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# Harnessing the Therapeutic Potential of Ginsenoside Rd for Activating SIRT6 in Treating a Mouse Model of Nonalcoholic Fatty Liver Disease

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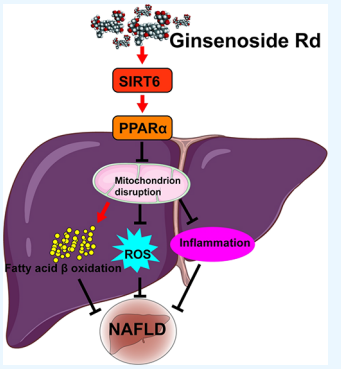
**ABSTRACT:** Nonalcoholic fatty liver disease (NAFLD) is a prevalent global condition and a common precursor to liver cancer, yet there is currently no specific medication available for its treatment. Ginseng, renowned for its medicinal and dietary properties, has been utilized in NAFLD management, although the precise underlying mechanism remains elusive. To investigate the effectiveness of ginsenoside Rd, we employed mouse and cell models to induce NAFLD using high-fat diets, oleic acid, and palmitic acid. We explored and confirmed the specific mechanism of ginsenoside Rd-induced hepatic steatosis through experiments involving mice with a liver-specific knockout of SIRT6, a crucial protein involved in metabolic regulation. Our findings revealed that administration of ginsenoside Rd significantly reduced the inflammatory response, reactive oxygen species (ROS) levels, lipid peroxide levels, and mitochondrial stress induced by oleic acid and palmitic acid in primary hepatocytes, thereby mitigating excessive lipid accumulation. Moreover, ginsenoside Rd administration effectively enhanced the mRNA content of key proteins involved in fatty acid oxidation, with a particular emphasis on SIRT6 and its target proteins. We further validated that ginsenoside Rd directly binds to SIRT6, augmenting its deacetylase activity. Notably, we made a significant observation that the protective effect of ginsenoside Rd against hepatic disorders induced by a fatty diet was almost entirely reversed in mice with a liver-specific SIRT6 knockout. Our findings highlight the potential therapeutic impact of Ginsenoside Rd in NAFLD treatment by activating SIRT6. These results warrant further investigation into the development of Ginsenoside Rd as a promising agent for managing this prevalent liver disease.




## 1. INTRODUCTION

NAFLD (nonalcoholic fatty liver disease) is a metabolic disease of unknown origins. The onset of this disease is often accompanied by other pathological changes, such as obesity, islet resistance, and so on. It is generally believed that nonalcoholic fatty liver disease is a serious metabolic disease. Without any intervention, there is a certain probability that nonalcoholic fatty liver disease can evolve into liver cirrhosis or even liver cancer. As globalization has intensified, the Western diet has spread all over the world. The incidence rate of nonalcoholic fatty liver disease is also increasing. According to reports, nearly a quarter of people in Europe suffer from nonalcoholic fatty liver and are subject to the inaccessibility of testing technology; there is an increase in the number of undetected nonalcoholic fatty liver disease cases.[1] Combined with recent research findings, so far, no drugs for the treatment of nonalcoholic fatty liver have been approved by world-class authorities, and managing the disease via lifestyle interventions, including weight loss and exercise, and reducing associated risk factors remain the primary treatment.[2] Therefore, there is an urgent need to find effective targets and drugs for the treatment of NAFLD.

In many studies on the pathogenesis of NAFLD, the multiple strike hypothesis is in the process of being established, and the hypothesis states that a variety of injuries, including nutritional factors, insulin resistance hormones, intestinal flora, and genetics, jointly induce NAFLD formation.[3,4] Among them, liver fat accumulation and inflammatory environment formation induced by multiple factors are indispensable pathological changes that occur throughout the disease.

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High-sugar and high-fat diets can induce changes in lipid deposition, insulin resistance, intestinal microbiota, etc. These changes can accelerate the synthesis of fatty acids in the body. When there are too many fatty acids, they are metabolized by the liver and eventually form fatty liver. When the liver accumulates too much fat, liver homeostasis fails, resulting in hepatocyte endoplasmic reticulum and mitochondrial stress; this in turn produces excess reactive oxygen species (ROS), increases the secretion of chemokines and inflammatory factors, and ultimately activates oxidative stress and inflammatory responses. Continuous chronic inflammation leads to structural damage and the functional destruction of liver tissue, which facilitates the development of liver fibrosis and eventually results in cirrhosis 3. Therefore, ROS play an important role in the pathogenesis of NAFLD. Reducing ROS may be one course of action for the treatment of NAFLD.

As a histone deacetylase dependent on nicotinamide adenine dinucleotide (NAD), SIRT6 is relatively conserved at the NAD-binding site. SIRT6 can deacetylate target proteins and is involved in the control of mammalian metabolic balance and stress response via specialized histone deacetylation.[5−7] Compared with the normal human body, the level of SIRT6 proteins in the adipose tissue of obese patients is significantly lower. Moreover, fat-specific SIRT6 knockout mice were more susceptible to obesity generated by a high-fat diet, with increased tissue inflammation. By increasing the phosphorylation and acetylation of FKHR, SIRT6 deletions can lower the transcriptional activity of FKHR relative to fatty triglyceride lipase, reduce lipolysis activity, and cause lipid buildup.[8] SIRT6 overexpression can reduce the excessive accumulation of fatty acids in the liver and insulin resistance. XBP1s is deacetylated by SIRT6 to protect the liver against oxidative-stress-induced damage, reducing steatosis-related pathological processes.[9] SIRT6 also responds to oxidative stress by activating NFE2L2, which increases the production of antioxidant enzymes and hence reduces inflammation.[10] In summary, many current studies have shown that the activation of SIRT6 can be a hopeful method in the intervention of NAFLD.

Ginseng is important in drug and food homology, and it is widely used for healthcare all over the world. Ginsenoside Rd, a component of Ginseng, has anti-inflammatory and antioxidant effects, and it is commonly used to treat cardiac hypertrophy, inflammatory bowel disease, ischemic brain injury, and other disorders.[11−13] Ginsenoside Rd can reduce oxidative stress, and this is related to inhibiting reactive oxygen species overproduction, increasing antioxidant enzyme activity, protecting mitochondrial membrane potential change, restoring calcium levels, and ameliorating mitochondrial dysfunction.[14] Furthermore, ginsenoside Rd can be used to treat diseases associated with metabolism; for example, it can reduce obesity induced by a high-fat diet in mice and ameliorate insulin resistance, which may be related to an increase in cyclic adenosine phosphate content in brown adipose cells as well as an increase in thermogenic genes expressed in brown adipose tissue, thus enhancing adaptive thermogenesis in mice.[15] However, current studies on the treatment of NAFLD by ginsenoside Rd are scarce, and its mechanism of action remains to be fully understood. Our study suggests that ginsenoside Rd can possibly act as an SIRT6 activator. By alleviating oxidative stress and inflammatory responses, the ginsenoside Rd treatment is effective in treating high-fat-diet-induced hepatic lipid metabolism disorders.

## 2. MATERIAL AND METHODS

**2.1. Ethics Statements.** The animal study was reviewed and approved by the animal experiment ethics committee of Guangzhou University of Traditional Chinese Medicine.

