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Research Paper

Hepatoprotective effects of geniposide in a rat model of nonalcoholic steatohepatitis

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Abstract

Objectives Nonalcoholic steatohepatitis (NASH), a metabolic disorder of the liver, may gradually evolve into fibrosis or cirrhosis. Recent studies have suggested that geniposide can effectively inhibit experimental liver fibrosis. Therefore, the aim of this study was to determine whether geniposide can influence the early phase of fibrogenesis in an animal model of NASH.

Methods Male Sprague–Dawley rats were given a high fat diet alone or the same diet combined with geniposide at doses of 25, 50 or 100 mg/kg for six weeks. Ten rats received corresponding solvent as a normal control.

Key findings Treatment with geniposide could improve liver histology through reducing the elevated liver index (liver weight/body weight), serum alanine aminotransferase and aspartate aminotransferase. Total cholesterol, triglycerides and free fatty acids in serum and liver decreased in geniposide-treated rats. Furthermore, geniposide increased serum insulin levels but reduced serum tumour necrosis factor-α level in high-fat diet rats. In addition, geniposide suppressed expression of CYP2E1 and increased peroxisome proliferator-activated receptor-α (PPARα) expression. These benefits may be associated with increased superoxide dismutase and decreased malondialdehyde in liver.

Conclusions Geniposide exerts protective effects against hepatic steatosis in rats fed with a high fat diet; the underlying mechanism may be associated with its antioxidant actions or regulation of adipocytokine release and expression of PPARα.

Keywords geniposide; lipid peroxidation; non-alcoholic steatohepatitis (NASH); rats

Introduction

With increased aging and weight-gain in our population, obesity and diabetes have become frequently associated with non-alcoholic fatty liver disease (NAFLD). NAFLD involves a wide spectrum of liver disease, ranging from simple steatosis to steatohepatitis and cirrhosis.[1–3] Non-alcoholic steatohepatitis (NASH) forms the borderline between a benign condition (steatosis) and a serious/morbid condition (cirrhosis).[4] Although the exact pathogenesis of NASH is yet to be understood, several studies have focused on strongly related markers, such as lipid peroxidation, reactive oxygen species (ROS) production, secretion of inflammatory cytokines and collagen deposition, to confirm the diagnosis of NASH or to develop new treatment strategies.[5] Among these strategies, the use of complementary and alternative medicines, such as natural antioxidants and hepatoprotective plant products, has been popular in the last decade.[6]

Geniposide is an iridoid glucoside extracted from *Gardenia jasminoides* Ellis. Fruits, which have been used as a herbal medicine to treat liver and gall bladder disorders, such as hepatitis and acute jaundice, as well as inflammation and fever, in Chinese medicine for many years.[7–9] A clinical study has shown that crude *Gardenia* extract rapidly lowers serum bilirubin and transaminase levels in jaundice-induced acute hepatitis. A recent pilot study has also illustrated the effectiveness of geniposide in reducing insulin resistance and plasma markers of liver fibrosis in patients with NAFLD.[10,11] However, no detailed analysis of the beneficial effect and mode of action of geniposide has ever been reported in the context of NASH. Thus, this study was conducted with the objective of evaluating the efficacy of the geniposide in protecting the liver against diet-induced NASH in rats.

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Taotao Ma and Cheng Huang contributed equally to this work.

Materials and Methods

Reagents

Geniposide was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Animals and treatment

Male Sprague–Dawley rats, 200 ± 20 g, were obtained from the Experimental Animal Center, Anhui Medical University. They were housed in plastic cages under standardized conditions of temperature (21–22°C) and humidity (40–60%) with a 12-h light–dark cycle. The study and procedures were approved by the Ethic Committee and the Animal Experimental Committee of Anhui Medical University. All rats received human care in compliance with the Animal Experiments Guidelines and Animal Care of Chinese Academy of Sciences. The rats were given a normal diet for one week while adapting to vivarium conditions and then randomly divided into five groups ($n = 10$ per group): normal control group, NASH group and three treatment groups (geniposide 25, 50 and 100 mg/kg). The NASH model was induced by a high-fat emulsion as previously reported with a slight modification.[12] Briefly, rats were orally treated with the high-fat emulsion (10 ml/kg) at 0800 h each day for six weeks, with the exception of the normal control group, which received an equal volume of saline alone. The macronutrients present in this emulsion were as follows: corn oil 400 g; saccharose 150 g; total milk powder 80 g; cholesterol 100 g; sodium deoxycholate 10 g; Tween 80 36.4 g; propylene glycol 31.1 g; vitamin mixture 2.5 g; cooking salt 10 g; mineral mixture 1.5 g; distilled water 300 ml. In the treatment groups, 25, 50 or 100 mg/kg of geniposide was given via gavage at 1800 h each day to the NASH rats after the first week. The normal control and NASH groups were given an equal volume of 0.5% CMC-Na solution (5 g CMC-Na powder dissolved in 1000 ml water). After six weeks, rats were sacrificed. Serum was collected and stored at −20°C. The liver and spleen were immediately removed and weighed to calculate the liver and spleen indexes. Specimens of rat isolated livers were fixed in formaldehyde and stored in liquid nitrogen.

