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Original Article

Effect and mechanism of gallic acid on oleic acid-induced steatosis in the Chang liver cell NAFLD model

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Abstract: Objective: To investigate the effect and underlying mechanism of gallic acid (GA) on oleic acid (OA)-induced triglyceride (TG) accumulation in the Chang liver cell non-alcoholic fatty liver disease (NAFLD) model. Methods: Chang liver cells were incubated with 0.2 mM OA to establish a NAFLD model. After intervention with different concentrations of GA, the treated cells were collected for determination of TG, SOD, GSH-PX, and MDA levels. Oil red O staining revealed the changes in intracellular TG accumulation after GA intervention. The levels of TNF-α and IL-8 in the culture medium were measured by ELISA. The levels of expression of intracellular AMPK, ACC2, and CPT-1A mRNA were evaluated by RT-PCR. The levels of phosphorylation of intracellular AMPK and ACC2 protein and the level of expression of CPT-1A protein were assessed by Western blot analysis. Results: GA intervention significantly decreased the TG level in the OA-induced NAFLD model, improved the status of steatosis, up-regulated the levels of SOD and GSH-PX, down-regulated the MDA level, and significantly reduced the levels of TNF-α and IL-8 in steatotic cells. In addition, the expression of AMPK and CPT-1A mRNA was up-regulated, the expression of ACC2 mRNA was down-regulated, the levels of phosphorylation of AMPK, ACC2 protein were enhanced, and the expression of CPT-1A protein was up-regulated. Conclusion: GA decreased TG accumulation in a NAFLD cell model, and was probably associated with an anti-oxidation effect. GA decreased cytokine levels and regulated the expression of enzymes related to lipid metabolism at the protein and mRNA levels.

Keywords: Gallic acid, lipid metabolism, NAFLD

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease and the primary cause of liver enzyme abnormalities in developed countries [1]. The prevalence of NAFLD in general adults is 20%-33% [2]. In China, NAFLD is the primary cause of chronic liver disease [3-5], with an increasing incidence and a younger age of onset [6, 7]. Moreover, NAFLD is closely correlated with type 2 diabetes mellitus (T2DM), metabolic syndrome (MS), and related cardiocerebral vascular events [8, 9]. The risk of DM and arteriosclerosis in NAFLD patients is equivalent to the risk of cirrhosis [10]. NAFLD has become a novel challenge in the treatment of liver disease. The underlying pathogenesis of NAFLD is extremely complex and remains elusive. The theory of "two hits" is widely recognized. The "first hit" is lipid accumulation in the liver caused by insulin resistance (IR), and the "second hit" is the incidence and progression of NAFLD induced by oxidative stress and lipid peroxide injury [11, 12].

Gallic acid (GA), also known as 3,4,5-trihydroxy benzoic acid, is a type of phenolic acid commonly found in a variety of Chinese herbal medicines, vegetables, tea, and fruits [13-16]. GA has a strong ability to eliminate free radicals, and also has an effect of anti-oxidative [17-20] and anti-inflammatory activities [21, 22]. In recent years, the role of GA in the prevention and treatment of fatty liver disease, liver cancer, and other hepatic diseases has drawn more and more attention amongst clinicians [22-26]. GA is considered to be associated with the treatment on the lipid metabolism

disease including NAFLD. However, the mechanism underlying the effect of GA on lipid metabolism remains poorly defined. This study was designed to evaluate the effect and potential mechanism of GA on lipids in the Chang liver cell NAFLD model.

Materials and methods

Main reagents and instruments

GA (97.5-102.5% [G7384]) was purchased from Sigma (St. Louis, MO, USA). RPMI-1640 culture medium, double antibiotics (100 mg/L of streptomycin plus 100 IU/mL of penicillin), trypsin, and PBS were purchased from HyClone (USA). Fetal bovine serum was supplied by Hangzhou Sijiqing Co., Ltd. (China). OA was purchased from Tianjin Fuchen Chemical Reagent Factory (China). Oil red O was bought from Sigma. Dimethyl sulfoxide (DMSO) was supplied by Biosharp (USA). A BCA protein quantitative kit, triglyceride (TG), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-Px) were purchased from Nanjing Jiancheng Bioengineering Institute (China). A tumor necrosis factor-α (TNF-α) kit was provided by Shanghai Excell Biology, Inc. (China). An interleukin-8 (IL-8) kit was purchased from eBioscience (USA). A ReverAid First Strand cDNA synthesis kit was purchased from Thermo (USA). A SYBR Select Master Mix kit was supplied by ABI (USA). The primer was designed by primer express 2.0 software and synthesized by Shanghai Sangon Biotech Co., Ltd. (China). Protein antibody was purchased from Abcam (UK).

