

Full-length article

Therapeutic effect of osthole on hyperlipidemic fatty liver in rats¹Yan ZHANG, Mei-lin XIE², Lu-jia ZHU, Zhen-lun GU*Department of Pharmacology, Medical School of Soochow University, Suzhou 215123, China***Key words**

osthole; hyperlipidemic fatty liver; pharmacology

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Abstract

Aim: To study the effects of osthole on hyperlipidemic fatty liver and investigate the possible mechanisms. **Methods:** A rat model with hyperlipidemic fatty liver was successfully established by feeding fatty milk for 6 weeks. The experimental rats were then treated with 5–20 mg/kg osthole for 6 weeks. The mouse hyperlipidemic model was induced by feeding fatty milk when they were treated with 10–20 mg/kg osthole for 3 weeks. **Results:** After treatment with osthole, the levels of rat serum total cholesterol (TC), triglyceride (TG) and low density lipoprotein-cholesterol significantly decreased as compared with the fatty liver model group ($P<0.05$ or $P<0.01$). Hepatic weight and its coefficient, the hepatic tissue contents of TC, TG, and malondialdehyde, also significantly decreased ($P<0.05$ or $P<0.01$). In fatty milk-induced hyperlipidemic mice, the post-heparin plasma activities of lipoprotein lipase (LPL), hepatic lipase (HL), and total lipase (TL) significantly increased after treatment with 10–20 mg/kg osthole for 3 weeks ($P<0.05$ or $P<0.01$). Importantly, the histological evaluation of rat liver demonstrated that osthole dramatically decreased lipid accumulation ($P<0.01$). **Conclusion:** Osthole was found to have therapeutic effects on fatty milk-induced rat fatty liver; the mechanisms might be associated with its anti-oxidation and the elevation of the activities of LPL and HL.

Introduction

Non-alcoholic fatty liver (NAFL) is one of the most common causes of chronic liver disease. The disease is a spectrum that is initiated with steatitis. It can progress to non-alcoholic hepatosteatitis and later fibrosis, cirrhosis, and potentially end-stage liver failure and/or hepatocellular carcinoma^[1–4]. Although several promising medications are on the horizon, there are currently no ideal pharmacological reagents that can prevent or reverse this disease^[5].

Osthole (7-methoxy-8-isopentenoxycoumarin) is an active constituent isolated from the fruit of *Cnidium monnieri* (L) Cusson, one of the Chinese herbal medicines which possesses a variety of pharmacological properties^[6,7] and has been administered to humans in clinics for many years. Modern pharmacological studies have proven that osthole has many functions such as anti-inflammation^[8], anti-oxidation^[9], anti-tumor^[10], anti-apoptosis^[11], estrogen-like effects, and so on. It is considered to have potential therapeutic applications, but there is no report about the treatment of

hyperlipidemic fatty liver. We found that the level of serum triglyceride (TG) in ovariectomized rats decreased after treatment with osthole for 12 weeks (0.30 ± 0.13 mmol/L in the model group vs 0.19 ± 0.07 mmol/L in the 10 mg/kg osthole group) accidentally, so we began to study the therapy for hyperlipidemic fatty liver. Lipanthyl can lower serum total cholesterol (TC), and TG, and be used for treatment of hyperlipidemia and hyperlipidemic fatty liver in clinics. Therefore, it acted as a positive drug in the experiment.

Materials and methods

Drugs and reagents Osthole was kindly provided by Dr Jia ZHOU (Xi'an Green Fount Natural Product Co Ltd, Xi'an, China); the purity was >98% as determined by HPLC. Lipanthyl was procured from Laboratories Fournier SA (Chenove, France). The assay kits for serum TC, TG, and high density lipoprotein-cholesterol (HDL-C) were purchased from Suzhou Tianneng Biology-Technology Company (Suzhou, China). The assay kits for liver tissue TC, TG,

malondialdehyde (MDA), superoxide dismutase (SOD), plasma lipoprotein lipase (LPL), and hepatic lipase (HL) were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals Sprague-Dawley rats (male, 200±20 g) and Kunming mice (male, 18±2 g) were obtained from the Animal Breeding Center of Soochow University (Suzhou, China). The animals were housed under standard conditions (20±1 °C room temperature, 60%±10% humidity, and light from 6:00 to 18:00) in regular cages and allowed free access to food and water. All animal studies were conducted according to the regulations for the use and care of experimental animals.