Biochemical determination

Serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), triglyceride (TG), free fatty acids (FFA), high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), liver homogenate FFA, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) were determined using commercial analysis kits obtained from the Jiancheng Institute of Biotechnology (Nanjing, China) and Furui Institute of Biotechnology (Beijing, China). Hepatic concentrations of TC and TG were also measured after chloroform–methanol extraction. Serum insulin and tumour necrosis factor-α (TNFα) levels were measured by using radioimmunoassay kits obtained from the Northern Bioengineering Institute (Beijing, China).

Histological staining

The liver specimens were fixed with 10% neutral formalin and embedded in paraffin. Hematoxylin and eosin (HE) staining was performed according to a standard procedure. The pathological changes were assessed and photographed under an Olympus BX-51 microscope. Liver biopsy was scored according to Brunt *et al*.[13] as follows: 0, no steatosis, normal liver; I, >25% of hepatocytes affected; II, 26–50% of hepatocytes affected; III, 51–75% of hepatocytes affected; IV, >76% of hepatocytes affected. Pathology was scored in a blinded manner by two independent pathologists with expertise in rodent liver.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of TNFα, CYP2E1, proliferator-activated receptor-α (PPARα) and β-actin in the liver were analysed by reverse transcriptase-polymerase chain reaction (RT-PCR) coupled with agarose gel electrophoresis. Briefly, total RNA was isolated from liver using an RNA isolation kit. Sequences and accession numbers of primers used are summarized in Table 1. The resulting single-stranded cDNA (5 ml) was denatured at 94°C for 5 min and, after the addition of the polymerase, subjected to 32 cycles of amplification, each consisting of 40 s at 94°C, 40 s at 51°C and 1 min at 72°C, with a 10-min final extension at 72°C during the last cycle. Four or five samples were chosen randomly from each group and the assay of every gene in each sample was replicated three times. The rat β-actin gene was amplified as a loading control. The PCR products were fractionated by electrophoresis on an agarose gel, visualized by ethidium bromide staining and photographed using a Kodak DC120 digital camera,

Table 1 Sequences of the primers used in the polymerase chain reaction measurements

Target mRNA	Accession No.	Primer	Sequence	Product length (bp)
β-actin	NM_031144	Sense	5′TGGAATCCTGTGGCATCCATGAAAC3′	350
		Antisense	5′ACGCAGCTCAGTAACAGTCCG3′	
TNFα	BC107671.1	Sense	5′AGAACTCCAGGCGGTGTCT 3′	485
		Antisense	5′TCCCTCAGGGGTGTCCTTAG 3′	
PPARα	NM_001113418.1	Sense	5′TGCATGTCCGTGGAGACCGTCAC 3′	523
		Antisense	5′ACTCGGTCTTCTTGATGACC 3′	
CYP2E1	NM_012775	Sense	5′GTCTGAGGCTCATGAGTTTG 3′	628
		Antisense	5′TCTGGAAACTCATGGCTG 3′	

Nucleotide sequence of primers used for RT-PCR. Position is defined as the 5-nucleotide of the respective primer related to the source sequence.

scanned by the Kodak Electrophoresis Documentation and Analysis System (EDAS 120), and quantified using Kodak Image Analysis Software.