**2.2. Screening of Effective Dose of Ginsenoside Rd.** We used OA&PA modeling in primary liver cells to build models and set three different dose gradients of Ginsenoside Rd: 5 mg/kg (low dose), 10 mg/kg (medium dose), 15 mg/kg (high dose). Finally, the levels of TC and TG in cells and the content of Lipid TOX were measured.

**2.3. Animals.** The Animal Laboratory Center of Guangdong Province (Guangzhou, China) provided C57BL/6J mice aged 6−8 weeks. To generate liver-specific SIRT6 knockout mice, mice hybridization was carried out by using Cre recombinase and floxed SIRT6 alleles. We housed the animals under standard laboratory conditions (20−25°, 40−60% dampness, light−dark cycle every half day), and these mice have access to water and food. The normal group($n$ = 10) and high-fat group ($n$ = 12)were fed food containing 9% fat and food containing 60% fat, respectively, for 12 weeks. Mice that were diagnosed with NAFLD received intraperitoneally injected Rd or normal saline (control group) for 5 weeks. According to the guiding principles of the Animal Ethics Committee of Guangzhou University of Chinese Medicine, all above animal treatments were approved. Ginsenoside Rd was purchased from Shanghai Yuanye biology Co., Ltd. (Cas#52705-93-8,Lot# G21S11Y125049, HPLC > 98%). The purity of ginsenoside Rd is 99.7%.

**2.4. Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT).** We treated mice with abdominal cavity glucose injections (1 g/kg) after they fasted for 16 h overnight, and blood glucose was measured in the caudal vein after they were injected with glucose. During an insulin tolerance test (ITT), mice were given abdominal cavity injections of human insulin (0.75 IU/kg) after fasting for six hours, and their blood glucose data were obtained by blood collection at the end of the mice's tail. In this experiment, each group repeated 3 times($n$ = 3).

**2.5. Measurement of Total Cholesterol and Total Triglycerides.** Kits for determining serum and hepatic TG and TC content (A111-1-1, Nanjing Jiancheng Bioengineering Institute) and triglyceride content (A110-1-1, Nanjing Jiancheng Bioengineering Institute) were used to detect the contents of TC and TG, respectively. The values of TC and TG were calculated according to the instructions on the kit. In this experiment, each group repeated 3 times ($n$ = 3).

**2.6. Staining Using Hematoxylin−Eosin.** The steps in this experiment included the following: fixing the liver tissue in 10% paraformaldehyde, embedding it in paraffin, slicing it, staining it with HE, dehydrating it, and sealing it with transparent and neutral gum. Observations of pathological changes in liver tissue were carried out under a light microscope.

**2.7. Purification and Culture of Mouse Primary Hepatocytes (MPHs).** In mice anesthetized with a perfusion solution containing type II collagenase (2275GR001, Biofroxx) via the inferior vena cava, collagenase was perfused. Cultured liver cells were isolated and cultivated in an RMI1640 medium with 10% fetal bovine serum.

**2.8. Staining with Oil Red O.** MPHs were isolated and cultured with 300 $\mu$M oleic acid (OA) and 100 $\mu$M palmitic acid (PA) for 24 h, and this was followed by Rd treatment for


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24 h, after which the cells were rinsed twice with PBS, mixed with 4% paraformaldehyde, soaked in 60% isopropyl alcohol, and dyed with Oil Red O working liquor; an electron microscope was used in observations.

**2.9. Mitochondrial DNA Copy Number.** The Universal Genomic DNA Tiss kit was used to obtain total DNA from the liver (Tiangen, Beijing, China). The ratio of COXII mitochondrial coding protein to nuclear coding protein RPS18, which was employed as a metric of mitochondrial density, determined the copy number of mitochondrial DNA. In this experiment, each group repeated 3 times ($n$ = 3).

**2.10. Determination of Intracellular Reactive Oxygen Species (ROS).** For this step, fluorescence probe DCFH-DA (Sigma-Aldrich, St. Louis, MO, USA) was utilized. Cells were added with 10 $\mu$m DCFH-Da and incubated for 20 min in the dark; then, they were washed with PBS in triplicate. Intracellular ROS levels were analyzed under a fluorescence microscope.

**2.11. Molecular Modeling and Docking Research.** For computation and result analysis, Autodock software was used. The structure of ginsenoside Rd was obtained from the PubChem database (PubChem CID 24721561), and majorization using molecular mechanics software was implemented to achieve the most stable structure possible. The detailed crystal structure of SIRT6 (PDB DOI: 10.2210/pdb5Y2F/pdb) was obtained from the RCSB PDB database and edited using hydrogenation, side chain repair, charge addition, and embedded ligand extraction. The effect of molecular docking is evaluated using a cross-comparison of the total score and C score value. The Molcad program was used to investigate the binding mode and interaction mechanism between proteins and small molecules by calculating their surface properties.

**2.12. COIP.** Liver primary cells were lysed using RIPA lysis buffer containing PMSF. SIRT6 antibody (PPAR$\alpha$ antibody) was added to the lysate to allow specific binding with SIRT6 protein (PPAR$\alpha$ protein). Prior to adding the antibody to the lysis buffer, the antibody was pre-incubated with Protein A/G agarose beads for better separation and collection of the precipitate. IgG of the same species as the antibody was used as a positive control. The immunoprecipitation complex was washed multiple times with PBS to remove nonspecifically bound proteins and other interferences. After washing, the immunoprecipitation complex was separated from the solution by centrifugation. The precipitate was subjected to protein immunoblotting.

**2.13. Fatty Acid Oxidation Detection.** Soochow Pukang Biotechnology Co., Ltd conducted the tests. The supernatant fluid was gathered and treated with 1.3 M perchloric acid to conclude the procedure. The supernatant underwent acid base neutralization using 2 M KOH and 0.6 M MOPS after centrifugation. Finally, [3H] radioactivity was detected using scintillation liquid.

**2.14. ELISA Detection.** The total protein content was standardized, and serum TNF (MEIMIAN,Jiangsu, China) and the ketone body were assessed using the specified assay kit (QY1105, Hangzhou qiyu biological science and technology Co., LTD). In this experiment, each group repeated 3 times ($n$ = 3).

**2.15. Neutral Lipid Staining of Cells.** The cells were mixed with 4% paraformaldehyde, and then, they were incubated via LipidTOX neutral lipid staining (Invitgen) at room temperature for 30 min, sealed with an anti-fluorescence quenching agent containing DAPI and observed under a fluorescence microscope.

**2.16. Experiments on Lipid Buildup and Lipid Peroxidation.** BODIPY 493/503 (D3922, Thermo Fisher) and 581/591 (D3861, Thermo Fisher) were used to assess intracellular lipid accumulation and lipid peroxidation, respectively. MPHS was rinsed twice with a serum-free medium before adding the probe's working solution and incubating at 37 °C for 30 min in order to protect it from light. Photographs were obtained under a fluorescence microscope after two washes using serum-free media.

