

Ganoderic acid A ameliorates non-alcoholic steatohepatitis (NASH) induced by high-fat high-cholesterol diet in mice

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Abstract. Non-alcoholic steatohepatitis (NASH) is becoming a huge global health problem. Previous studies have revealed that ganoderic acids have hepatoprotective and hypocholesterolemic effects. In the present study, to evaluate the anti-NASH activity of ganoderic acid A (GAA), male 6-week-old C57BL/6J mice were divided into the following four groups, which were administered different diets: Normal diet (ND group), high-fat high-cholesterol diet (HFHC group), HFHC diet supplemented with 25 mg/kg/day (GAAL group) or 50 mg/kg/day of GAA (GAAH group). After 12 weeks of GAA treatment, histopathological results revealed that compared with that of the HFHC group, GAA significantly inhibited fat accumulation, steatosis, inflammation and fibrosis in the liver. GAA effectively reduced serum aspartate transaminase and alanine transaminase levels compared with the HFHC model. Furthermore, the endoplasmic reticulum (ER) stress-responsive proteins, including glucose-regulated protein 78, phosphorylated (p)-eukaryotic initiation factor-2 α and p-JNK, were significantly suppressed by GAA, while ERp57, p-MAPK and p-AKT were significantly increased after GAA treatment. Taken together, it was concluded that GAA could resist HFHC diet-induced NASH. In terms of its underlying mechanism, GAA could improve liver inflammation and fibrosis by inhibiting hepatic oxidative stress and the ER stress response induced by HFHC.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a disease that involves the ectopic accumulation of fat in hepatocytes, which is usually caused by simple steatosis (simple fatty liver),

insulin resistance, type 2 diabetes mellitus (T2DM) and dyslipidemia (1-3). Non-alcoholic steatohepatitis (NASH), which is fatty liver disease with inflammatory damage and/or fibrosis of hepatocytes, is a more aggressive and irreversible disease that is associated with an increased risk of end-stage liver disease [including cirrhosis and hepatocellular carcinoma (HCC)] (4). In recent years, the prevalence of NAFLD and NASH has been increasing. Estimates of the global prevalence of NAFLD ranged from 24 to 30% in 2018, and patients tend to be younger (5). As a chronic multisystem disease, NASH can also lead to numerous complications in other organs, such as T2DM, cardiovascular, chronic kidney and heart disease. However, there is no effective treatment for NASH. The only effective way to prevent NASH is moderate exercise and lifestyle improvement (6,7). Therefore, it is very urgent to find a new and effective therapeutic regimen.

At present, 'multiple-hit model theory' is widely regarded as the pathogenesis of NASH (8). The 'first hit' usually involves excessive accumulation of fat in the liver and insulin resistance. Subsequently, 'subsequent multiple hits' occur, showing an interaction of oxidative stress, endoplasmic reticulum (ER) stress, inflammatory cytokines and numerous other factors. Pro-inflammatory cytokines play a key role in the development of NASH. For example, TNF- α overexpression results in upregulation of sterol regulatory element binding protein-1, a key nuclear transcription factor in lipid metabolism (9). In pathogenesis, this cytokine also leads to lipid metabolism disorder. ER stress leads to the activation of three unfolded proteins, blocking the protein-response signaling pathways for novel protein synthesis, ER chaperone production and misfolded protein degradation (10,11). Under certain conditions, these events can lead to inflammation and even cell death, suggesting that ER stress is closely associated with inflammation and lipid metabolism disorders (10,11).

The active natural products extracted from herbs are one of the vital sources for anti-metabolic disease drug development. *Ganoderma lucidum* (*G. lucidum*) is one of the momentous Asian fungi known as Ling Zhi in China and Korea, and Reishi mushroom in Japan (12). *G. lucidum* is not only used in traditional medicine to improve health and promote longevity, but also potentially treats a variety of diseases, such as tumors, HIV, hypoglycemia, sedation and myocardial ischemia; it also participates in the regulation of blood lipids

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and liver protection (13,14). It was also identified that the powdered mycelium of *G. lucidum* caused plasma cholesterol to decrease in rats (15). Several oxygenated lanostane-type triterpenoids isolated from *G. lucidum* have been shown to inhibit β -hydroxy β -methylglutaryl-CoA (HMG-CoA) reductase activity *in vitro* (15-17). For instance, ganoderiol F and ganodermic acid Q demonstrated inhibitory activity against HMG Co-A reductase and against acyl-CoA acyltransferase (18,19). Although ganoderic acid A (GAA) has been revealed to improve metabolic syndromes such as obesity, hyperlipidemia and insulin resistance induced by high-fat diet, its anti-NASH effect has not been reported to date. In addition, its anti-NASH mechanism is not clear. Therefore, further elucidation of the anti-NASH effect of GAA and its underlying molecular mechanisms is highly warranted. Consequently, the present study was conducted to investigate whether GAA is able to alleviate the development of NASH in high fat-high cholesterol (HFHC)-fed mice with experimentally induced NASH and which are the underlying molecular mechanisms of the effects of GAA in the aforementioned model.