Cell culture and treatment

Live Chang liver cells were cultured in RPMI-1640 culture medium containing 1% double antibiotics and 10% fetal bovine serum at 37°C with 5% CO_2. The cells were passaged using 0.25% trypsin at a cell confluence of 80%-90%. Cells in the logarithmic growth phase were sampled for subsequent experiments. After cell counting, the cells were seeded into 6-well plates at a density of 1×10^6 cells per well. In the control group, RPMI-1640 culture medium containing 0.48% DMSO was prepared (A solution). In the model group, a solution was mixed with 0.2 mmol/L OA and designated as B solution. In the intervention group, B solution was supplemented with different concentrations of GA (12.5, 25.0, or 50.0 μg/mL). The cells in all groups were incubated for 24 h and cell incubation was repeated 6 times in each group.

Determination of the intracellular TG level

The culture medium was discarded from the 6-well plate. Cells were supplemented with trypsin, centrifuged at 1000 r/min at 4°C for 3 min, and the cells were harvested and subjected to 3 cycles of freezing and thawing. The cells were then lysed using ultrasound, centrifuged at 13000 r/min for 10 min at 4°C, and the supernatant was collected. The protein concentration was measured using the BCA assay. The TG level was determined according to the manufacturer's instructions. The results were standardized as total protein per gram of cells.

Oil red O staining

Oil red O (5%) was prepared with isopropanol and mixed with ultrapure water at a ratio of 3:2 at room temperature for subsequent use. The culture medium in the 6-well plate was discarded, fixed with 10% paraformaldehyde overnight, and stained with oil red O for 20 min. The staining solution was discarded, stained with hematoxylin for 0.5 min, twice-irrigated with PBS, dried in a solution of hydrochloric acid: 75% ethanol, irrigated with PBS, and observed and photographed under a fluorescence microscope.

Determination of intracellular levels of SOD, MDA, and GSH-Px

The culture medium in the 6-well plate was discarded, supplemented with pancreatin, centrifuged at 1000 r/min at 4°C for 3 min. The collected cells were subjected to three cycles of freezing and thawing, lysed by ultrasound, centrifuged at 13000 r/min for 10 min at 4°C, and the supernatant was collected. The protein concentration was measured using the BCA assay. SOD, MDA, and GSH-Px levels were determined according to the manufacturer's instructions. The results were standardized as the total protein per gram of cells.

Determination of TNF-α and IL-8 levels in cell culture medium after treatment

With 50 μg/mL of GA for 24 h, TNF-α and IL-8 levels were determined according to the manufacturer's instructions.

Table 1. PCR primer pairs used to amplify AMPK, ACC, and CPT-1A fragments

Target		Oligonucleotide (5' to 3')	bp
ACC	F	TCAGCCTACAAAACCGCCCA	150
	R	AAGGCCGTCCACGATGTAGG	
AMPK	F	TTGAAACCTGAAAATGTCCTGCT	113
	R	GGTGAGCCACAACTTGTTCTT	
CPT-1A	F	TGCTGATGACGGCTATGGTG	115
	R	GTGCCTTCCAAAGCGATGAG	
β-actin	F	TGACGTGGACATCCGCAAAG	205
	R	CTGGAAGGTGGACAGCGAGG	

F: Forward primer; R: Reverse primer; bp: Melting temperature.

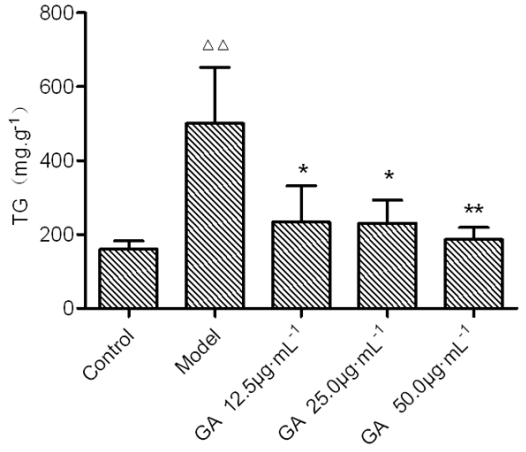

Figure 1. The effect of GA on the TG level in the NAFLD model cell. $^{\Delta\Delta}P<0.01$ vs. control, $*P<0.05$; $**P<0.01$ vs. model.