**2.17. Real-Time PCR Assays.** Total RNA was tissued using TRIzol, and the experimental system was set up to carry out reverse transcription and cDNA synthesis using All-In-One RT MasterMix (G490, abcam). Universal SYBR Green Fast qPCR Mix (RK21203) was used to run real-time quantitative PCR with actin as a blank control group, and $2^{-\Delta\Delta Ct}$ was utilized to compute the levels of target genes. The gene primer sequences used in this study are shown in Table 1. In this experiment, each group repeated 3 times ($n$ = 3).

**Table 1. Primers Used for Real-Time qRT-PCR**

| primer | sequences |
|---|---|
| Srebp-1c (m) | F1: GGAGCCATGGATTGCACATT |
| | R1: GGCCAGGGAAGTCACTGT |
| Fas (m) | F1: CTTGGGTGCTGACTACAACC |
| | R1: GCCCTCCCGTACACTCACTC |
| Acc (m) | F1: AGGAAGATGGCGTCCGCTCTG |
| | R1: GGTGAGATGTGCTGGGTCAT |
| SIRT1 (m) | F1: CAGTAGCACTAATTCCAAGTTCC |
| | R1: AATGCAGATGAGGCAAAGG |
| $\beta$-actin (m) | F1: ATGACCCAAGCCGAGAAGG |
| | R1: CGGCCAAGTCTTAGAGTTGTTG |

**2.18. Immunofluorescence Assay.** The samples were mixed for 20 min at room temperature with 4% paraformaldehyde and then treated for 15 min with 0.3% Triton before being incubated for 12 h at 4 °C in a dark environment using the primary antibody. The samples were incubated for 60 min at normal temperatures with a fluorescently tagged secondary antibody, and images were acquired using a fluorescence microscope on the next day.

**2.19. RNA Sequencing.** RNA sequencing was conducted at Berry Genomics Co., Ltd. Briefly, RNA was first transcribed into cDNA, and then, the cDNA transcribed by RNA was cloned by constructing a library. The sequencing platform can read the corresponding matched library and take out the base sequence of the fragment in each library. After assembly analysis, a complete RNA sequence was obtained.

**2.20. Western Blotting.** In the RIPA buffer with protease inhibitors, the samples were homogenized. BCA was used to determine protein concentrations, and a loading buffer was supplied in proportion. The protein was cooked for 5 min in a metal bath at 100 °C. Wet transfer was used to transfer the protein to the polyvinylidene fluoride PVDF membrane. SIRT6 (Proteintech, 67510-1-IG, 1:1000) or $\beta$-actin (Bioword, AP0060, 1:1000) antibodies were incubated on the membrane overnight at 4 °C. The levels of a second antibody increased, and it was incubated for 60 min at normal temperatures. The Bole Bio-RadXR Gel Imaging analysis system was used to visualize the proteins.



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**2.21. Statistic Analysis.** The data were provided as a mean with standard deviation (SD). An unpaired $T$ test or a nonparametric test was applied to compare the two groups (Mann−Whitney $U$ test). One-way ANOVA was utilized to examine the comparison between various groups. It was determined that $P < 0.05$ was statistically significant.

## 3. RESULTS

**3.1. In Primary Hepatocytes from Mice, Ginsenoside Rd Increases Lipid Metabolism.** In primary hepatocytes from mice, high dose ginsenoside Rd (15 mg/kg) increases lipid metabolism. OA&PA-incubated MPHs were employed to observe the contribution of ginsenoside Rd in aberrant fat metabolism. However, there was no significant difference in the effect of low and medium doses of ginsenoside Rd on fatty liver cells compared with the model group. High-dose ginsenoside Rd administration effectively lowered intracellular triglycerides (TGs) and total cholesterol (TC) levels in OA&PA-resultant MPHs, confirming our conjecture (Figure 1A). The lipid TOX assay confirmed the good pharmacological action of high-dose ginsenoside Rd against lipid deposition, demonstrating a dose-dependent increase in lipid catabolism in MPHs after the ginsenoside Rd treatment (Figure 1B). Following this, subsequent mechanism analysis revealed that the high ginsenoside Rd treatment validly suppressed the expression of lipogenic proteins, particularly fatty acid synthase (Fas) and acetyl CoA carboxylase (Acc), while increasing the content of proteins with respect to fatty acid oxidation, such as Cpt1b and Cpt1a, giving rise to a reduction in lipid accumulation in MPHs (Figure 1C). Overall, our findings suggest that high-dose ginsenoside Rd therapy reduces MPH lipid accumulation caused by OA&PA.

**3.2. In MPHs, Ginsenoside Rd Reduces Peroxidative Damage and Inflammation Caused by OA and PA.** The aberrant lipid peroxidation—which inhibited fatty acid oxidation, significantly accelerated the formation of ROS, and disrupted mitochondrial biogenesis—was found to be a major contributor to excessive intracellular lipid accumulation in recent research. One of the most important risks associated with NAFLD is lipoperoxidation. As a result, we investigated ginsenoside Rd's effects on the cellular lipoperoxidation system. The ginsenoside Rd treatment resulted in a decrease in lipid deposition and lipoperoxidation in OA&PA-incubated MPHs and a reduction in cellular ROS levels, implying a reduction in cellular redox dysfunction (Figure 2A). Furthermore, we discovered that ginsenoside Rd-treated OA&PA-incubated MPHs had improved mitochondrial biogenesis (Figure 2B, C). Furthermore, the ginsenoside Rd treatment successfully reduced the nuclear expression of NFκb-

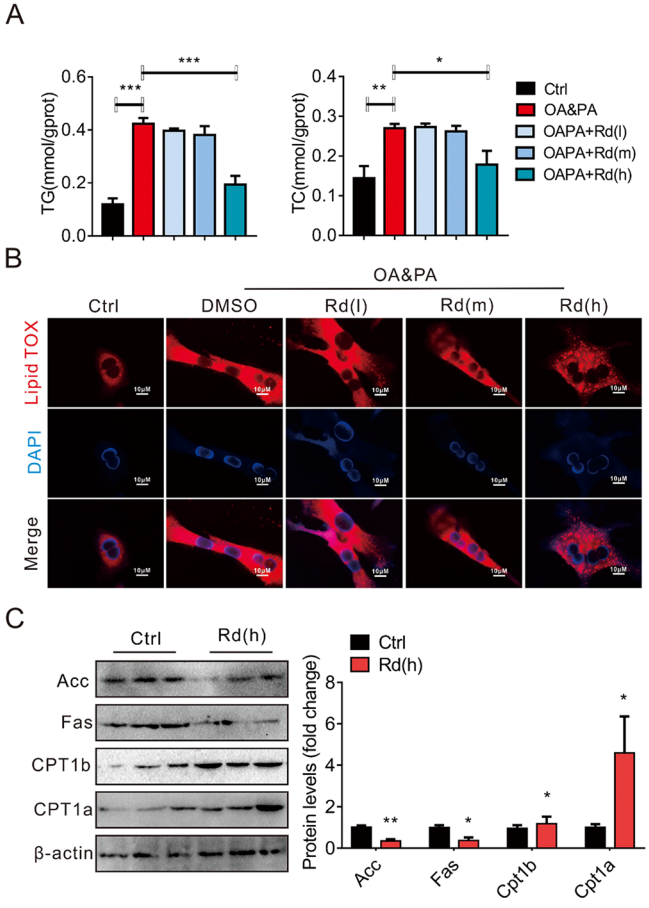


**Figure 1.** Ginsenoside Rd administration promotes lipid breakdown in primary hepatocytes from mice. (A) In OA&PA-incubated MPHs, ginsenoside Rd therapy lowered triglyceride and total cholesterol levels. (B) Representative lipid-TOX-stained pictures demonstrated that ginsenoside Rd therapy reduced lipid deposition in MPHs. (C) Ginsenoside Rd therapy affects lipid-metabolism-related protein expression levels. The mean ± SD of three independent experiments is used to show all data. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