Establishment of rat hyperlipidemic fatty liver model^[12-14] A hyperlipidemic fatty liver rat model was generated by feeding fatty milk (containing 6% cholesterol, 15% lard, 0.2% propylthiouracil, 2% bile salt, 20% propylene glycol, and 20% Tween-80) at 1 mL/100 g (body weight per d) for 6 weeks. Three rats were then killed and the livers were taken for assessment of fatty hepatic development. After the model developed, the rats were randomly divided into 5 groups ($n=10$): the fatty hepatic model group, the 5, 10, and 20 mg/kg osthole groups, and the 20 mg/kg lipanthyl group. Control and model animals were treated with an equivalent volume of 0.5% sodium carboxymethyl cellulose solution. These medications were taken orally for 6 weeks. Finally, all of the rats were sacrificed, blood was obtained, and hepatic tissues were collected for measurement.

Establishment of the mouse hyperlipidemic model^[15] The hyperlipidemic mouse model was generated by feeding fatty milk (containing 10% cholesterol, 20% lard, 1% thimecil, 2% bile salt, 20% propylene glycol, and 20% Tween-80) at 0.2 mL/10 g (body weight per d). The experimental mice were randomly divided into 5 groups, ($n=10$): the control group, hyperlipidemic model group, 10 and 20 mg/kg osthole groups, and the 40 mg/kg lipanthyl group. These mice were administered in the morning and treated with fatty milk in the afternoon for 3 weeks. Control and model animals were treated with an equivalent volume of 0.5% sodium carboxymethyl cellulose solution. After 3 weeks, all mice received an injection of heparin (312.5 U/kg, iv) 15 min before being sacrificed. Post-heparin plasma was obtained for the measurement of LPL and HL activities.

Measurement of serum TC, TG, HDL-C, and the coefficient of hepatic weight in rats Rat blood was obtained after 12 h of overnight fasting. Serum TC, TG, and HDL-C were determined by colorimetric methods according to the procedure provided. Low density lipoprotein-cholesterol (LDL-

C) was obtained by the Friedewald calculation, namely, $LDL-C = TC - (TG/2.2 + HDL-C)$. The coefficient of hepatic weight was calculated according to liver weight (g) divided by body weight (100 g).

Measurement of plasma LPL, HL, and total lipase (TL) activities in mice Activities of LPL and HL in mouse post-heparin plasmas were determined by enzymatic methods according to the procedure provided, and the TL was obtained by LPL plus HL.

Measurement of TC and TG in rat liver tissue^[16] The hepatic lipid was extracted from the liver tissue using a chloroform/methanol mixed solution (1/1, v/v). The prepared sample was then centrifuged at 1200×g for 10 min; the obtained supernatant was used for the measurement of TC and TG according to colorimetric methods.

Measurement of SOD, MDA, and protein in rat liver tissue The liver tissue was taken at the time of slaughter and rapidly broken to pieces in ice-cold saline, then the tissue homogenate (10%, w/v) was prepared. The contents of SOD and MDA were determined by colorimetric methods according to the procedure provided, respectively. Protein in liver was measured by colorimetric methods.

Histological observation^[17] For the histological study, rat liver specimens were fixed in 10% formaldehyde and embedded in paraffin for hematoxylin (HE) staining. The degree of fatty degeneration is generally determined by evaluating the proportion of hepatocytes containing fat droplets and graded and expressed as “–, +, ++, +++”; no fat present as “–”, less than 1/3 of the hepatic lobule as “+”, 1/3 to 2/3 as “++”, and more than 2/3 as “+++”. The histological evaluation of the liver sections was performed blindly.