Materials and methods

Animals and treatment. All experiments and animal care were conducted in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by the Ethics Committee of Science and Technology Department of Jiangsu [approval no. SYXK (SU) 2016-0011]. A total of 30 Male C57BL/6 mice (6-8 weeks old; weight: 18-22 g) were purchased from Nanjing Biomedical Research Institute and were randomly maintained on a basal diet [the normal diet (ND group); 360 kcal/100 g; comprising 13.3 g/100 g fat, 26.2 g/100 g protein and 60.5 g/100 g carbohydrate] or a HFHC diet (506.8 kcal/100 g; comprising 10 g/100 g lard, 2 g/100 g cholesterol, 5 g/100 g egg yolk power, 10 g/100 g sucrose, 2 g/100 g propylthiouracil and basal diet, 72.8 g/100 g). Animals were housed at 24°C and 55% relative humidity in a barrier facility under a 12 h light/dark cycle with free access to food and water. The basal diet and HFHC diet were provided by the Jiangsu Xietong Medical and Biological Corporation. Subsequently, mice were randomly divided into the following three groups (n=6-8 per group): Vehicle-treated chow group, vehicle-treated HFHC group and GAA-treated HFHC group (25 or 50 mg/kg/day). The administration concentration was determined to have therapeutic effect in a previous study (20). HFHC-fed mice were gavaged with GAA, which was purchased from Shanghai Yousi Biotechnology Co., Ltd. and was dissolved in 0.5% sodium carboxymethyl cellulose (Sigma-Aldrich; Merck KGaA) for 12 weeks. All mice were sacrificed by cervical dislocation after overnight fasting at the termination of the experiment. Subsequently, mouse liver tissue was obtained. Before euthanasia, 300 μ l blood was collected in the 3% isoflurane-anaesthetized (Forene™; Abbott Laboratories SA; oxygen flow rate of 4 l/min) mice by retroorbital venous plexus method. All animal experiments were performed in accordance with the approved guidelines.

Serum biochemical analysis. Serum was collected after sacrifice instantaneously by centrifugation at 1,200 x g for

15 min at room temperature. The serum lipids, including total cholesterol (TC; cat. no. A111-1-1), total triglycerides (TG; cat. no. A110-1-1), low-density lipoprotein cholesterol (LDL-c; cat. no. A113-1-1) and high-density lipoprotein cholesterol (HDL-c; cat. no. A112-1-1) were detected. Aspartate transaminase (AST; cat. no. C010-2-1) and alanine transaminase (ALT; cat. no. C009-2-1) levels were evaluated to assess the hepatic injury. All serum lipids were evaluated by commercial kits (Nanjing Jiancheng Bioengineering Institute). ELISA measurements of serum IL-1 β (cat. no. EMC001b.96), TNF- α (cat. no. EMC102a.96) and IL-6 (cat. no. EMC004.96) were performed following the manufacturer's protocol (Neobioscience Technology Co., Ltd.).

Histopathological analysis. Histopathological analysis was performed with standardized specimens from specified portions of liver. Liver tissues were fixed in 4% paraformaldehyde for 4 h at room temperature, dehydrated in a series of ethanol and embedded in paraffin wax. Sections (4-mm-thick) were stained with hematoxylin-eosin (H&E) at room temperature for 15 min before being analyzed under a light microscope (BX53; Olympus Corporation). The NAFLD activity score (NAS) of each group was calculated as previously described (21). Lipid droplets of the fresh liver samples were stained by Oil Red O (cat. no. O8010; Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at room temperature and analyzed to quantify lipid content by cell imaging under an Olympus-BX53 light microscope. Sirius Red (SR; cat. no. ab150681; Abcam) staining, anti-CD68 (cat. no. ab31630; Abcam; 1:200) and anti-F4/80 antibodies (cat. no. ab16911; Abcam; 1:200) were used for histological analysis under a light microscope (BX53; Olympus Corporation). Areas of stained droplets (Sirius Red staining for 1 h at room temperature) or SR and the intensity of immunohistochemical staining were determined using ImageJ 1.8.0 (National Institutes of Health).

Hepatic lipid and hepatocellular oxidative stress analysis of the liver. Lipids extracted from murine liver tissue were dissolved in isopropanol, after which hepatic TG and TC contents were detected as aforementioned. Hepatic malondialdehyde (MDA) was measured using a MDA assay kit (TBA method; cat. no. A003-1-2; Nanjing Jiancheng Bioengineering Institute) in accordance with the manufacturer's protocols. Briefly, 0.1 ml sample was mixed with 1,1,3,3-tetramethoxypropane, 0.75 ml TBA working solution (0.37%) and perchloric acid. The resulting solution was incubated at 95°C for 45 min. After cooling (10 min in ice water bath), the flocculent precipitate was removed by centrifugation (4,000 x g 10 min at room temperature). The supernatant was analyzed at 532 nm using a multi-scan spectrum microplate spectrophotometer at room temperature. Hepatic superoxide dismutase (SOD) levels was measured using a SOD assay kit (WST-1 method; cat. no. A001-3-2; Nanjing Jiancheng Bioengineering Institute). Briefly, the tissue were lysed with No-nidet P-40 lysis buffer (1% NP-40, 50 mmol/l Tris-HCl [pH 7.5], 0.05 mmol/l ethylenediamine tetra-acetate) for 20 min at 4°C. The lysates were centrifuged at 300 g for 10 min, and 20 μ l of this sample solution was used for determination of SOD enzyme activity according to the manufacturer's instructions. The value for each treatment group was converted to the percentage of control.