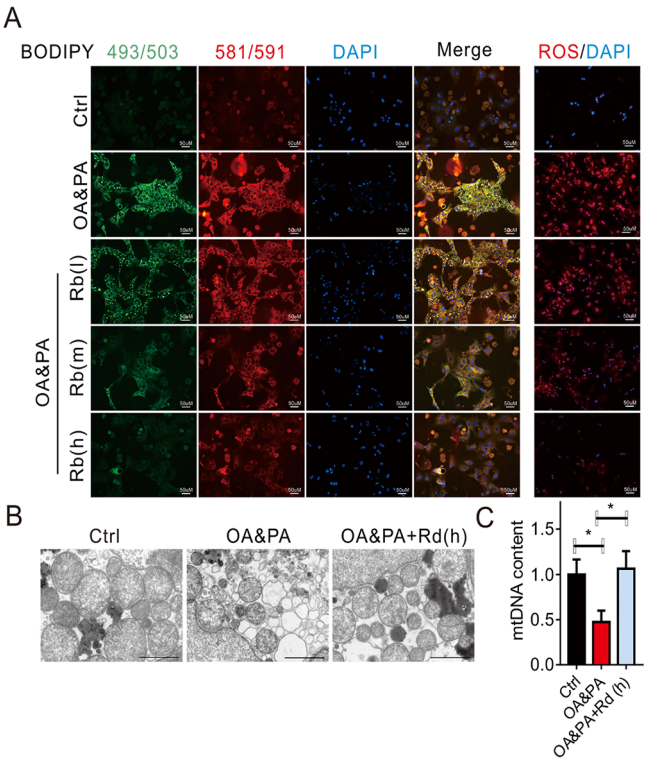


**Figure 2.** Ginsenoside Rd protects MPHs against OA&PA-induced peroxidative degradation from reducing ROS. (A) In OA&PA-incubated MPHs, ginsenoside Rb therapy reduced hepatic lipid accumulation (BODIPY 493/503), lipoperoxidation (BODIPY 581/591), and ROS levels. In MPHs, treatment with ginsenoside-Rd-enhanced mitochondrial structure (B) and biogenesis (C). The mean ± SD of three independent experiments is used to show all data. *$P < 0.05$.



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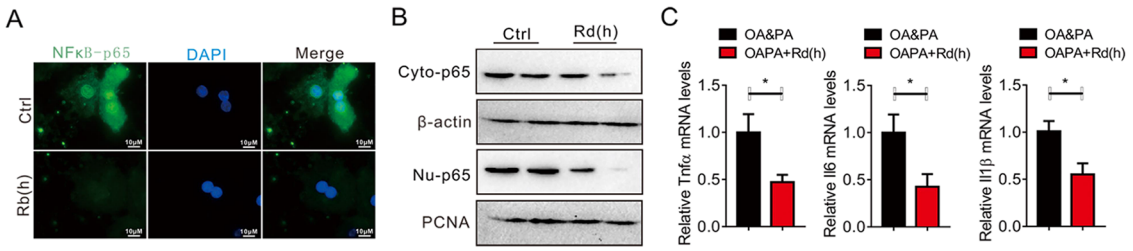


**Figure 3.** Ginsenoside Rd decreases inflammation in MPHs. (A and B) Ginsenoside Rb therapy lowered nuclear NFκb-p65 levels in MPHs, as exhibited by IF (A) and WB (B). (C) In OA&PA-incubated MPHs, ginsenoside Rd therapy reduced inflammation-related genes. The mean ± SD of three independent experiments is used to show all data. *$P < 0.05$.

p65, reducing the inflammatory response in OA&PA-incubated MPHs, as evidenced by lower inflammatory gene expression (Figure 3A, B). Ginsenoside Rd can also detect TNF $\alpha$, mRNA content of IL6, and IL1B (Figure 3C).Overall, these findings indicated that ginsenoside Rd might successfully reverse the peroxidative and inflammatory damage caused by OA&PA in MPHs.

**3.3. Ginsenoside Rd Has a Candidate Target in SIRT6.** In addition, our RNA sequencing and immunofluorescence study revealed that ginsenoside Rd administration improved nuclear SIRT6 expression in MPHs (Figure 4A,B). We performed a docking study between ginsenoside Rd and SIRT6 to carry out further efforts for exploring and refining the precise mechanism of ginsenoside-Rd-derived protective effects, and our results revealed a good binding affinity via hydrophobic contact (Figure 4C). Meanwhile, Western blotting results revealed that ginsenoside Rd therapy effectively improves SIRT6 deacetylase activity, as evidenced by lower H3K9 and H3K56 acetylation levels (Figure 4D). These findings suggest that ginsenoside Rd may operate as a novel SIRT6 activator by altering its expression and enzyme activity to promote lipid degradation. The COIP results suggested that ginsenoside Rd could increase the expression of Sirt6 and PPAR$\alpha$ and their binding to each other (Figure 4E).

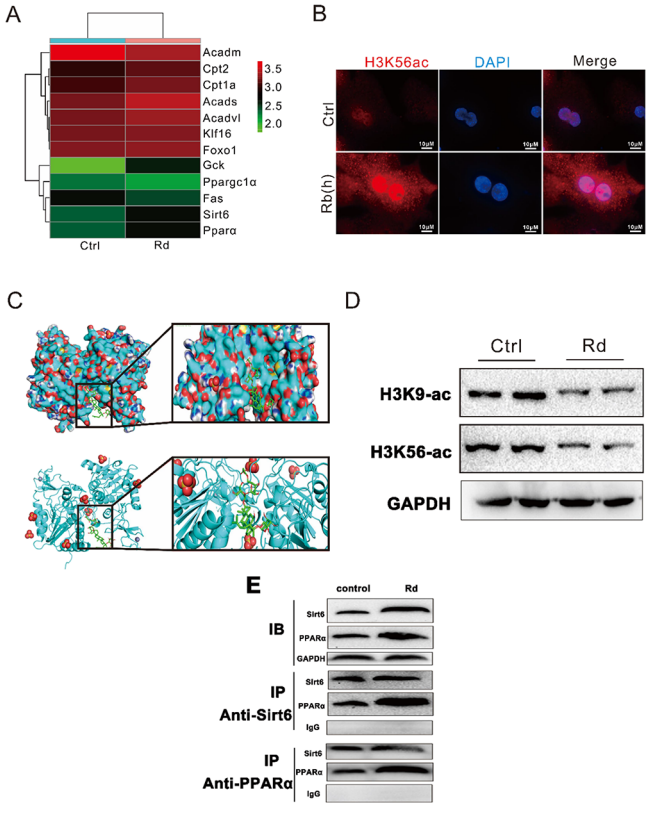


**Figure 4.** SIRT6 is a possible target for Ginsenoside Rd. (A) Heatmap of RNAseq on OA&PA-incubated MPHs with or without ginsenoside Rd treatment. (B) Treatment with ginsenoside Rd enhanced nuclear SIRT6 in MPHs. (C) Ginsenoside Rd and SIRT6 molecular docking model. (D) Ginsenoside Rd increases SIRT6 enzyme activity in MPHs, as evidenced by reduced H3K9 and H3K56 acetylation. (E) COIP results suggested that ginsenoside Rd can activate the protein expression of Sirt6 and PPAR$\alpha$ as well as the expression of the complex of these two proteins.