Statistical analysis Data are expressed as mean±SD. One-way ANOVA was performed to determine difference between the experimental groups and χ^2 -test was used to evaluate the significances of histopathological evaluation. For all tests, $P<0.05$ was considered statistically significant.

Results

General condition of the rats At the end of the experiment, the rats' body weight and hepatic weight were measured and the coefficient of hepatic weight was calculated. Hepatic weight and its coefficient in the model group were significantly higher than those in the control group, but the rat's body weight had no difference among the groups. After treatment with 5–20 mg/kg osthole for 6 weeks, compared with the model group, the coefficient of hepatic weight in the osthole groups significantly decreased ($P<0.05$ or $P<0.01$), hepatic weight also decreased, especially in the 10

and 20 mg/kg osthole groups ($P<0.05$ or $P<0.01$). In the lipanthyl group, hepatic weight and its coefficient inversely increased ($P<0.01$; Table 1).

Table 1. Body weight, hepatic weight, and coefficient of hepatic weight after treatment with osthole for 6 weeks in hyperlipidemic fatty liver rats. $n=10$. Data are presented as mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control. ^e $P<0.05$, ^f $P<0.01$ vs model.

Group	Body weight (g)	Liver weight (g)	Coefficient of hepatic weight (g/100 g)
Control	433.8 \pm 27.6	13.4 \pm 1.7	3.08 \pm 0.30
Model	430.3 \pm 38.7	15.2 \pm 1.4 ^b	3.53 \pm 0.15 ^c
5 mg/kg osthole	409.4 \pm 40.8	13.6 \pm 2.1	3.30 \pm 0.30 ^e
10 mg/kg osthole	410.5 \pm 41.5	12.9 \pm 1.8 ^f	3.15 \pm 0.25 ^f
20 mg/kg osthole	428.2 \pm 36.9	13.4 \pm 1.6 ^e	3.13 \pm 0.23 ^f
20 mg/kg lipanthyl	422.7 \pm 44.0	19.1 \pm 2.4 ^f	4.51 \pm 0.29 ^f

Effects on serum levels of TC, TG, HDL-C, and LDL-C in fatty liver rats The serum levels of TC, TG, and LDL-C in the model group were significantly higher than those in the control group ($P<0.05$ or $P<0.01$); the level of HDL-C between the different groups had no difference. After 6 weeks of administration, serum TC, TG, and LDL-C levels were significantly lower in the osthole groups than in the model group ($P<0.05$ or $P<0.01$). The serum HDL-C level increased to some degree, especially in the 10 mg/kg osthole group ($P<0.01$). In the lipanthyl group, the serum levels of TC, TG, and LDL-C significantly decreased, but the serum level of HDL-C was not increased (Table 2).

Effects on plasma activities of LPL, HL, and TL in hyperlipidemic mice The post-heparin plasma activities of LPL, HL, and TL in the mouse model group were markedly higher

than those in the control group ($P<0.01$). After administration of osthole for 3 weeks, post-heparin plasma LPL, HL, and TL activities in the mice further increased ($P<0.05$ or $P<0.01$); the percentages increased by 11.8%–30.1% for LPL, 41.3%–77.5% for HL, and 20.4%–44.0% for TL, respectively. In the lipanthyl group, mice plasma activities of LPL, HL and TL also increased significantly ($P<0.01$; Table 3).

Table 3. Post-heparin plasma activities of plasma lipoprotein lipase (LPL), hepatic lipase (HL), and total lipase (TL) after treatment with osthole for 3 weeks in hyperlipidemic mice. $n=10$. Data are presented as mean \pm SD. ^c $P<0.01$ vs control. ^e $P<0.05$, ^f $P<0.01$ vs model.