Immunoblot analysis

Liver samples were homogenized in extraction buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1% NP-40 and a protease inhibitor fluid. The extract was centrifuged at 10 000*g* for 10 min at 4°C, and protein concentration was determined in the supernatant. Proteins of liver homogenates were separated by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride membrane using a Transblot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in a buffer containing 5% non-fat milk, 50 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20 (TBS-T) for 1 h and then incubated with specific antibodies: β-actin (Sigma-Aldrich, St Louis, MO, USA), CYP2E1 and PPARα (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were subsequently washed, incubated with specific secondary horseradish peroxidase-conjugated antibodies and revealed with the enhanced chemiluminescence (ECL) kit by Pierce (Thermo Fisher Scientific, Rockford, IL, USA). The band intensity was analysed by scanning densitometry.

Statistical analysis

Data were expressed as mean ± SD. All comparisons were done by one-way analyses of variance with Dunn's test for post-hoc analysis. The Kruskal–Wallis test was used for the analysis of degree of histopathological grades. Differences were considered significant when $P < 0.05$.

Results

Effect of geniposide on liver indices and serum levels of alanine transaminase and aspartate aminotransferase

The relative liver indices were significantly increased compared with the normal control group. In contrast, treatment with geniposide (50 and 100 mg/kg) significantly reduced the liver weights compared with the NASH group. Similarly, the NASH group had elevated serum ALT and AST levels. Administration of geniposide (50 and 100 mg/kg) attenuated high-fat diet-induced increase in ALT and AST activity ($P < 0.05$) (Figure 1a). Geniposide, interestingly, reverses these changes, suggesting that it has the potential to prevent liver injury.

Effect of geniposide on lipid metabolism

To analyse the possible role of geniposide in lipid metabolism, which is key factor relative to fatty liver formation, TG, TC, FFA, HDL and LDL-C were investigated. As shown in Figure 1b–e, the increased serum and liver levels of TG, TC, FFA as well as serum LDL-C, were significantly suppressed by geniposide, whereas decreased serum HDL-C levels were obviously elevated by treatment with geniposide. The data suggest that geniposide can prevent hepatosteatosis via downregulation of accumulation of lipid.

Effect of geniposide on serum insulin and tumour necrosis factor-α

Because insulin and TNFα disorder has been documented in hepatic steatosis, the levels of insulin and TNFα were studied. As shown in Figure 2, the high-fat diet did not significantly change the levels of insulin compared with the normal control group; however, the expression of TNFα was enhanced after the high-fat feeding. Serum insulin level was increased while TNFα level was inhibited by geniposide. These results suggest that the preventive effects of geniposide on hepatosteatosis are associated with upregulation of insulin and downregulation of TNFα.

Effect of geniposide on the levels of hepatic malondialdehyde, superoxide dismutase, glutathione peroxidase in rats with nonalcoholic steatohepatitis

As shown in Figure 3, the antioxidant activity of geniposide was estimated by examination of hepatic MDA content and activity of SOD and GSH-Px. High-fat feeding caused an increase in the liver MDA level but a decrease in the liver SOD and GSH-Px activity compared with the control group. The abnormal changes in these indexes in the geniposide (50 and 100 mg/kg) groups were pronouncedly ameliorated.

Effect of geniposide on the pathological changes of rats with nonalcoholic steatohepatitis

Liver tissues were collected to assess the effect of geniposide on liver pathological changes. Rats fed with a high-fat diet for six weeks developed a high degree of steatosis, showing hepatocytes with severe cytoplasmic vacuoles and swelling (Figure 4b), whereas no histological abnormalities were observed in normal control rats (Figure 4a). Administration of geniposide prevented the fatty deposition in hepatocytes. Especially, geniposide at a dose of 100 mg/kg almost blocked the formation of steatosis (Figure 4c and Table 2).

Effect of geniposide on the expression of liver peroxisome proliferator-activated receptor α, CYP2E1 and tumour necrosis factor-α

Previous studies showed that alterations of PPARα and CYP2E1 contributed to NAFLD. Therefore, we analysed the expression of PPARα, CYP2E1 and TNFα in the liver. As shown in Figure 5, during the six weeks of diet treatment, the expression of CYP2E1 and TNFα were significantly increased in the NASH group, whereas the PPARα expression was gradually reduced compared with that in the normal control group. Geniposide (100 mg/kg) treatment reduced the expression of CYP2E1 and TNFα and increased the expression of PPARα significantly.