Reverse transcription-quantitative (RT-q) PCR. Total RNA extraction was performed using TRIzol® (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. The purity and concentration of RNA was determined by spectrophotometric analysis via absorbance at 260/280 and 260/230 nm. RNA concentrations were equalized and converted to cDNA using Hiscript II reverse transcriptase (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. Gene expression was measured using a LightCycler 96 Real-Time PCR System (Roche Diagnostics) using SYBR-green dyes (Roche Diagnostics). The thermocycling conditions were as follows: Initial denaturation at 95°C for 10 min followed by 40 cycles for 10 sec at 95°C, 10 sec at 55°C and 30 sec at 72°C, with a final elongation step at 72°C for 7 min. All data were analyzed using GAPDH gene expression as an internal standard. Relative gene expression levels were analyzed using the $2^{-\Delta\Delta C_q}$ method (22,23). The sequences of the murine PCR primers were as follows: TNF- α forward, 5'-AAGGGAGAGTGGTCA GGTG-3' and reverse, 5'-TCTGTGAGGAAGGCTGTGC-3'; IL-1 β forward, 5'-AACCTGCTGGTGTGTGACGTTTC-3' and reverse, 5'-CAGCACGAGGCTTTTTTGTGT-3'; IL-6 forward, 5'-CGGAGAGGAGACTTCACAGAG-3' and reverse, 5'-CATTTCCACGATTTCCAGA-3'; α -smooth muscle actin (α -SMA) forward, 5'-CCCTGAAGTATCCGATAGAACA-3' and reverse, 5'-TGCCTGGGTACATGGTAGTG-3'; TGF- β forward, 5'-CTTTGTACAACAGCACCCGC-3' and reverse, 5'-TAGATTGCGTTGTTGCGGTC-3'; MMP-13 forward, 5'-GTGACTCTTGCGGGAATCCT-3' and reverse, 5'-CAG GCACTCCACATCTTGGT-3'; and GAPDH forward, 5'-AAC AGCAACTCCCACCTCTTC-3' and reverse, 5'-CCTGTTGCT GTAGCCGTATT-3'.

Western blot analysis. Liver tissues were homogenized at 4°C with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA and protease inhibitors). After sonication, the samples were centrifuged for 10 min at room temperature at 13,000 x g. The protein concentration were then detected by performing a BCA assay (Beyotime Institute of Biotechnology). 10% SDS-PAGE was conducted by loading equal amounts of protein per lane (40 μ g). Gels were then transferred to PVDF membranes (EMD Millipore) and blocked with 5% non-fat milk in TBST buffer for 1 h at room temperature. The membranes were then incubated with the indicated primary antibodies overnight at 4°C, and washed three times with TBST (containing 0.1% Tween 20 in TBS) for a total of 10 min. Thereafter, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at room temperature and washed three times with TBST. The blots were visualized with the Amersham ECL Plus (Amersham; Cytiva) western blotting detection reagents according to the manufacturer's protocol. The primary antibodies (all dilutions were 1:500) including glucose-regulated protein 78 (GRp78; cat. no. 3177), phosphorylated (p)-eukaryotic initiation factor-2 α (eIF-2 α ; cat. no. 3398), eIF-2 α (cat. no. 5324), p-JNK (cat. no. 4668), JNK (cat. no. 9252), ERp57 (cat. no. 2887S), p-AKT (cat. no. 4060), AKT (cat. no. 9272) p-MAPK (cat. no. 4370), MAPK (cat. no. 9102) and GAPDH (cat. no. 8884) were obtained from Cell Signaling

Technology, Inc. Immunoreactive signals were detected with Chemi-Lumi One Ultra (Tanon Science and Technology Co., Ltd.). The densitometric analysis of the blots was performed by Image Pro Plus 6.0 software (Media Cybernetics, Inc.).

Statistical analysis. Statistical analysis was performed using Prism version 7.0 statistical software (GraphPad Software Inc.). All quantitative values are presented as the mean \pm SEM. Statistical data were analyzed using one-way ANOVA with Dunnett's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GAA improves the metabolic profiles in HFHC-fed mice. The effect of GAA on the weight gain and metabolic features of serum lipids in C57BL/6J mice is demonstrated in Fig. 1. Mice fed with a HFHC diet had significantly increased body weights, without significant difference in food intake, compared with the ND-fed mice (Fig. 1A and B). Weight gain was also accompanied by serum lipid disorder, including higher TG, TC, LDL-cholesterol (LDL-c) and HDL-cholesterol (HDL-c) in the HFHC group compared with the ND-fed group (Fig. 1C-F). In addition, serum ALT and AST levels of mice were significantly increased in the HFHC group compared with the ND group, which revealed hepatic injury in model group (Fig. 1G and H). The chemical structure of GAA is presented in Fig. 1I.

Compared with the model group, the body weight of HFHC-fed mice treated with GAA was significantly reduced (Fig. 1A). Serum lipid disorders (increased levels of TG, TC, LDL-c) were also reversed by GAA treatment in HFHC-fed mice (Fig. 1C-F). Additionally, the serum ALT and AST levels were significantly lower in both GAA groups compared with the HFHC group, demonstrating a hepatoprotective role of GAA against liver injury (Fig. 1G and H). These results suggested that GAA improved the metabolic profiles of HFHC-fed mice.

GAA alleviates hepatic steatosis in HFHC-fed mice. Hepatic lipid accumulation was evaluated by Oil Red O staining and biochemical parameter assays. As revealed in Fig. 2A and B, compared with the ND group, the Oil Red O staining positive area in HFHC group mice was increased significantly. By contrast, the positive area was reduced by GAA in a dose-dependent manner compared with the model group (Fig. 2A and B). Similar results were observed following liver biochemical parameter assays. The ratio of liver weight to body weight and the levels of hepatic TG and TC were significantly increased in the HFHC group compared with the ND group. Conversely, oral administration of 25 or 50 mg/kg of GAA reduced the HW/BW, TG and TC levels (Fig. 2C-E). These results indicated that GAA alleviated hepatic steatosis in HFHC-fed mice.

