient MPHs, this mechanism did not operate because NRF2's degradation was rather fast despite the treatment with ginsenoside Rc. Taken together, the hepatoprotective mechanisms that ginsenoside Rc operates are tightly associated with the regulation of the SIRT6/NRF2 signaling pathway.

After intake, ethanol and its metabolite acetaldehyde alter the intestinal barrier function, promote the absorption of endotoxins, such as LPS,[64,65] and induce Kupffer cells to release ROS and an array of proinflammatory cytokines and chemokines including TNF-α, IL-1β, and IL-6.[13] The stimulation of Kupffer cells and the production of oxidative stress by alcohol metabolites could exacerbate the inflammation in ALD. As reported by Kim et al., SIRT6 plays a key role by reducing hepatic inflammation, thereby alleviating ALD. Then again, NRF2, as a stress-sensing genetic transcription factor, also plays a critical protective role against inflammation.[66,67] In this work, through the results of immunofluorescence and of qPCR analyses, we also showed that the treatment with ginsenoside Rc increased SIRT6's anti-inflammatory action.

Reports indicate that under the action of hepatic drug-metabolizing enzymes, alcohol induces the generation of free radicals, which consume the antioxidants present in liver cells, causing the rapid accumulation of large amounts of TG in the same cells and, hence, liver steatosis.[41,42] Moreover, PPAR-α and SREBP1-c, i.e., the major genes controlling fatty acid synthesis and oxidation, regulate the hepatic lipid metabolism in ALD.[14,15] Interestingly, SIRT6 improves glucose and lipid metabolism by regulating PPAR-α and SREBP1-c.[40,68] After observing H&E- and Oil Red O-stained slides and measuring the expression of β-oxidation- and lipid synthesis-related genes, we found that ginsenoside Rc reversed alcohol-induced hepatic steatosis. In keeping with our findings, the ginsenoside Rc-induced mitigation of lipid accumulation was abolished by SIRT6's deficiency.

Additionally, ginsenoside Rc acted as a therapeutic agent also in a mouse model of acute alcoholic liver injury by inhibiting inflammation and oxidative reactions. Conversely, its effects on acute alcohol-elicited hepatic lipids accumulation were not so obvious. This might be related to the acute alcohol intake-induced production of ROS, which could activate autophagy and prevent lipid accumulation in the early stages of ALD.[56] At the same time, the therapeutic effects of ginsenoside Rc were remarkably attenuated in SIRT6 knockout mice. In summary, ginsenoside Rc exerts certain therapeutic effects on both acute and chronic alcoholic liver injuries.

A growing body of research showed that ginsenoside Rc plays an important role in the treatment of many diseases; however, it has not yet been applied to the clinical treatment of diseases in any way. Although we have verified that ginsenoside Rc could modulate SIRT6-NRF2 interaction to alleviate alcoholic liver disease first, our experiments lacked validation of clinical case samples and was just verified on mice and cells. Moreover, intraperitoneal injection is conducive to ginsenoside

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Rc accumulation in the liver, but it is worth further studying whether the absorption of alcohol in the intestine can be inhibited by intragastric administration of ginsenoside Rc. Huang et al. reported that ginsenoside Rc could promote energy metabolism in cardiomyocytes and neurons by activating SIRT1, and whether ginsenoside Rc can regulate SIRT1 to alleviate alcoholic liver disease deserves further study.

In conclusion, we show that ginsenoside Rc prevented liver injury and hepatic oxidative stress and alleviated inflammation and lipid accumulation induced by alcohol metabolism in chronically alcohol-treated mice and MPHs. What is more, we found that by activating SIRT6, ginsenoside Rc could directly deacetylate NRF2, afterward elevating NRF2 expression and reinforcing the latter's transcriptional activity, thereby exerting antioxidant effects and alleviating ALD in mice. Moreover, the treatment with ginsenoside Rc also improved acute alcohol injury by inhibiting oxidative stress and inflammatory damage in a SIRT6-dependent manner. Therefore, our studies confirm the view that ginsenoside Rc is a promising compound to treat or relieve ALD.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.2c06146.

Supplementary figures and tables (PDF)

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○Z.P., K.T., and J.G. contributed equally to this work. Y.G., C.S., X.Y., and Y.H.Z. contributed to the conception of the work. Z.P., K.T., J.G., Y.L.C., X.G., X.X., S.D., T.C., and G.Z. assisted with experimental preparation and data collection. Y.J.C. and Y.D.Z. conducted animal feeding. Z.P., J.G., and Y.L.C. drafted the manuscript. Y.G., C.S., X.W., and X.Y. revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Notes

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■ ABBREVIATIONS

ALD, alcoholic liver disease; ROS, reactive oxygen species; Nrf2, nuclear factor erythroid 2-related factor 2; Ho-1, heme oxygenase 1; Sod2, superoxide dismutase 2; NQO1, NAD(P)H-quinone oxidoreductase 1; MPHs, mouse primary hepatocytes; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AKP, alkaline phosphatase; MDA, malonal-

dehyde; TG, triglycerides; GSH, glutathione; SOD, superoxide dismutase; ADH, alcohol dehydrogenase; MEOS, microsomal ethanol oxidizing system; TNFα, tumor necrosis factor alpha; Il-1β, interleukin 1 beta; Il-6, interleukin 6; Nfκb, nuclear factor kappa B; Cyp2e1, cytochrome P450 family 2 subfamily E member 1; Aldh1a1, aldehyde dehydrogenase 1 family member A1; Aldh1b1, aldehyde dehydrogenase 1 family member B1

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