Discussion

NASH refers to the excessive accumulation of lipids within hepatocytes due to imbalance of lipid formation and lipid degradation.[14,15] Hypercholesterolaemia, hypertriglyceridaemia, a low level of HDL-C and a high level of LDL-C are the most common impairments in lipid homoeostasis in patients with steatosis.[16] In this study, we demonstrated that

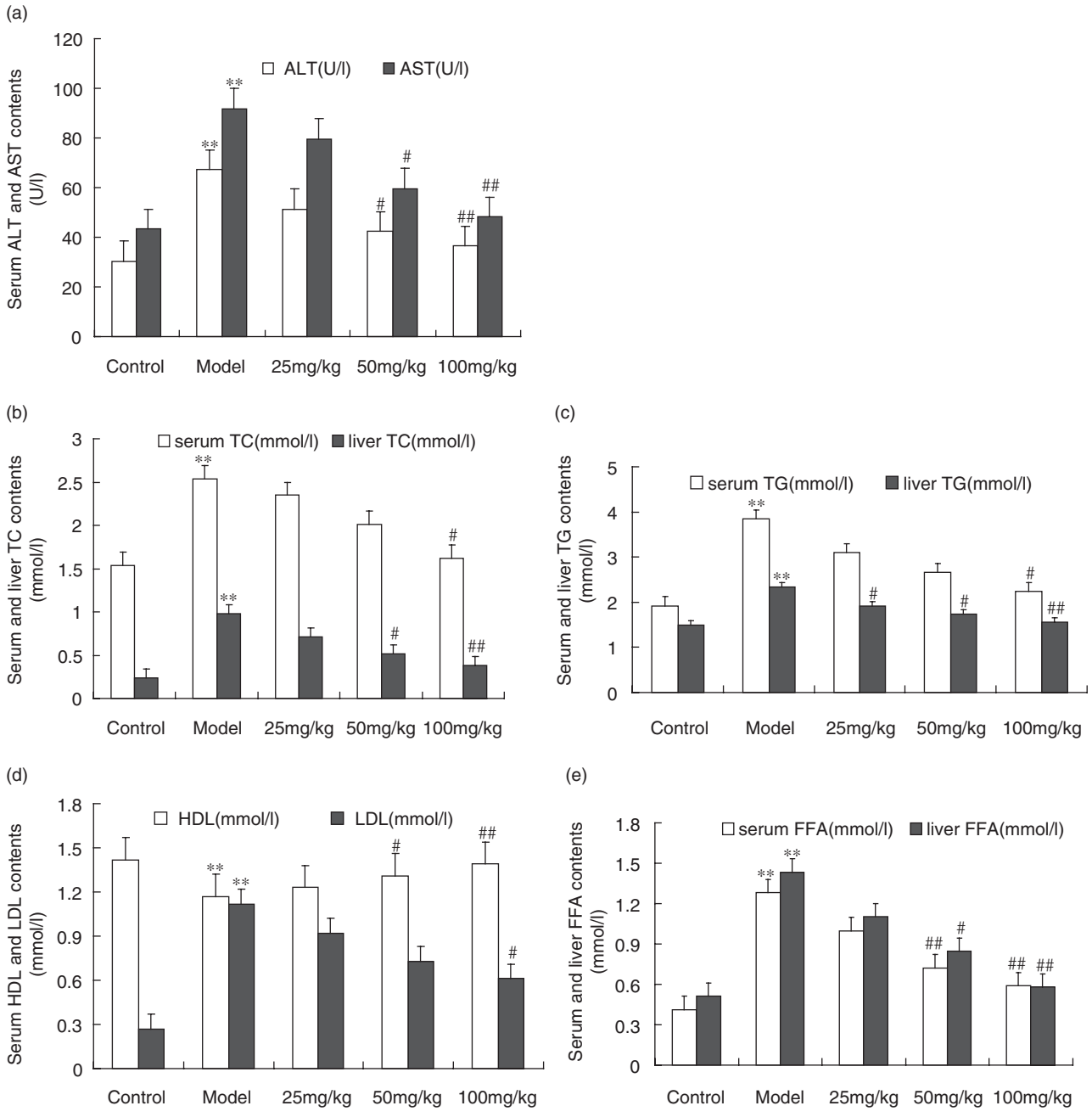

Figure 1 Effect of geniposide on serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels and lipid metabolism parameters in rats with nonalcoholic steatohepatitis (NASH). ALT, AST and lipid metabolism parameters were measured as described in Materials and Methods. Each group consists of 10 rats. Data were expressed mean ± SD $^{**}P < 0.01$ vs normal group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs NASH model group.

the preventive effect of geniposide is mediated through down-regulation of the levels of TG, TC and LDL-C and through elevation of HDL-C synthesis. Histological examination showed that histological changes of liver injury were also remarkably ameliorated after geniposide treatment. These results indicate that geniposide attenuated the disorder of lipid metabolism in liver following high-fat feeding.