Fluorescent quantitative RT-PCR

After treatment with 50 μg/mL of GA, the changes in the levels of expression of adenosine monophosphate-activated protein kinase (AMPK), acetyl-CoA carboxylase 2 (ACC 2), and carnitine palmitoyl transferase-1A (CPT-1A) mRNA were detected. All primers are listed in **Table 1**.

Western blot analysis

Following treatment with 50.0 μg/mL of GA for 24 h, total protein was extracted for determination of the protein concentration using the BCA assay. Western blot analysis was used to measure the levels of protein expression of AMPK, p-AMPK, ACC2, p-ACC2, and CPT-1A. A ChemiScopemini chemiluminescence analyzer (Clinx Science Instruments Co., Ltd, China) was used for detection and photography. The integral optical density value of the target protein was calculated and statistically compared with that of β-actin. The relative expression of the target protein was calculated.

Statistical analysis

The data are expressed as the mean ± s. SPSS 17.0 software was utilized for one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

Changes in the TG level in Chang liver cells after GA treatment

As illustrated in **Figure 1**, the TG level in the model group was significantly elevated by OA incubation compared with the control group ($P<0.01$). Different concentrations of GA (12.5, 25.0, or 50.0 μg/mL) significantly reduced the increasing TG accumulation in Chang liver cells induced by OA incubation (all $P<0.01$), suggesting that GA decreases the intracellular accumulation of TG.

Oil red O staining results

After a 24-h GA treatment, the NAFLD Chang liver cell model was subjected to oil red O staining, observation, and photography under a fluorescence microscope. In the model group, oil red O-stained lipid deposition was frequently observed within the cells. Under the same visual field, the steatosis rate in the model group was significantly higher compared with the control group, suggesting that 0.2 mM of OA incubation for 24 h significantly enhanced intracellular lipid accumulation. The quantity of intracellular orange lipid in the three GA dose groups was decreased compared with the model group, as illustrated in **Figure 2**.

Determination of intracellular levels of SOD, MDA, and GSH-Px after GA treatment

After OA incubation, the levels of SOD and GSH-PX were down-regulated, whereas the level of MDA was up-regulated. After GA treatment, the intraocular levels of SOD and GSH-Px were significantly enhanced (both $P<0.01$). The MDA levels in the 12.5 and 25.0 μg/mL GA groups were significantly lower than the model group (both $P<0.01$), suggesting that GA possesses

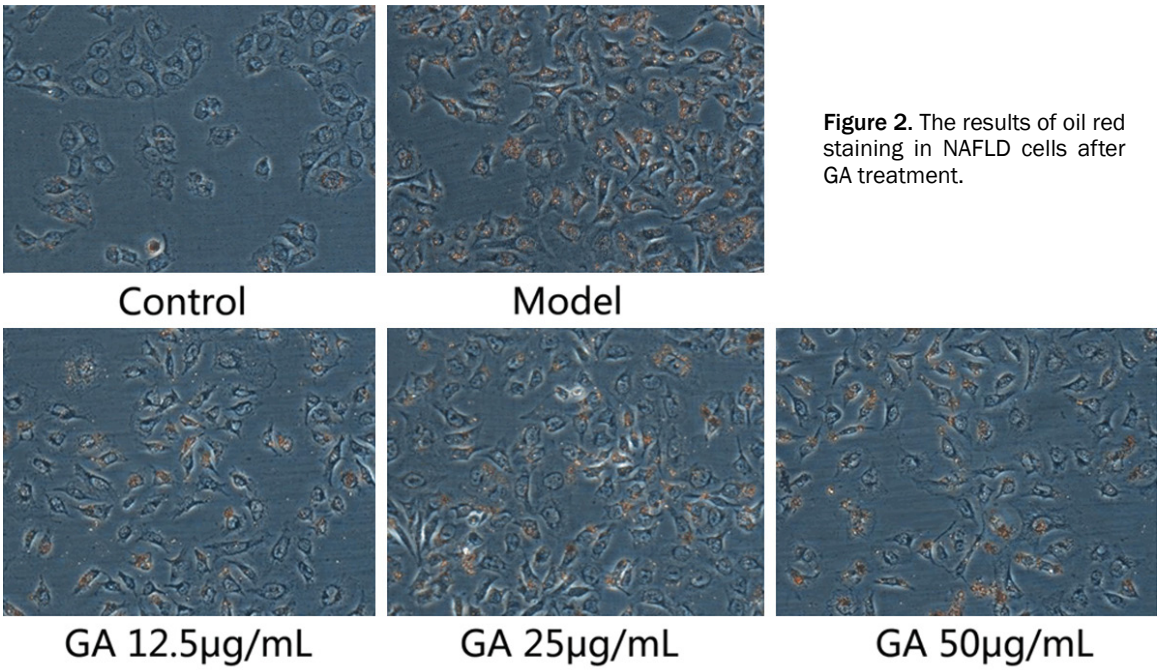

Figure 2. The results of oil red staining in NAFLD cells after GA treatment.