GAA reduces the hepatic inflammation response in HFHC-fed mice. To evaluate the effect of GAA on the hepatic injury process from NAFLD to NASH, liver histopathology was detected by typical H&E staining (Fig. 3A). Compared with mice in the ND group, the typical pathological phenomenon

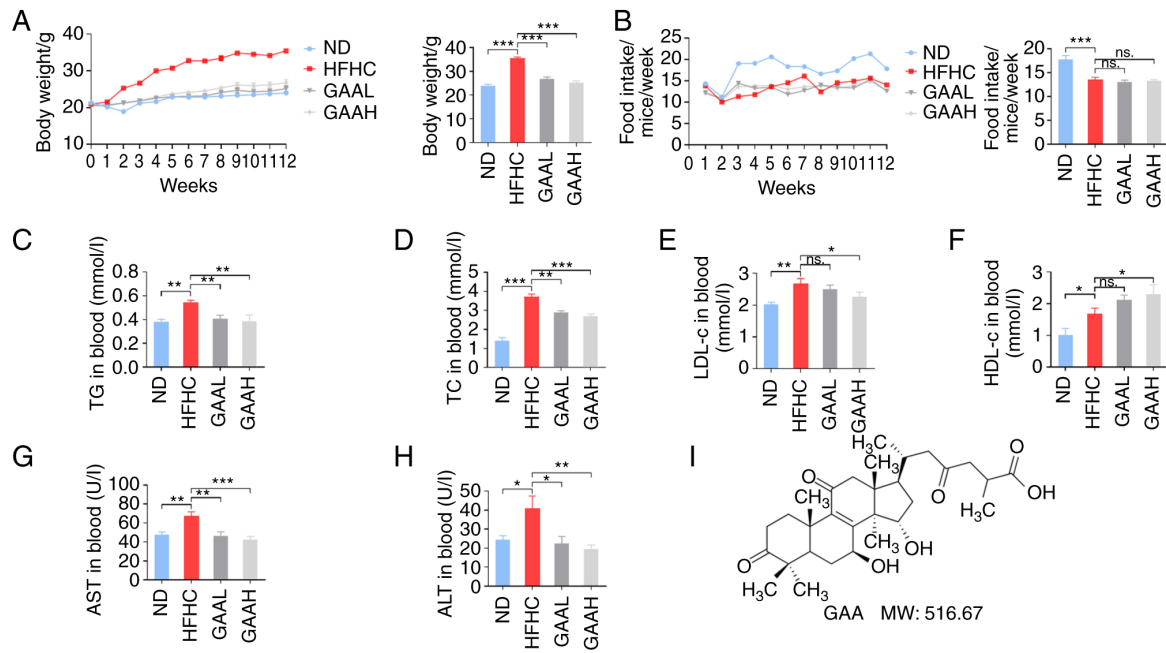


Figure 1. GAA improves the metabolic profiles of HFHC-fed mice. (A) Body weight, (B) food intake, (C) TG, (D) TC, (E) LDL-c, (F) HDL-c, (G) AST and (H) ALT levels were determined in murine blood. Mice were fed an ND or HFHC diet with or without indicated doses of GAA treatment (n=6-8). (I) The chemical structure and molecular weight of GAA. Assays were repeated three times. Data are expressed as the mean \pm SEM. * P <0.05, ** P <0.01 and *** P <0.001 vs. the HFHC group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GAA, ganoderic acid A; GAAH, GAA 50 mg/kg/day; GAAL, GAA 25 mg/kg/day; HDL-c, high density lipoprotein-cholesterol; HFHC, high-fat high-cholesterol; LDL-c, low density lipoprotein-cholesterol; MW, molecular weight; ns, not significant; ND, normal diet; TC, total cholesterol; TG, triglyceride.

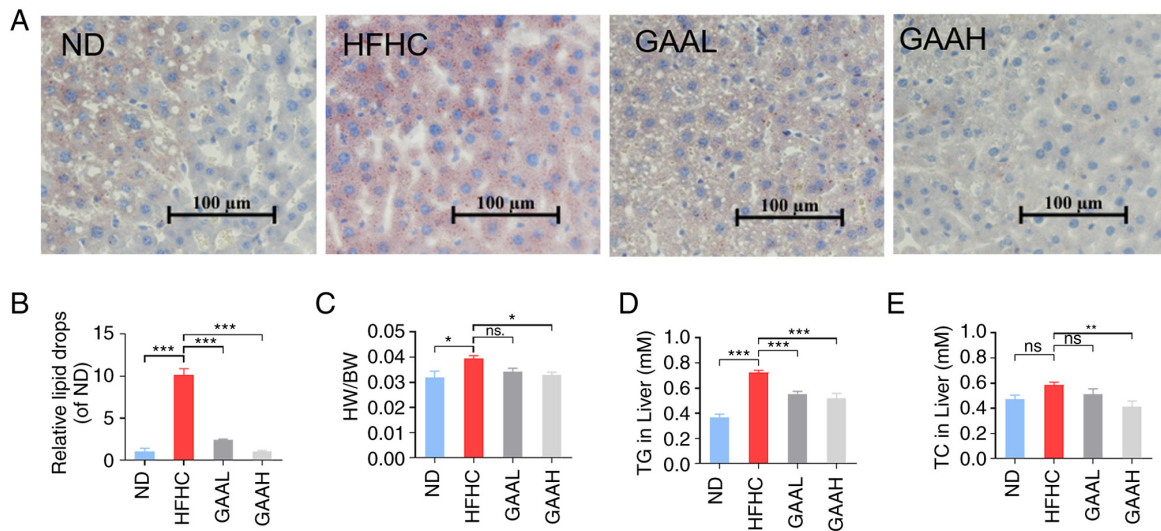


Figure 2. GAA alleviates hepatic steatosis in HFHC-fed mice. (A) Livers were stained with Oil red O. (B) The area of Oil red O was determined using ImageJ 1.8.0 software. (C) The ratio of HW/BW was determined. (D and E) Hepatic TC and TG levels were evaluated. Mice were fed an ND or HFHC diet with or without indicated doses of GAA treatment (n=6-8). Assays were repeated three times. Data are expressed as the mean \pm SEM. * P <0.05, ** P <0.01 and *** P <0.001 vs. the HFHC group. GAA, ganoderic acid A; GAAH, GAA 50 mg/kg/day; GAAL, GAA 25 mg/kg/day; HFHC, high-fat high-cholesterol; HW/BW, liver weight to body weight; ND, normal diet; ns, not significant; TC, total cholesterol; TG, triglyceride.