Although NASH represents a form of lipotoxicity, its pathogenesis remains poorly understood.[5] Insulin resistance was found to be a strong predictor of NAFLD.[17] With insulin resistance, the elevated plasma concentrations of fatty acids promotes hepatic fatty acid synthesis and impairs β-oxidation, which further leads to hepatic steatosis. In obese and diabetic patients, increased plasma FFA levels were observed, due to abnormal release by insulin-resistant adipocytes. FFAs appear to be important mediators of lipotoxicity, both as potential cellular toxins and by inducing lipid over-accumulation through insulin resistance.[18,19] Furthermore, FFAs not only promote hepatic lipotoxicity by stimulating TNFα expression via a lysosomal pathway[20] but also are the most important

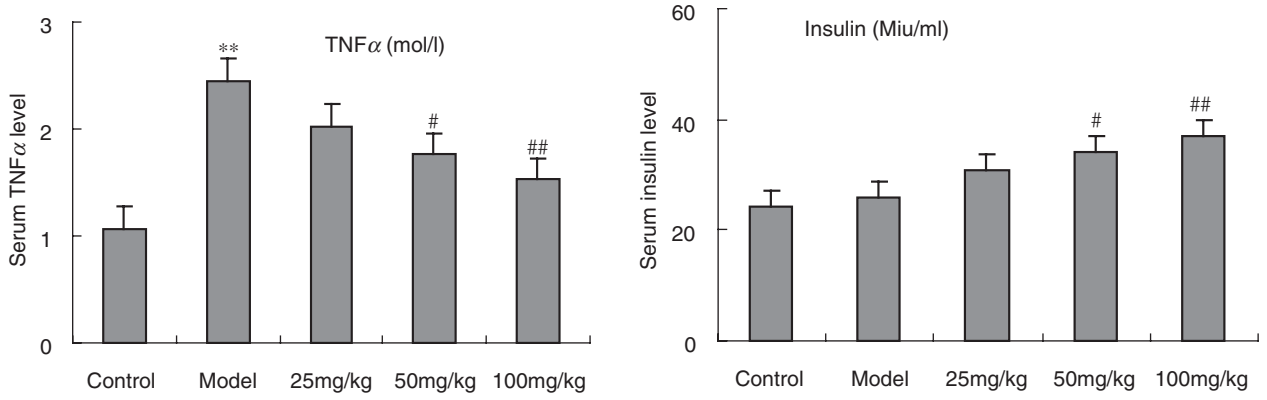

Figure 2 Effect of geniposide on serum insulin and tumour necrosis factor-α (TNFα) levels in rats with nonalcoholic steatohepatitis (NASH). Insulin and TNFα levels were measured as described in Materials and Methods. Data were expressed mean ± SD from five separate experiments. $^{**}P < 0.01$ vs normal group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs NASH model group.

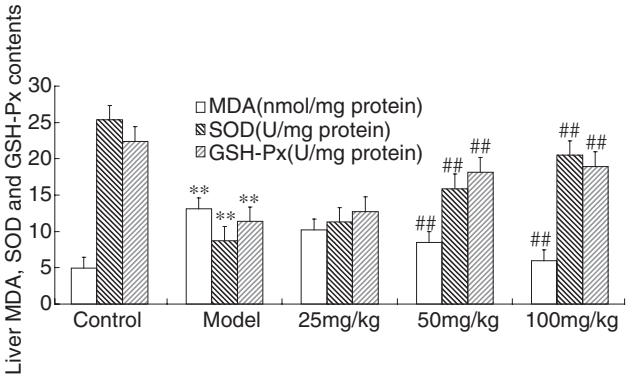

Figure 3 Effect of geniposide on liver malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels in rats with nonalcoholic hepatosteatosis (NASH). MDA, SOD and GSH-Px levels were measured as described in Materials and Methods. Data were expressed mean ± SD from five separate experiments. $^{**}P < 0.01$ vs normal group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs NASH model group.