Table 2. The effect of GA on SOD, GSH-Px, and MDA levels in the NAFLD model cell (mean ± s, n=6)

Group	SOD (U/mg protein)	GSH-PX (U/mg protein)	MDA (nmoL/mg protein)
Control	23.60±3.14	290.68±73.04	0.07±0.02
Model	4.96±1.39$^{\Delta}$	158.79±40.07$^{\Delta}$	0.20±0.03$^{\Delta\Delta}$
GA 12.5 μg/mL	22.32±8.11*	323.60±43.11**	0.09±0.02**
GA 25.0 μg/mL	23.95±6.08*	363.00±28.67**	0.10±0.02**
GA 50.0 μg/mL	18.54±2.14*	509.02±126.44**	0.12±0.02**

$^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 vs. control; *P<0.05, **P<0.01 vs. model.

strong oxidizing characteristics, as shown in **Table 2**.

Changes in the intracellular levels of TNF-α and IL-8 after GA treatment

After treatment with 50.0 μg/mL of GA, the levels of TNF-α and IL-8 (cytokines closely related to NAFLD) in the model group were significantly decreased (P<0.05 and P<0.01), as illustrated in **Figure 3**.

Effect of GA upon the relative mRNA expression of enzymes related to lipid metabolism

As shown in **Figure 4**, the relative levels of expression of AMPK and CPT-1A mRNA in the model group were significantly reduced compared with the control group (both P<0.01). After GA treatment, the relative expression of AMPK and CPT-1A mRNA in the control group was significantly elevated than the model group (P<0.05 and P<0.01). Compared with the values in the control group, the relative expression of ACC2 mRNA was considerably enhanced (P<0.01). After treatment with GA, the relative expression of ACC2 mRNA in the control group was dramatically decreased compared to the model group (P<0.01), suggesting that GA probably regulates the TG level via the AMPK/ACC2/CPT-1A signaling pathway.

Effect of GA upon protein expression of enzymes related to lipid metabolism

The level of phosphorylation of AMPK in the model group was significantly decreased compared with the control group (P<0.01). Following GA treatment, the level of phosphorylation of AMPK protein was considerably up-regulated (P<0.01). The level of phosphorylation of AMPK in the model group was significantly decreased compared with the control group, whereas it was significantly elevated after GA treatment (P<0.01). The level of expression of CPT-1A protein in the model group was dramatically de-

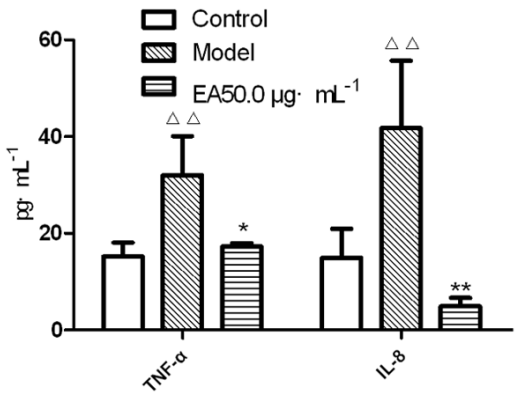

Figure 3. The effect of GA on the TNF-α and IL-8 levels in NAFLD model cell. $^{\Delta\Delta}P<0.01$ vs. control, $*P<0.05$; $**P<0.01$ vs. model.