**3.4. Ginsenoside Rd Had No Effect on the Lipid Accumulation and Redox Imbalance Caused by OA&PA in SIRT6-Deficient MPHs.** Primary liver cells were extracted from the liver of SIRT6 knockout mice and cultured in a medium containing OA and PA for 24 h before being treated with ginsenoside Rd to further demonstrate the ginsenoside Rd function via the SIRT6-mediating pathway. SIRT6 deficiency, as observed in Fat TOX staining and TG content detection in primary liver cells, prevents resultant ginsenoside-Rd therapeutic effects on lipid buildup in primary liver cells (Figure 5A,B). Furthermore, in SIRT6-deficient primary liver cells, ginsenoside Rd administration has no effect on the expression of genes implicated in the oxidation of fatty acid (Figure 5C). Furthermore, due to deficiency, the effective pharmacological action of ginsenoside Rd on lipoperoxidation and ROS generation was reduced in these MPHs, as were the effects on mitochondrial structure and biogenesis (Figure 5D−F). Furthermore, ginsenoside Rd administration had no effect on inflammatory stress in SIRT6-deficient MPHs, as evidenced by the expression of NFκb-p65 and the constancy of other inflammatory genes (Figure 6−C). Overall, our findings showed that ginsenoside Rd has an SIRT6-dependent protective effect in MPHs against OA&PA-induced cellular damage.

**3.5. Ginsenoside Rd Protects Mice from Metabolic Harm Caused by a Fatty Diet.** In MPHs, we detected a considerable increase in PPAR$\alpha$ and fatty acid oxidation rate following ginsenoside Rd, indicating that ginsenoside Rd may have a potential therapeutic effect on NAFLD (Figure 7A,B). Our findings showed that ginsenoside Rd therapy lowered the body weight in mice on the HFD while also increasing serum ketone body levels, implying that ginsenoside Rd may increase fatty acid oxidation in these mice (Figure 7C,D). Our biochemical investigation also revealed lower hepatic and serum TG and TC content in ginsenoside-Rd-treated HFD animals, implying decreased lipid accumulation in the liver, as indicated by H&E and Oil Red O staining (Figure 7E,F). In terms of mechanisms, we discovered that the ginsenoside Rd


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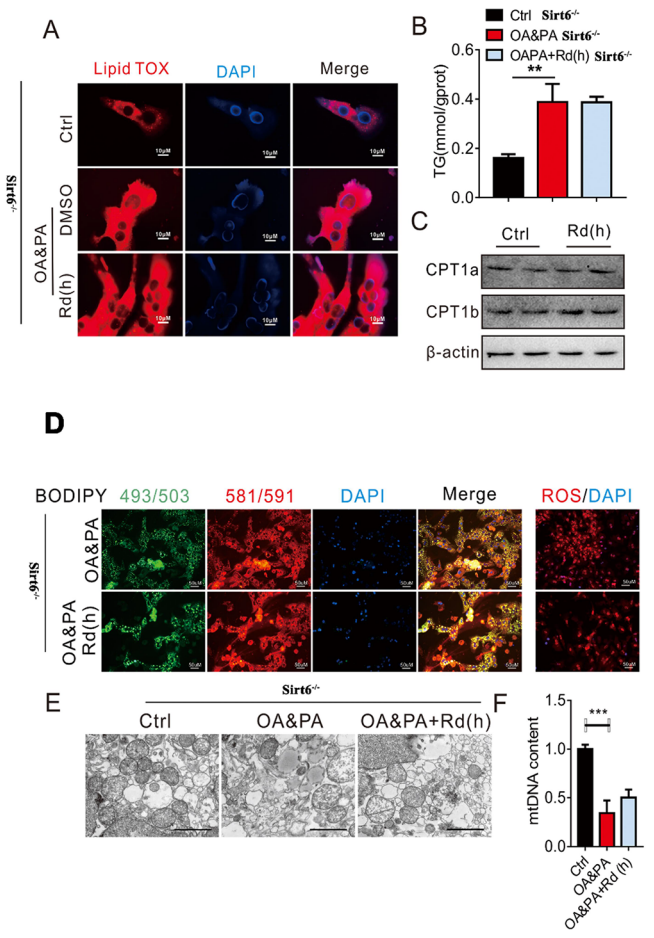


**Figure 5.** Ginsenoside Rd has no effect on the lipid metabolism's abnormality caused by OA&PA in SIRT6-deficient MPHs. (A) SIRT6 deficiency eliminates the therapeutic benefits of ginsenoside Rd on lipid accumulation in MPHs. (B) Treatment with ginsenoside Rd fails to lower TG levels in MPHs that are generated by SIRT6-deficient mice. (C) Ginsenoside Rd administration has little effect on SIRT6-deficient MPHs' fatty acid oxidation genes. (D) Ginsenoside Rb therapy has no effect on hepatic lipid accumulation (BODIPY 493/503), lipoperoxidation (BODIPY 581/591), or ROS levels in SIRT6-deficient MPHs treated with OA&PA. In OA&PA-incubated SIRT6-deficient MPHs, ginsenoside Rd administration had no effect on the mitochondrial structure (E) or biogenesis (F). The mean ± SD of three independent experiments is used to show all data. ***$P < 0.001$.

treatment significantly boosted lipogenic genes and fatty acid oxidation genes in HFD mice, and a significant upregulation of SIRT6 was observed (Figure 7G,H). Furthermore, after ginsenoside Rd therapy, our biochemical examination of serum ALT and AST revealed an improvement in hepatic function in HFD mice (Figure 8A). Furthermore, ginsenoside Rd therapy decreased glucose intolerance and insulin resistance in HFD animals (Figure 8B,C). Notably, ginsenoside Rd therapy successfully reduces inflammatory stress in HFD rats, as evidenced by lower hepatic F4/80 expression and serum TNF levels (Figure 8D,E). These findings suggested that ginsenoside Rd could help mice with HFD-induced NAFLD.