Group	LPL (U/mL)	HL (U/mL)	TL (U/mL)
Control	9.56 \pm 1.74	2.93 \pm 1.18	12.58 \pm 1.73
Model	13.92 \pm 3.75 ^c	5.77 \pm 1.35 ^c	19.69 \pm 4.62 ^c
10 mg/kg osthole	15.56 \pm 2.35	8.15 \pm 1.94 ^f	23.71 \pm 2.59 ^e
20 mg/kg osthole	18.11 \pm 1.98 ^f	10.24 \pm 3.35 ^f	28.35 \pm 4.05 ^f
40 mg/kg lipanthyl	17.99 \pm 2.67 ^f	9.35 \pm 1.43 ^f	27.34 \pm 2.78 ^f

Effects on hepatic tissue TC, TG, SOD, and MDA contents in fatty liver rats The contents of TC, TG, and MDA in the hepatic tissue in the model group were significantly higher than those in the control group. After treatment with 5–20 mg/kg osthole for 6 weeks, the contents of TC, TG, and MDA in the hepatic tissue were significantly lowered as compared with the model group ($P<0.05$ or $P<0.01$) and decreased by 12.5%–32.2%, 25.0%–34.3%, and 20.6%–26.5%, respectively. In the lipanthyl group, the levels of TC, TG, and MDA also decreased. However, the activity of SOD between the model group and the drug-treated group was not significantly different (Table 4).

Rat liver histological changes The degree of liver fatty degeneration was severer in the model group than in the

Table 2. Serum levels of cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), and low density lipoprotein-cholesterol (LDL-C) after treatment with osthole for 6 weeks in hyperlipidemic fatty liver rats. $n=10$. Data are presented as mean \pm SD. ^b $P<0.05$, ^c $P<0.01$ vs control. ^e $P<0.05$, ^f $P<0.01$ vs model.

Group	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
Control	1.63 \pm 0.19	0.69 \pm 0.32	0.54 \pm 0.09	0.77 \pm 0.27
Model	2.08 \pm 0.41 ^c	1.02 \pm 0.23 ^b	0.54 \pm 0.13	1.08 \pm 0.36 ^b
5 mg/kg osthole	1.52 \pm 0.50 ^e	0.79 \pm 0.23 ^e	0.72 \pm 0.42	0.44 \pm 0.28 ^f
10 mg/kg osthole	1.28 \pm 0.27 ^f	0.67 \pm 0.26 ^f	0.72 \pm 0.09 ^f	0.26 \pm 0.22 ^f
20 mg/kg osthole	1.09 \pm 0.50 ^f	0.51 \pm 0.26 ^f	0.67 \pm 0.23	0.19 \pm 0.22 ^f
20 mg/kg lipanthyl	0.92 \pm 0.16 ^f	0.66 \pm 0.19 ^f	0.45 \pm 0.07	0.16 \pm 0.11 ^f

Table 4. Contents of TC, TG, superoxide dismutase (SOD), and malondialdehyde (MDA) in hepatic tissue after treatment with osthole for 6 weeks in hyperlipidemic fatty liver rats. *n*=10. Data are presented as mean±SD. ^b*P*<0.05, ^c*P*<0.01 vs control. ^e*P*<0.05, ^f*P*<0.01 vs model.

Group	TC (mg/g wet tissue)	TG (mg/g wet tissue)	SOD (U/mg protein)	MDA (nmol/mg protein)
Control	5.76±0.74	23.77±6.03	61.74±4.78	3.65±0.37
Model	8.97±0.77 ^c	41.24±8.88 ^c	61.62±5.47	4.08±0.46 ^b
5 mg/kg osthole	7.85±1.25 ^f	30.91±8.25 ^e	57.19±3.95	3.24±0.48 ^f
10 mg/kg osthole	6.97±1.01 ^f	28.52±8.39 ^f	58.58±1.91	3.16±0.43 ^f
20 mg/kg osthole	6.08±1.06 ^f	27.11±8.88 ^f	60.36±4.37	3.00±0.55 ^f
20 mg/kg lipanthyl	6.33±0.94 ^f	30.95±7.37 ^e	57.17±5.46	3.05±0.60 ^f