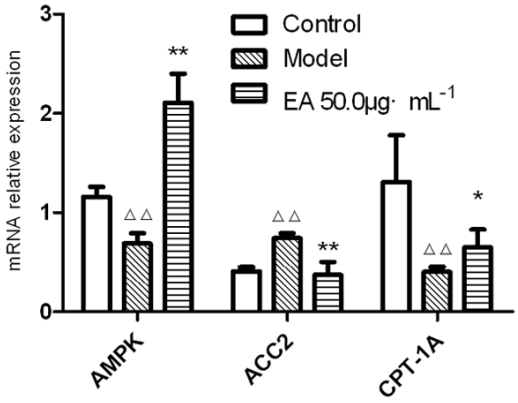

Figure 4. The effect of GA on AMPK, ACC2, and CPT-1A mRNA expression in the NAFLD model cell. $^{\Delta\Delta}P<0.01$ vs. control, $*P<0.05$; $**P<0.01$ vs. model.

creased in the control group, while the level of expression of CPT-1A protein was significantly elevated following GA treatment ($P<0.01$), as illustrated in **Figure 5**.

Discussion

The hepatic steatosis in NAFLD mainly results from excessive absorption and storage of nutrients, which increases the synthesis of fatty acids and transforms into TG accumulating in the liver. Recently, it has been reported that gallic acid had ability to ameliorate impaired glucose and lipid homeostasis in high fat diet-induced NAFLD mice [27]. However, the underlying mechanism of gallic as a compound on NAFLD is not well elucidated. In current study, gallic acid decreased TG accumulation in a NAFLD cell model, and was probably associated with an anti-oxidation effect. Furthermore, gallic acid decreased cytokine levels and regulated the expression of enzymes related to lipid metabolism at the protein and mRNA levels.

As a rate limiting enzyme of fatty acid synthesis, ACC2 is able to catalyze the carboxylation of acetyl-CoA to malonyl-CoA and suppresses the enzymatic activity of CPT-1A, another rate limiting enzyme of fatty acid oxidation [28]. The level of expression of ACC2 is regulated by AMPK. The activity of ACC2 is enhanced after the phosphorylation level of AMPK is elevated. Subsequently, phosphorylated ACC2 is capable of decreasing AMPK activity, reducing the inhibition on CPT-1A, strengthening fatty acid oxidation [29-31], and decreasing TG accumulation. In this experiment, GA effectively reduced the intracellular TG level in the NAFLD model, enhanced the level of phosphorylation of AMPK and ACC2, up-regulated the expression of CPT-1A, which suggested that GA enhanced the effect of lipid metabolism through regulation of the AMPK/ACC2/CPT-1A signaling pathway.

Oxidative stress and lipid peroxide are vital factors related to the incidence and progression of NAFLD. In the presence of IR, an increase in free fatty acids induces the elevated expression of the cytochrome enzyme, P4502E1, in liver microsomes, enhances β oxidation in mitochondria, and generates a substantial quantity of reactive oxygen species (ROS), which exceeds the elimination ability of the anti-oxidative system represented by GSH-PX. Then, the peroxidation chain is triggered to form MDA and alternative lipid peroxides (LPO) [32, 33]. MDA can form Mallory-Denk bodies via protein cross-linking, induce autoimmunoreaction, propel chemotaxis of HNE towards neutrophilic leukocytes, and cause inflammatory cell infiltration. In addition, accumulation of peroxide hydrolyzes ApoE100, reduces the transformation of TG into VLDL discharged from the liver, arouses biomembrane lipid peroxide, DNA oxidation and modification, and intracellular enzyme and protein denaturation, thus leading to nucleic acid and protein injury and even liver cell death [34]. In addition, the increase in LPO inhibits the anti-oxidative system, enhances the sensitivity of the host and cells to exogenous peroxide, increases the concentration of endogenous ROS and toxicity, forms a vicious cycle, aggravates inflammation