of NASH, including NAS, hepatocellular ballooning, lobular inflammation and inflammatory cell infiltration were observed in the livers of HFHC-fed mice (Fig. 3A). Furthermore, the number of CD68 positive marking Kupffer cells and F4/80 positive marking macrophage infiltration were significantly enhanced in the livers of HFHC-fed mice, compared with the ND group (Fig. 3B-E), which suggested that hepatic inflammation had occurred in the model group. Circulating

inflammation factors, such as IL-1 β , TNF- α and IL-6, were also significantly increased in the model group (Fig. 3F-H). In addition, the mRNA expression of inflammation factors, IL-1 β , TNF- α and IL-6, was significantly increased, compared with the corresponding control group (Fig. 3I-K).

The typical pathology of NASH, including NAS, hepatocellular ballooning, lobular inflammation and inflammatory cell infiltration, was significantly decreased in the liver of

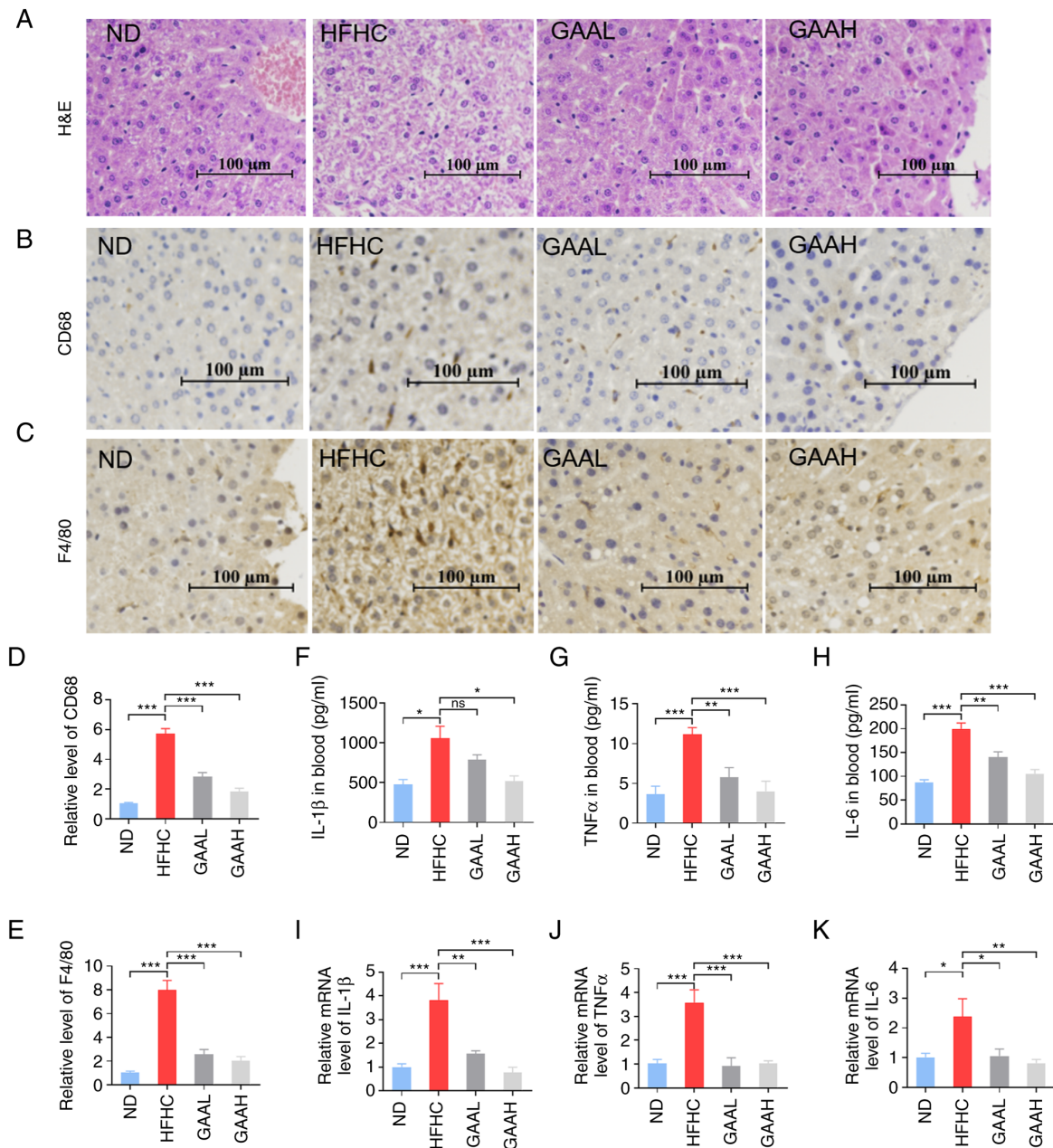


Figure 3. GAA reduces the hepatic inflammation response in HFHC-fed mice. (A) H&E staining of liver tissue. (B and C) CD68 and F4/80 levels in liver were detected by immunohistochemistry. (D and E) Relative levels of CD68 and F4/80 were measured. (F) IL-1 β , (G) TNF α and (H) IL-6 levels in murine blood. mRNA levels of (I) IL-1 β , (J) TNF α and (K) IL-6 levels in liver tissue. Mice were fed an ND or HFHC diet with or without indicated doses of GAA treatment (n=6-8). Assays were repeated three times. Data are expressed as the mean \pm SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. the HFHC group. GAA, ganoderic acid A; GAAH, GAA 50 mg/kg/day; GAAL, GAA 25 mg/kg/day; H&E, hematoxylin and eosin; HFHC, high-fat high-cholesterol; ND, normal diet; ns, not significant.