control group. After administration of osthole for 6 weeks, the degree of fatty degeneration in the liver was significantly improved (*P*<0.01), especially in the 10 and 20 mg/kg osthole groups; liver fatty degenerations in partial rats were reversed. In the lipanthyl group, the severity of liver fatty degenera-

tion also significantly decreased. A histological evaluation of the liver specimen demonstrated osthole dramatically decreased lipid accumulation (Table 5; Figure 1). Thus, our results demonstrated a therapeutic effect of osthole on fatty milk-induced fatty liver.

Table 5. Histopathological changes of hepatic fatty degeneration after treatment with 5–20 mg/kg osthole for 6 weeks in fatty milk-induced fatty liver rats. *n*=10. *P* value for medicine-treated or control groups vs model group.

Degree of fatty degeneration	Control	Model	5 mg/kg	Osthole 10 mg/kg	20 mg/kg	Lipanthyl 20 mg/kg
-	10	0	0	3	3	2
+	0	1	6	4	6	7
++	0	7	4	3	1	1
+++	0	2	0	0	0	0
<i>P</i>	<0.01		<0.01	<0.01	<0.01	<0.01

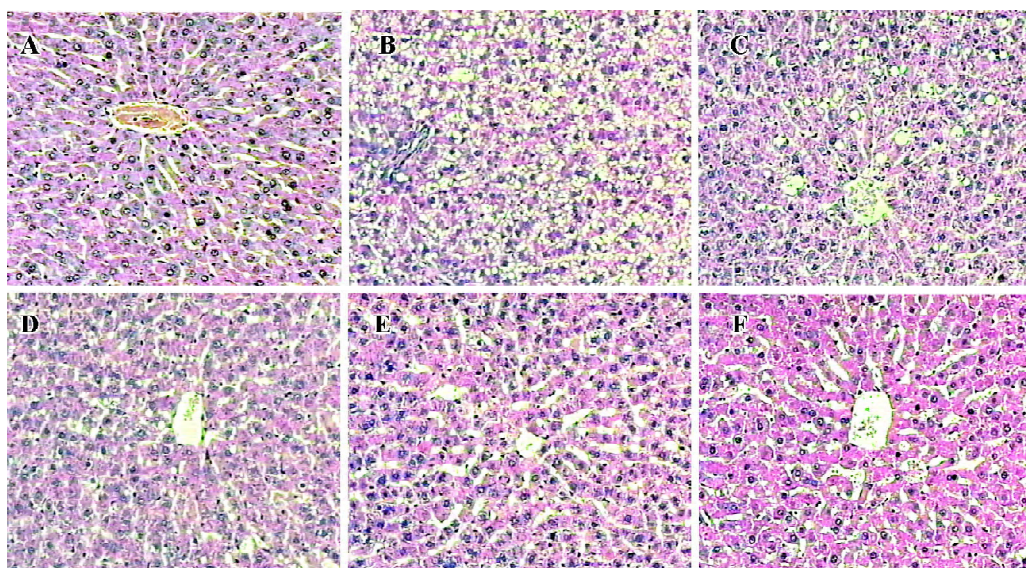


Figure 1. Histopathological changes of rat liver (HE staining, ×25). No steatosis was observed in the control group (A) and the fatty degeneration as seen in the model group (B). Fatty degeneration of the liver was alleviated after treated with 5 mg/kg osthole (C), 10 mg/kg osthole (D), 20 mg/kg osthole (E), and 20 mg/kg lipanthyl (F) for 6 weeks.