**3.6. Hepatic SIRT6 Deficiency Reduces the Protective Effects of Ginsenoside Rd against Hepatic Metabolic Damage Caused by the HFD.** In SIRT6-deficient MPHs, ginsenoside Rd administration has no effect on the cellular expression of PPAR or fatty acid oxidation rate, showing that ginsenoside Rd actions are abolished after SIRT6 deletion (Figure 9A). When Sirt6 was knocked out, the effects of high doses of ginsenoside Rd on fatty acid oxidation rate, body weight, serum ketone bodies, and serum and liver TC and TG contents were not significantly changed (Figure 9B−E), while the hepatic pathology of fatty liver mice was not significantly alleviated (Figure 9F). After SIRT6 knockdown, ginsenoside Rd did not significantly improve the mRNA of genes related to glucolipid metabolism and oxidative stress (Figure 9G,H). Furthermore, serum ALT and AST levels remained unaltered, indicating that ginsenoside Rd did not improve hepatic function in SIRT6-deficient animals fed an HFD (Figure 10A). Ginsenoside Rd had no effect on glucose intolerance or insulin resistance in these mice due to the SIRT6 gene's deletion (Figure 10B,C). In SIRT6-deficient mice, treatment with ginsenoside Rd failed to change the inflammatory condition (Figure 10D,E).

## 4. DISCUSSION

NAFLD is a prevalent liver condition that is one of the leading causes of cirrhosis and hepatocellular cancer.[16] However, there is currently no specific medicine for the treatment of NAFLD.[17] Our findings demonstrated that ginsenoside Rd could reduce lipid accumulation in an NAFLD model, and the mechanism is linked to the SIRT6 signaling pathway. An NAFLD in vivo and in vitro model was created to investigate the therapeutic mechanism of ginsenoside Rd on NAFLD. Ginsenoside Rd at high doses reduced lipid buildup, ROS levels, inflammation, and increased fatty acid oxidation in vitro. Ginsenoside Rd promoted mitochondrial DNA replication. Ginsenoside Rd, on the other hand, inhibited the production

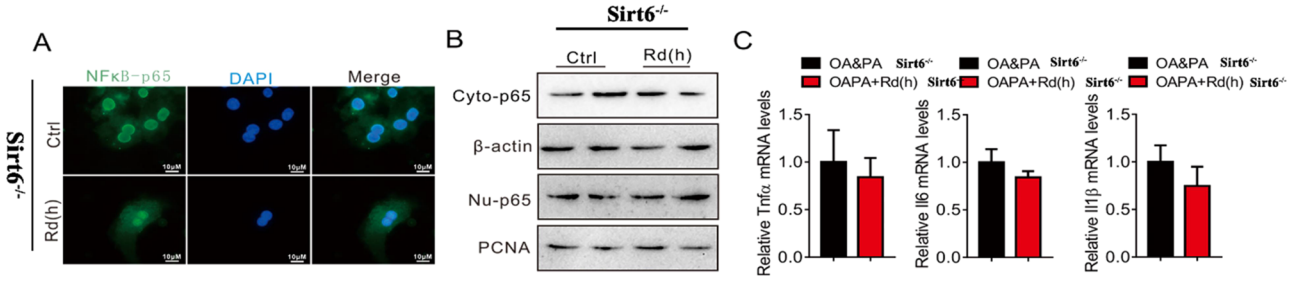


**Figure 6.** Ginsenoside Rd had no effect on inflammatory levels in SIRT6-deficient MPHs. (A and B) In OA&PA-incubated SIRT6-deficient MPHs, the ginsenoside Rd treatment lowered nuclear NFκb-p65 levels, as exhibited by IF (A) and WB (B). (C) After OA&PA therapy, ginsenoside Rd has no effect on genes associated with inflammation in SIRT6-deficient MPHs. The mean ± SD of three independent experiments is used to present all data.



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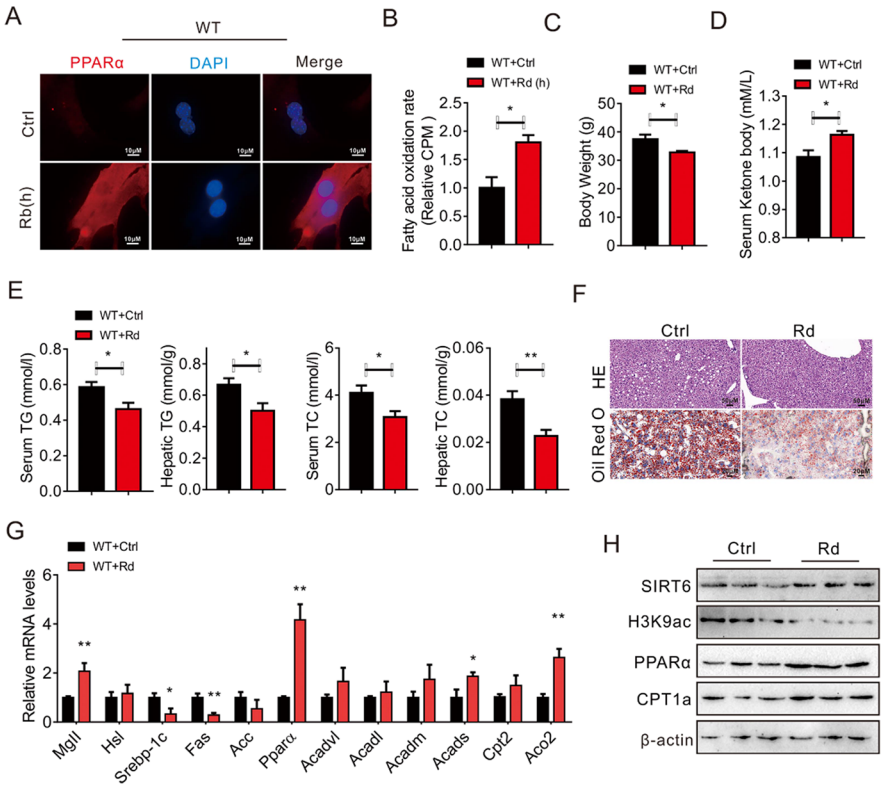


**Figure 7.** Ginsenoside Rd alleviates high-fat-diet-induced lipid disorders in mice by increasing fatty acid $\beta$ oxidation. (A and B) Ginsenoside Rd treatment increased both PPAR$\alpha$ levels (A) and the rate of cellular fatty acid $\beta$ oxidation (B) in MPHs. (C and D) Ginsenoside Rd treatment reduced body weight (C) and increased serum ketone body levels (D) in mice fed a high-fat diet. (E) Ginsenoside Rd treatment decreased serum and hepatic TG levels while slightly decreasing serum and hepatic TC content in HFD mice. (F) H&E and Oil Red O staining revealed that the ginsenoside Rd treatment reduced HFD-induced lipid deposition. (G and H) Ginsenoside Rd treatment alters genes related to lipid metabolism in mRNA (G) and protein levels (H). The mean ± SD of three independent experiments is used to show all data. *$P < 0.05$; **$P < 0.01$.

of inflammatory proteins. Furthermore, transcriptome sequencing revealed that in the ginsenoside-Rd-treated group, the SIRT6 glucolipid metabolic protein was substantially upregulated. In addition, ginsenoside Rd decreased the acetylation of the SIRT6 substrate in the liver, and molecular docking revealed that ginsenoside Rd and SIRT6 were well docked. Ginsenoside Rd reduced lipid buildup, increased lipid oxidation and liver function, decreased blood glucose, and enhanced key protein indicators of glucolipid metabolism in wild mice. Furthermore, the aforesaid effects of ginsenoside Rd were clearly eliminated when primary liver cells from mutant mice SIRT6 were employed. High-quality studies[7,8] have shown that SIRT6 can regulate PPAR$\alpha$ to reduce the level of reactive oxygen species and inflammation in NAFLD cells. This study showed that ginsenoside Rd alleviated mitochondrial metabolism under stress and reduces the production of ROS via the SIRT6/PPAR$\alpha$ signal. The decrease in cellular ROS levels and inflammatory reactions, as well as the enhancement of fatty acid $\beta$ oxidation, can ameliorate the symptoms of nonalcoholic fatty liver.