GAA-treated (25 or 50 mg/kg) HFHC-fed mice (Fig. 3A). As demonstrated in Fig. 3B-E, GAA (25 or 50 mg/kg) treatment markedly reduced the number of Kupffer cells and macrophage infiltration in the livers of HFHC-fed mice. Additionally, the upregulated serum inflammation factors in HFHC-fed mice were significantly reduced by GAA treatment for 12 weeks (Fig. 3F-H). In consistency with these results, the mRNA expression of inflammation factors, such as IL-1 β , TNF- α and IL-6, was significantly decreased in HFHC-fed mice treated with GAA (Fig. 3I-K). These results suggested that GAA reduced the hepatic inflammation response in HFHC-fed mice.

GAA decreases the hepatic fibrosis in HFHC-fed mice. To certify the pathological progression from NASH to hepatic fibrosis, SR staining was used to indirectly reflect activated stellate cells in the livers of HFHC-fed mice. As presented in Fig. 4A and B, the hepatic collagen formation of model mice was increased, compared with the ND group. Additionally, the mRNA levels of α -SMA, TGF- β and MMP-13 were also significantly upregulated in HFHC-fed mice (Fig. 4C-E).

Administration of GAA (25 or 50 mg/kg) reduced the number of activated stellate cells in the livers of HFHC-fed mice, as determined by SR staining (Fig. 4A and B). The

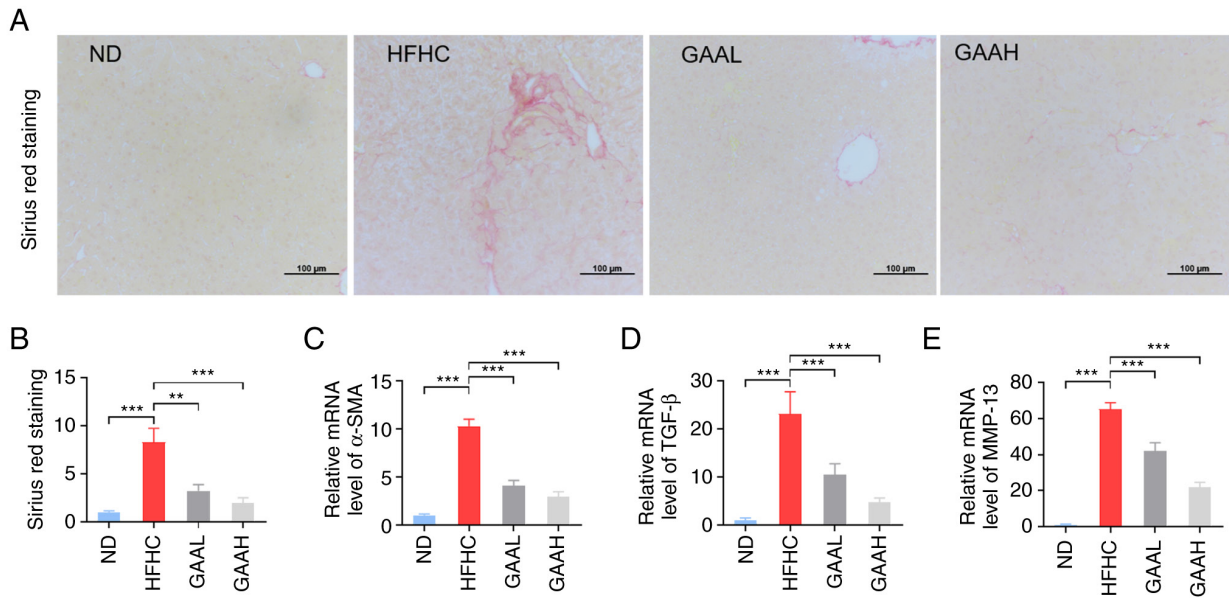


Figure 4. GAA decreases the hepatic fibrosis in HFHC-fed mice (A) SR staining of liver tissue. (B) The relative intensity of SR staining. mRNA levels of (C) α -SMA, (D) TGF- β and (E) MMP-13 levels in murine liver tissue. Mice were fed an ND or HFHC diet with or without indicated doses of GAA treatment (n=6-8). Assays were repeated three times. Data are expressed as the mean \pm SEM. **P<0.01 and ***P<0.001 vs. the HFHC group. GAA, ganoderic acid A; GAAH, GAA 50 mg/kg/day; GAAL, GAA 25 mg/kg/day; HFHC, high-fat high-cholesterol; ND, normal diet; α -SMA, α -smooth muscle actin; SR, Sirius Red.