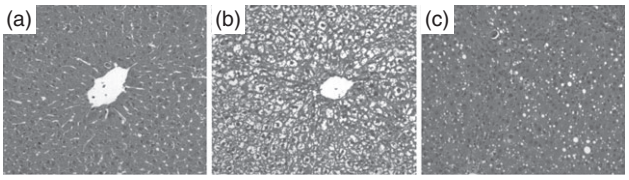

Figure 4 Effect of geniposide on liver histological examination of rats with nonalcoholic steatohepatitis (NASH) induced by high-fat emulsion. (a) Normal control group; (b) NASH model group; (c) geniposide (100 mg/kg) treatment group; (HE stains, ×200).

source of ROS.[21] In this study, insulin levels were elevated and FFA contents were reduced by geniposide, indicating that geniposide may be a potential candidate to prevent high-fat-induced hepatic steatosis.

TNFα plays a central role in the development of metabolic syndrome in hepatic steatosis.[22] Increased expression of TNFα is associated with insulin resistance, obesity and hypertriglyceridaemia.[23,24] Levels of TNFα have been shown to be raised in patients with NAFLD.[25] Anti-inflammatory drugs

Table 2 Effect of geniposide on hepatic steatosis in rats subjected to high-fat diet

Group	Dose (mg/kg)	0	I	II	III	IV	*P*
Control	n.a.	10	0	0	0	0	
Model	n.a.	0	0	1	2	7	**
Geniposide	25	0	1	8	1	0	
	50	2	3	5	0	0	#
	100	5	2	3	0	0	##

n.a., not applicable. 0, no steatosis, normal liver; I, >25% of hepatocytes affected; II, 26–50% of hepatocytes affected; III, 51–75% of hepatocytes affected; IV, >76% of hepatocytes affected. $^{**}P < 0.01$ vs normal control group. $^{\#}P < 0.05$ vs nonalcoholic steatohepatitis (NASH) model group. Each group consists of 10 rats and figures represent number of rat per grade.

such as vitamin E and anti-TNFα antibodies improve both insulin resistance and fatty liver disease. Masaki found that pretreatment with adiponectin attenuated the GalN/LPS-induced increases in serum and hepatic TNFα levels and increased PPARα messenger RNA expression in the liver.[26] PPARα, a nuclear receptor, plays an important role as a target for adiponectin in lipid metabolism. Investigators have demonstrated that PPARα agonists can prevent or improve hepatic steatosis in animal models of NAFLD.[27,28] PPARα regulates the inflammatory process by affecting the production of cytokines, such as TNFα, and the actions of cytokine signalling pathways. The administration of a PPARα agonist was shown to impair the production of TNFα.[29] Conversely, PPARα-null mice were shown to be more sensitive to the effects of LPS.[30] The present result shows that the expression of PPARα in fatty liver is lower than that of normal rats, and it is upregulated by geniposide; conversely, TNFα is decreased by geniposide, suggesting that the regulation of lipid metabolism by geniposide may be associated with the increased expression of PPARα and regulation of adipocytokine release.

It is believed that the steatotic liver is more vulnerable to oxidative stress and resulting lipid peroxidation leading to inflammation, necrosis and fibrosis.[5,31,32] The pro-inflammatory, profibrogenic effects of the aldehyde end