There is an association between nonalcoholic fatty liver disease (NAFLD) and certain types of cancer. The progressive form of NAFLD, known as nonalcoholic steatohepatitis (NASH), increases the risk of hepatocellular carcinoma (HCC).[18] Additionally, NAFLD may be linked to an elevated risk of bladder, pancreatic, colorectal, and breast cancers.[19−22] Controlling NAFLD can potentially reduce the prevalence of these cancer types. Therefore, it is crucial to address NAFLD to mitigate the associated cancer risks.

In addition to Sirt6, the Sirt family encompasses members Sirt1 through Sirt7, each playing multiple important roles within cells. These roles include cellular metabolic regulation, participation in apoptosis and cell survival, involvement in DNA repair processes, influence on stem cell function, and relevance to aging regulation[23] For instance, Sirt4 is involved in metabolic regulation, particularly regarding glucose and lipid metabolism. It influences energy metabolism by inhibiting the gluconeogenesis pathway and fatty acid oxidation.[24] Furthermore, Sirt4 affects islet function by suppressing insulin secretion and regulating the insulin signaling pathway.[24] Lastly, Sirt4 exhibits anti-oxidative stress effects by safeguarding cells through the regulation of mitochondrial antioxidant stress responses.[24]

SIRT4 is generally considered to have antioxidant effects, thereby reducing reactive oxygen species (ROS) production in hepatocytes. However, there are studies indicating that SIRT4 can increase ROS levels.[25] Possible reasons for this discrepancy include variations in the role of SIRT4 across different cell types, tissue environments, and experimental conditions. Additionally, different experimental models and methods used in studies may contribute to differences in results. It is important to note that the majority of studies support the notion that SIRT4 has an antioxidant effect, reducing ROS production.[26] It achieves this through the regulation of mitochondrial function, maintenance of oxidative phosphorylation homeostasis, and inhibition of ROS production pathways. Nonetheless, further research is required to fully comprehend the precise mechanisms underlying SIRT4's role


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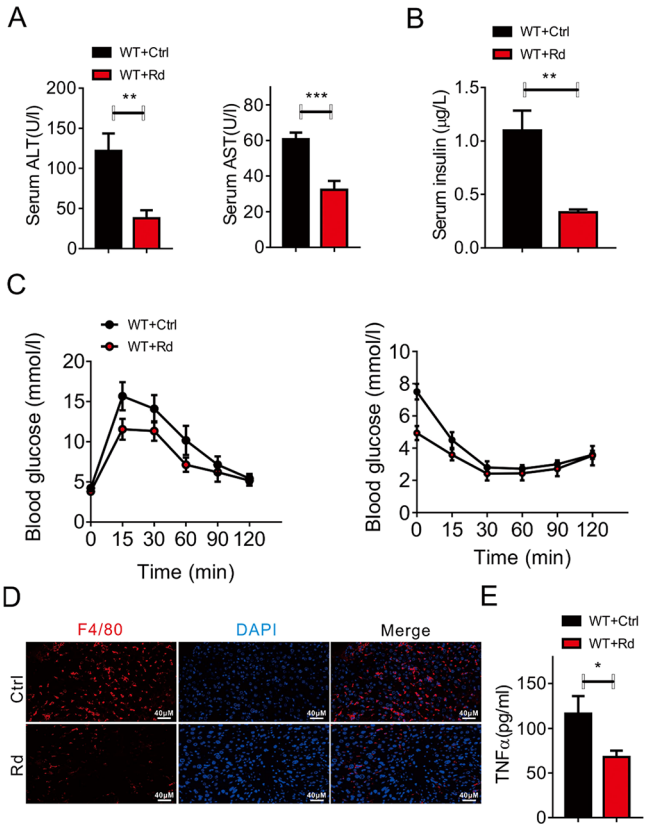


**Figure 8.** Ginsenoside Rd improves hepatic function and insulin resistance in HFD mice. (A) In HFD mice, ginsenoside Rd treatment decreased serum ALT and AST levels. (B) Ginsenoside Rd treatment reduced HFD mice's serum insulin levels. (C) Treatment with ginsenoside Rd improved HFD mice's glucose tolerance and insulin sensitivity. (D and E) In HFD mice, ginsenoside Rd treatment reduced hepatic inflammatory F4/80 expression (D) and serum TNF levels (E). The mean ± SD of three independent experiments is used to present all data. $^{*}P < 0.05$; $^{**}P < 0.01$.

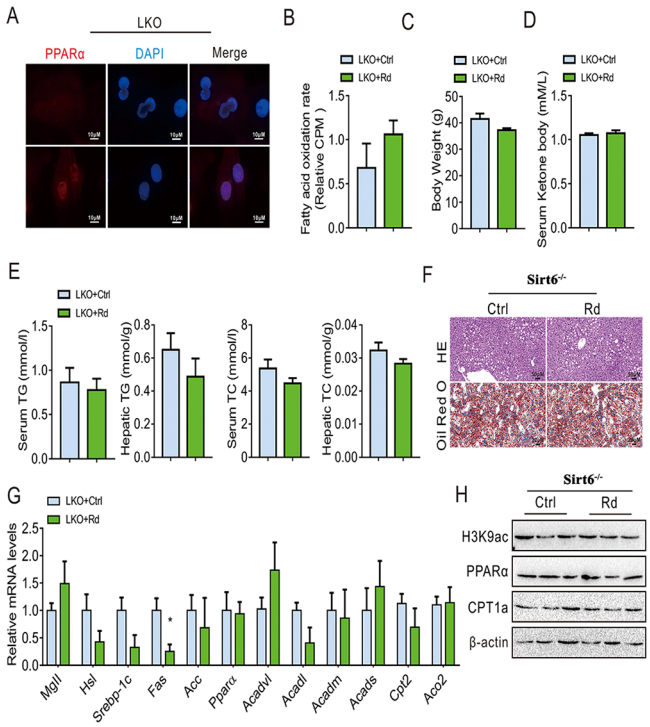


**Figure 9.** Due to SIRT6 deficiency, ginsenoside Rd failed to affect lipid catabolism in vivo and in vitro. (A and B) Ginsenoside Rd treatment does not change PPARα expression (A) or fatty acid oxidation rate (B) in SIRT6-deficient MPHs. (C and D) In SIRT6-deficient mice on HFD, ginsenoside Rd treatment has no effect on body weight (C) or serum ketone body levels (D). (E) With respect to SIRT6 deficiency, ginsenoside Rd treatment has no effect on hepatic and serum TG and TC contents. (F) HE and Oil Red O staining show that ginsenoside Rd treatment has no effect on lipid deposition in the liver of SIRT6-deficient mice. (G and H) Ginsenoside Rd treatment fails to change genes involved in lipid metabolism at both the mRNA (G) and protein (H) levels in mice.

in ROS regulation and its functional performance under various conditions.