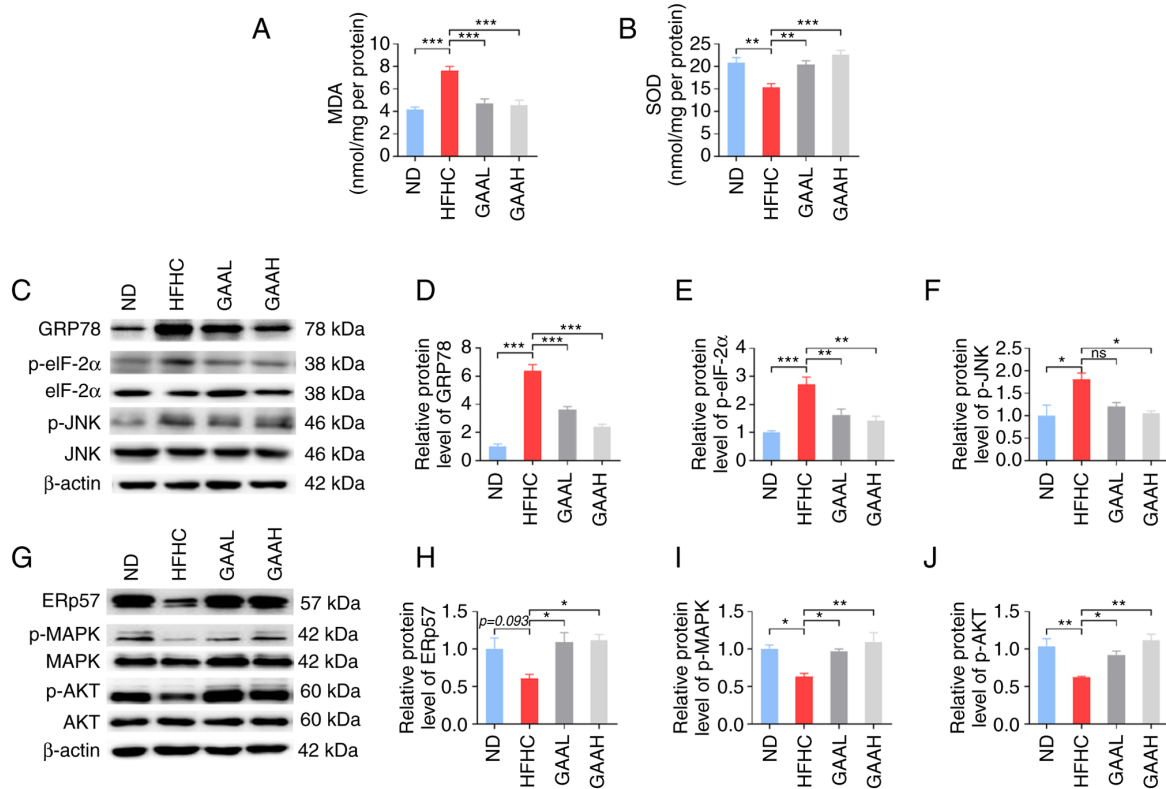


Figure 5. GAA hepatoprotection is associated with hepatic oxidative stress and the ER stress response. (A) MDA and (B) SOD levels were detected in murine livers. (C and G) Western blotting was performed to determine the relative protein levels of (D) GRP78, (E) p-eIF-2 α , (F) p-JNK, (H) ERp57, (I) p-MAPK and (J) p-AKT. Mice were fed an ND or HFHC diet with or without indicated doses of GAA treatment (n=6-8). Assays were repeated three times. Data are expressed as the mean \pm SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. the HFHC group. eIF-2 α , eukaryotic initiation factor-2 α ; GAA, ganoderic acid A; GAAH, GAA 50 mg/kg/day; GAAL, GAA 25 mg/kg/day; GRP78, glucose-regulated protein 78; HFHC, high-fat high-cholesterol; MDA, malondialdehyde; ND, normal diet; ns, not significant; p, phosphorylated; SOD, superoxide dismutase.

transcription of fibrosis-related genes, including α -SMA, TGF- β and MMP-13, was also decreased by GAA treatment

(Fig. 4C-E). These results indicated that GAA could decrease the hepatic fibrosis in HFHC-fed mice.

GAA hepatoprotection is associated with the hepatic oxidative stress and ER stress response. It has been reported that oxidative stress plays a key role in the occurrence and development of NASH (24). To explore the underlying mechanisms of GAA, the contents of MDA and SOD in the livers of mice fed with an HFHC diet were evaluated. As revealed in Fig. 5A and B, the hepatic level of MDA was significantly increased in HFHC-fed mice compared with the ND group, whereas it was decreased in HFHC-fed mice treated with GAA when compared with the HFHC group. Conversely, the hepatic level of SOD was significantly decreased in HFHC-fed mice compared with the ND group, whereas it was increased in HFHC-fed mice treated with GAA.

Subsequently, the expression levels of essential ER stress-related genes were detected. As demonstrated in Fig. 5C-F, administration of the HFHC diet to mice resulted in an increase in GRp78, p-eIF-2 α and p-JNK protein expression, which was significantly reversed by GAA treatment (25 or 50 mg/kg). Furthermore, compared with the ND group, the levels of ERp57, p-Akt, and p-MAPK were decreased in HFHC-fed mice, whereas GAA treatment increased their levels (Fig. 5G-J). These results suggested that the reduction of hepatic oxidative stress and the ER stress response by GAA may be related to the prevention of NASH progression in mice.

Discussion

As an important herbal medicine, *G. lucidum* is widely used in the treatment of multiple diseases due to its anti-inflammatory and antioxidant activities, including inflammation-associated diseases, cancer, cardiovascular and cerebrovascular diseases (25). The main category of biologically active compounds produced in *G. lucidum*, are the triterpenoids, which are known as ganoderic acids (26). GAA, which is derived from *G. lucidum* mushrooms, is considered to be a potential therapeutic candidate for the treatment of a variety of diseases, such as obesity, NAFLD, cancer and hepatic toxicity, but is focused on alternative or complementary therapies for these diseases (27-30). A previous study has revealed that ganoderic acids have hepatoprotective and hypocholesterolemic effects. In addition, a previous study revealed that GAA improved lipid accumulation and insulin resistance by inhibiting the sterol regulatory element-binding protein signaling pathway (20). Liu *et al* (28) also found that GAA attenuated high-fat-diet-induced liver injury in rats by regulating lipid oxidation and liver inflammation. However, in the present study, HFHC-induced mice were used to construct NAFLD and NASH models and for the first time, to the best of our knowledge. The results revealed that GAA significantly reduced liver lipid accumulation and markedly improved liver inflammation and fibrosis. In terms of the underlying mechanism of action, the results of the present study preliminarily demonstrated that GAA improved NASH by inhibiting hepatic oxidative and ER stress. The in-depth mechanism of GAA in the improvement of NASH should be investigated in subsequent studies.