Discussion

At present, there are many different therapeutic approaches for NAFL, but the strategies of therapy include two different directions; one is towards the risk factors, which are considered to be involved in the etiology of NAFL, such as obesity, dyslipidemia and diabetes, and the another is intervention in the pathogenetic mechanism of the disease itself, which remains unclear. Therefore, in clinical hypocaloric diets and physical exercise, treatment of associated insulin resistance, lipid-lowering medications, anti-oxidants and so on are used for the disease^[18], but no treatment has definitely evidence-based NAFL lesion-ameliorating or progression-avoiding effects. There are also no ideal drugs that can prevent or reverse the disease^[19].

Our experimental results show that after treatment with osthole for 6 weeks, the levels of serum TC, TG, LDL-C, hepatic weight and its coefficient, and the hepatic tissue contents of TC and TG decreased significantly in fatty milk-induced fatty liver rats. The histological evaluation of rat liver demonstrated osthole dramatically decreased lipid accumulation. More importantly, we found that osthole might enhance the activities of LPL and HL and decrease the content of MDA. These results suggest a new function of osthole that protects the liver from fat accumulation, and its mechanism might be associated with the increment of LPL, HL activities and anti-oxidation.

LPL and HL are essential in the hydrolysis of TG-rich lipoproteins^[20]. By proteoheparan sulfate, active LPL is attached to capillary endothelium and active HL is attached to sinusoids and capillaries, respectively, and they can be released to the blood stream by an injection of heparin. NAFL is caused by the excessive accumulation of TG droplets within hepatocytes. Sources of hepatic TG are transported via CM from the intestines, and then secreted into blood as VLDL. Accordingly, LPL plays a major role in lipid metabolism by regulating the catabolism of TG-rich lipoprotein particles that convert lipoprotein TG to free fatty acid. So LPL is a rate-limiting enzyme for the hydrolysis and removal of CM and VLDL-TG from the circulation. LPL can also act as a structural cofactor facilitating the cellular uptake of whole lipoprotein particles and selective cholesterol ester^[21]. HL activity is present mainly in the liver. It plays a major role in lipoprotein metabolism as a lipolytic enzyme that catalyzes the hydrolysis of TG and phospholipids in CM remnants, intermediate-density lipoprotein, and HDL^[22]. HL also serves as a ligand that facilitates the uptake of lipoproteins and lipoprotein lipids by cell surface receptors or proteoglycans, thereby directly affecting cellular lipid delivery^[23].

Our experimental results demonstrated that in fatty milk-induced fatty liver rats, osthole significantly decreased the levels of serum TC, TG, LDL-C, and the hepatic tissue contents of TC and TG. In fatty milk-induced hyperlipidemic mice, osthole dramatically increased the post-heparin plasma activities of LPL, HL, and TL. High LPL and HL activities lead to more TG and phospholipids hydrolyzed, which may contribute to the reduction of serum TG, TC, and LDL-C concentrations and improvement of hepatocyte lipid accumulation. From these results, we assumed that the therapeutic effect of osthole on fatty milk-induced fatty liver was probably due to its lipid-modifying effects.

In very recent studies, it was proved that oxidative stress and subsequent lipid peroxidation played a pivotal role in the development of fatty liver. Mitochondria are thought to be the source of the reactive oxygen species (ROS); overproduction of ROS triggered steatohepatitis by oxidizing the accumulated hepatic lipid and causing lipid peroxidation, contributed significantly to hepatocyte injury^[24,25]. The present results demonstrate that in the fatty milk-induced fatty liver rats, osthole significantly depressed MDA production and reduced the degree of hepatocellular steatosis in the liver tissue. These results suggested that osthole possessed a therapeutic effect on fatty milk-induced fatty liver, which was partly attributable to its anti-oxidation against lipid peroxidation.

In summary, our present study demonstrated that osthole was effective in alleviating fatty milk-induced fatty liver in rats. Notably, it also elevated the activities of LPL and HL, and the effect, together with its anti-oxidation, might be the main mechanisms for its therapeutic effects on NAFL.

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