Steatosis, lipotoxicity, inflammation, and insulin resistance are important pathological factors aggravating NAFLD.[27,28] In this research study, we discovered that ginsenoside Rd reduced the inflammatory response of fatty hepatocytes. Inflammatory responses increase the production of ROS and insulin resistance. However, these in turn aggravate the inflammatory response.[29] Ginsenoside Rd lessened fatty acid synthesis, promoted gluconeogenesis, and significantly improved glucose tolerance. Lipid accumulation in the liver can aggravate inflammatory responses, which in turn increase lipid accumulation and form a vicious circle.[30] A large number of research studies showed that PPARα can improve insulin resistance and dyslipidemia in NAFLD.[31−34] Hence, PPARα agonists have been clinically tested as therapeutic targets in the treatment of NAFLD.[35] Mitochondria can not only produce ROS but also clear ROS. Mitochondria play an important role in scavenging ROS in cells.[36] Mitochondrial dysfunction can lead to the excessive production of ROS and oxidative stress.[37] Oxidative stress is considered a key mechanism in the pathogenesis of many diseases, including NAFLD.[38] In conclusion, mitochondria play a key role in maintaining the physiological level of intracellular ROS. Moreover, many studies have shown that mitochondria play an antioxidant role via PPARα.[39−41] Coincidentally, our findings suggested that ginsenoside Rd strengthened fatty acids β-oxidation by PPARα. The present study confirmed the findings that the PPARα-mediated signaling pathway is involved in the antioxidant effect of mitochondria. The results showed that ginsenoside Rd increased the copy number of mitochondrial DNA, promoted the oxidative decomposition of liver lipids, and reduced the level of ROS.

SIRT6 has important functions in regulating liver glucolipid metabolism.[42] SIRT6 knockout mice are prone toward exhibiting disordered glucolipid metabolism.[43] A study showed that the expression of SIRT6 in obese patients was significantly lower than that in normal people.[44] The tolerance of SIRT6 knockout mice to HFD was significantly lower than the normal group, and they were more likely to gain metabolic diseases such as NAFLD.[45] Further studies have proved that SIRT6 activates PPARα to promote fatty acids, and β oxidizes and inhibits pyruvate oxidation.[46]

In our study, we demonstrated that ginsenoside Rd can interact with SIRT6 and inhibit its ubiquitination and degradation. Then, we established SIRT6 KO mice and treated them with HFD. The results showed that ginsenoside Rd ameliorated hepatocyte glucose, lipid metabolism, and inflammation. In contrast, the above-mentioned pharmacological effects of ginsenoside Rd were significantly weakened when SIRT6 KO mice were used. Together, the present


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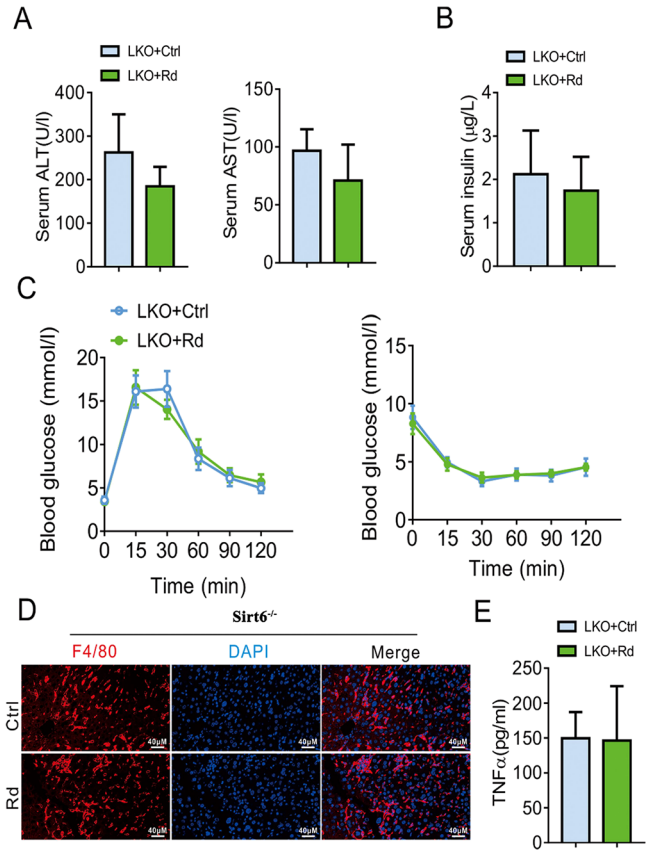


**Figure 10.** Ginsenoside Rd's protective effects against HFD-induced hepatic metabolic damage and insulin resistance are diminished in the presence of hepatic SIRT6 deficiency. (A) Ginsenoside Rd does not lower serum ALT and AST levels in SIRT6-deficient mice fed a high-fat diet. (B) Because of SIRT6 deficiency, ginsenoside Rd treatment fails to affect serum insulin levels in HFD mice. (C) Ginsenoside Rd treatment does not improve glucose tolerance and insulin sensitivity in SIRT6-deficient mice fed a high-fat diet. (D and E) Ginsenoside Rd treatment does not affect hepatic F4/80 levels (D) or serum TNF levels (E) in HFD mice due to SIRT6 deficiency. The mean ± SD of three independent experiments is used to present all data.

findings confirm that SIRT6 is an important mediator of ginsenoside Rd in the treatment of NAFLD.

Recently, the role of ginsenosides in glycolipids has attracted extensive attention. Studies have proved that ginsenosides Rg2, Rg3, Rg5, Rh1, Rh2, and Rh4 play a role in ameliorating abnormal glucose, lipid metabolism, and insulin resistance.[47−52] Our previous research studies found that ginsenoside Rg2 and ginsenoside Rc treat NAFLD via SIRT1 and SIRT6, respectively.[46,53] Perhaps the SIRT family may be a common target of ginsenosides in metabolic diseases.

## 5. CONCLUSIONS

This study provides evidence on how ginsenoside Rd ameliorates abnormal liver glycolipid metabolism and provides liver protection. It was confirmed that the above effects of ginsenoside Rd were mediated by SIRT6. Therefore, ginsenoside Rd is expected to be a new drug for the treatment of glucose and lipid metabolism diseases, including NAFLD. However, the detailed pathogenesis of NAFLD remains to be uncovered, and a more rigorous clinical experimental design of ginsenoside Rd needs to be applied in our future studies.

## ■ ASSOCIATED CONTENT

### Data Availability Statement

Data supporting the results of this study are available from the corresponding author upon reasonable request.

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### Author Contributions

In this article, T.C. is responsible for data statistics and writing; X.X., Z.P., and K.T. are responsible for the experiment; Y.Z., Y.C., J.G., and S.D. are responsible for image processing; G.Z., T.L., and X.L. are responsible for language proofreading; M.W., C.L., and D.Z. guided the entire experiment and were involved in writing. T.C., X.X., and Z.P. contributed equally to this work.

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### Notes

The authors declare no competing financial interest.

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