The first step in the progression of NASH is the accumulation of excessive TG in the liver. Therefore, suppression of hepatic TG accumulation may be a potential therapeutic

approach for NASH (31). In the present study, the role of GAA was investigated using a HFHC diet-induced NASH model that simulates the development of human NASH. The results demonstrated that oral administration of GAA effectively inhibited lipid accumulation in the liver, markedly reduced the oil red O staining area and largely reduced liver TG.

The HFHC diet is high in fat (~41%) and cholesterol (~0.21%). A deficiency of choline and methionine, two essential nutrients, leads to a reduction in the production of very-LDL particles, which leads to fat accumulation in the liver. Therefore, unlike the high-fat diet model, the HFHC diet model does not lead to excessive fat absorption in the gut, but induces fat accumulation via the lack of choline and methionine, thus blocking the TG transfer pathway to the liver (32). In a previous study, it has been demonstrated that GAA does not affect intestinal fat absorption in mice with high-fat diet-induced fatty liver (20). Therefore, the beneficial effects of GAA are not due to changes in intestinal fat absorption.

During the development of NASH, lipid disturbances can increase inflammation. TNF- α is a key factor in the progression of NASH, as it induces key molecules in hepatic lipid metabolism associated with inflammation and fibrosis (31). GAA has an effective anti-inflammatory effect (33). In the present study, the data revealed that GAA treatment significantly suppressed the HFHC diet-induced upregulated expression of IL-6 and TNF α in the serum and liver of NASH mice. At present, evidence supports the view that inhibition of IL-6 improves the symptoms of HFHC-induced NASH. For example, it has been reported that IL-6 inhibition suppresses NASH induced inflammation and liver damage (34). Consistent with other current pharmacological studies, resveratrol, a natural product, reduces liver steatosis and inflammation in NASH mice, indicating a reduction in serum IL-6 levels (35-37). The transition from steatosis to steatohepatitis represents an important step in liver damage progression, which eventually culminates in hepatic fibrosis and cirrhosis (38). Therefore, the effect of GAA on liver fibrosis was also investigated. The results demonstrated that GAA also inhibited the expression of α -SMA, TGF- β and MMP-13 induced by HFHC.

Pathological analysis further confirmed the anti-inflammatory effect of GAA, reflecting the aggregation of inflammatory cells; however, the anti-inflammatory mechanism of GAA is not clear. Recent studies demonstrated that activation of the ER stress pathway can trigger or aggravate fat accumulation and the inflammatory response, eventually leading to hepatocyte damage or even cell death in certain conditions. These are important factors in the pathogenesis of NASH (39,40). In the present study, it was found that GAA effectively reduced ER stress responses induced by the HFHC diet in NASH mice. Meanwhile, the decrease of GRp78 also decreased the expression of p-JNK, thereby inhibiting the expression of IL-6 and TNF- α to improve the inflammatory response, and increasing p-AKT to avoid cell damage. Evidence has demonstrated the role of GRp78 in promoting cell survival by activating MAPK (41). In the present study, GAA largely upregulated p-MAPK levels, suggesting that GAA may block hepatocyte death. Importantly, a previous study has shown that ERp57 is a protective factor against ER stress (42). GAA markedly

upregulated ERp57 levels in the liver tissue of NASH mice, serving a protective role and indicating that GAA may be associated with ERp57 and ER stress.

Oxidative stress occurs as a result of the imbalance between antioxidant response and the pro-oxidation reaction. The accumulation of excessive free radicals in the body lead to DNA oxidative damage and the abnormal expression of various cytotoxic related proteins, which is closely associated the occurrence of NAFLD (43-45). In the present study, the effects of GAA on hepatic oxidative stress were investigated from the two aspects of scavenging and producing reactive oxygen species. The hepatic level of MDA was significantly upregulated in HFHC-fed mice and this effect was reversed in HFHC-fed mice treated with GAA. Conversely, the hepatic level of SOD was significantly downregulated in HFHC-fed mice and this effect was reversed in HFHC-fed mice treated with GAA. The results demonstrated that GAA improved NASH-related oxidative stress injury by increasing hepatic anti-oxidase activity and suppressing the activities of free radical generating enzymes.

The present study had certain limitations. For example, there is no specific drug for the treatment of NASH on the market. It was therefore difficult to locate a recognized positive drug as a reference when pharmacodynamic evaluation was conducted. Therefore, no positive control was used in the experimental design of the present study. Furthermore, it was identified that GAA improved HFHC-induced NASH; however, this experimental data was produced using a mouse model, meaning that it is difficult to hypothesize whether GAA will exert similar effects in patients with NASH. The promotion of the clinical pharmacodynamic validation of GAA requires at least the conduction of pharmacodynamic, pharmacokinetic and pharmaco-toxicological experiments in a variety of different animal experiments to ensure the safety and effectiveness of GAA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ and JJ conceived and designed the current study. JZ and JD acquired the data. JZ and SL analyzed and interpreted the

data. JZ and JJ confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All experiments and animal care were conducted in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and approved [SYXK (SU) 2016-0011] by the Science and Technology Department of Jiangsu Province.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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