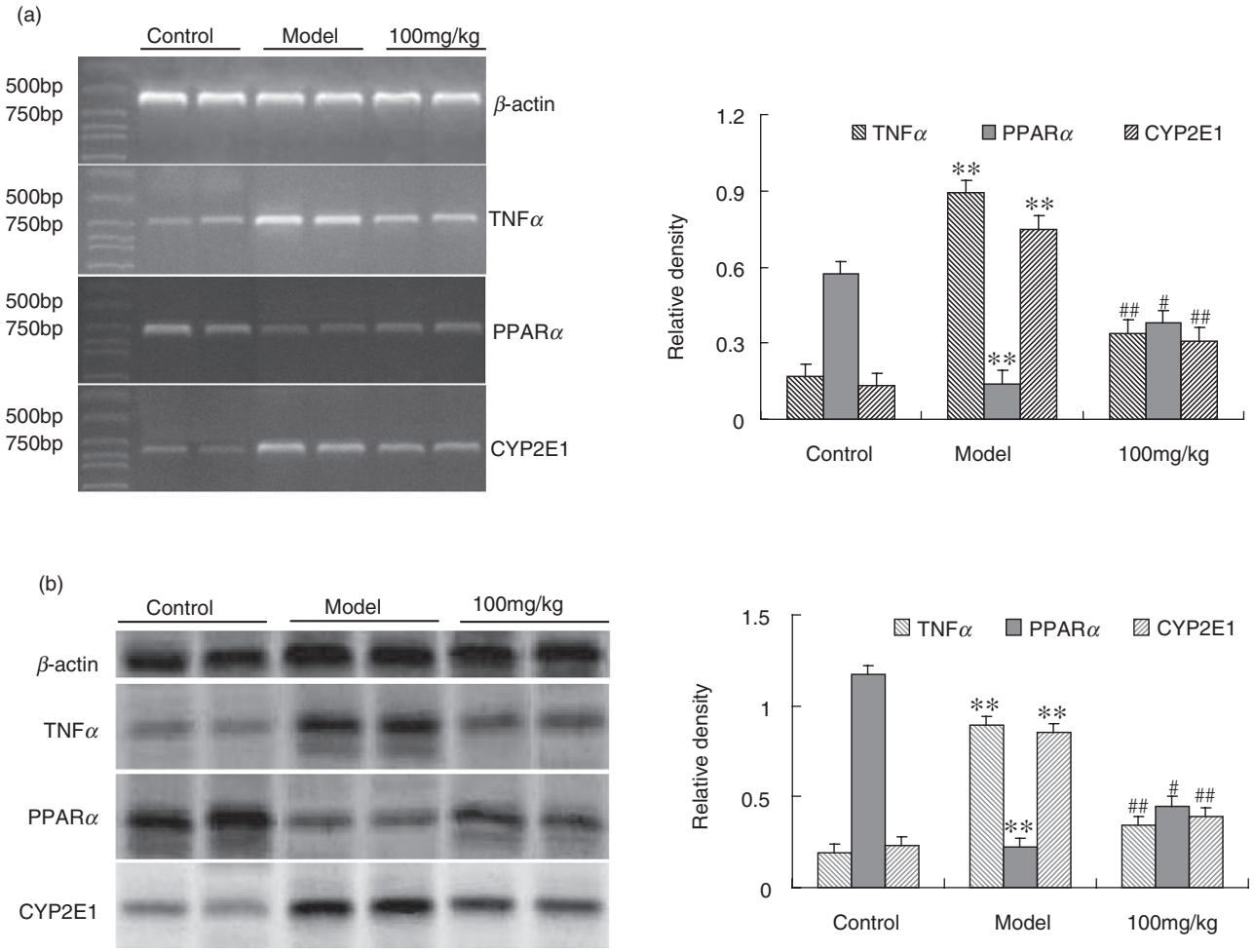

Figure 5 (a) Effect of geniposide on tumour necrosis factor-α (TNFα), CYP2E1 and proliferator-activated receptor-α (PPARα) mRNA in rats with nonalcoholic steatohepatitis (NASH). The mRNA was corrected by β-actin. (b) Effect of geniposide on TNFα, PPARα and CYP2E1 in NASH rats. The protein was corrected by β-actin. Values are means ± SD ($n = 6$ per group). $^{**}P < 0.01$ vs normal control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs NASH model group.

products of lipid peroxidation (MDA and 4-hydroxynonenol) potentially account for all of the typical histological features observed in NAFLD.[33] CYP2E1 is a major microsomal source of oxidative stress. In-vitro inhibition studies demonstrated that CYP2E1 is the major catalyst for the formation of lipid peroxides in mice.[34] GSH-Px, along with SOD, is one of the body's endogenous antioxidants, and is well known to protect liver cells against oxidative damage through chemical or enzymatic reactions. In our study, geniposide (50 or 100 mg/kg) markedly increased SOD and GSH-Px, decreased MDA and decreased CYP2E1 activity in the liver of rats with high-fat diet. The antioxidant properties of geniposide may be related to its ability to reduce free radical formation or through its free radical scavenging activity.

Conclusion

Our investigation for the first time proved the preventive effect of geniposide in rats with hepatic steatosis. Its anti-steatotic property may be associated with the regulation of lipid metabolism through regulation of adipocytokine release and increasing the expression of PPARα as well as its antioxidant ability. These results have shed some light on the clinical therapeutic potential of geniposide.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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