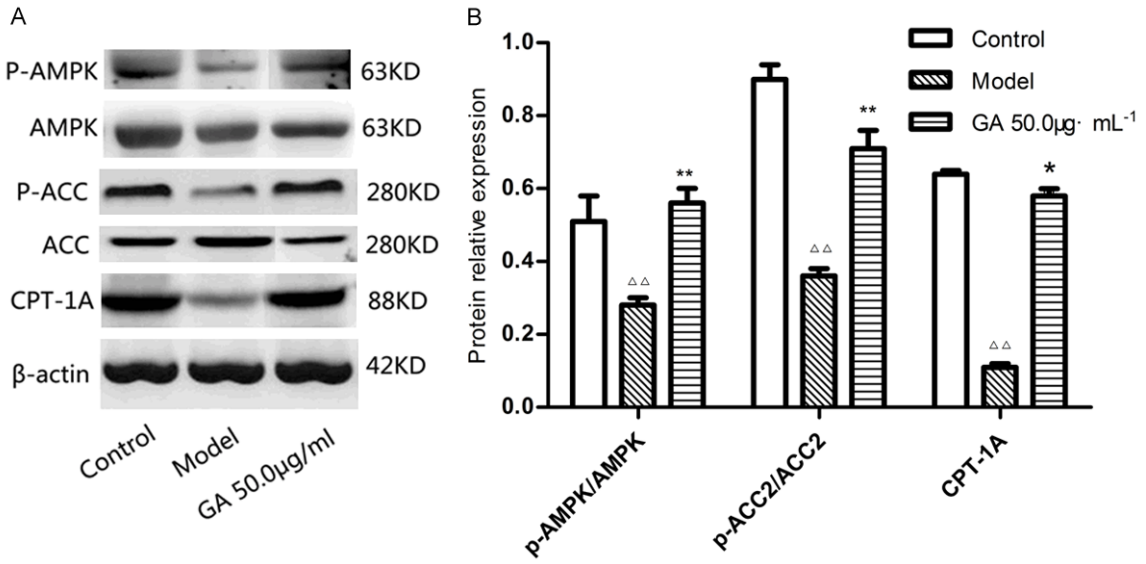

Figure 5. The effect of GA on p-AMPK/AMPK, p-ACC2/ACC2, and CPT-1A protein expression in the NAFLD model cell.

and necrosis, and activates Kupffer cells, which allows for the transformation of liver sternzellen into liver fibrocytes and promotes NAFLD progression into NASH and fibrosis [35, 36]. The anti-oxidizing ability of GA has been frequently reported. In current study, GA up-regulated the levels of SOD and GSH-PX in the NAFLD model, and reduced the concentration of MDA characterized by high anti-oxidizing ability, suggesting that GA probably serves as the second hit of the incidence and development of NAFLD.

A number of cytokines and proteins play a pivotal role in the incidence of NAFLD. A previous study has demonstrated that a high level of TNF-α is an independent risk factor for NAFLD [37]. The production of TNF-α is accompanied by increased FFA synthesis and the incidence of IR, which is considered to be a major cytokine of the progression of fatty liver disease into fatty hepatitis [38] and is intimately associated with inflammation and fibrosis [39]. TNF-α is able to reduce the activity of insulin receptor tyrosine kinases, and aggravates the severity of IR. TNF-α also participates in lipid peroxide and oxidative stress, induces chemotaxis to neutrophilic leukocytes, thus leading to a hepatic inflammatory response. In addition, TNF-α causes injury of liver cells via lipid peroxide and eventually leads to necrosis and the inflammatory response of liver cells. TNF-α has a crucial role in the development of simple fatty liver disease secondary to fatty hepatitis and liver fibrosis [40]. Wang et al. [41] reported that use of pioglitazone can alleviate fatty cell denaturation induced by a high-fat diet in the NAFLD rat model. IR mitigation was correlated with the reduction in the TNF-α level. Moreover, the correlation between IL-8 and NAFLD, liver injury, and hepatic cancer has been frequently reported in recent years [42, 43]. IL-8 is regarded as a vital cytokine for the development of a NAFLD medication. It has been demonstrated that the serum level of IL-8 is positively correlated with liver changes by laboratory and histologic examination and the severity of hepatic injury [44]. TNF-α, endotoxin, and multiple types of inducers lead to release of a large amount of IL-8, stimulates the release of cytokines via chemotaxis to inflammatory cells, and affects the pathologic progression of hepatic fibrosis indirectly by activating sternzellen. As a type of multi-functional chemokine of inflammatory reactions, IL-8 possesses the function of chemotaxis and activation of neutrophilic leukocytes, releases neutrophilic leukocyte elastase to directly damage hepatocytes, induces distortion, degranulation, and the lysosome release reaction of liver cells, generates ROS metabolites, arouses tissue and cellular infiltration, and gives rise to an inflammatory reaction [45, 46]. In the NAFLD model, GA can significantly down-regulate the intracellular levels of TNF-α and IL-8, suggesting that GA is capable of miti-

gating the incidence and progression of NAFLD by decreasing the levels of relevant cytokines.

In conclusion, the results of this study show that GA improves lipid metabolism in NAFLD model and investigate the potential mechanism of GA's effect. Therefore, we suggest that GA may prove useful as a therapeutic agent for lipid metabolism disorder like NAFLD. The findings that regulation of the AMPK/ACC2/CPT-1A signaling pathway and relevant cytokines by GA and its anti-oxidizing ability contribute to our current understanding of the mechanism underlying the effects of GA on lipid metabolisms.

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Disclosure of conflict of interest

None.

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