Coix lacryma-jobi Seed Oil Reduces Fat Accumulation in Nonalcoholic Fatty Liver Disease by Inhibiting the Activation of the p-AMPK/SePP1/apoER2 Pathway

Liangzhen Gu1#, Yanan Zhang1,3#, Shuang Zhang1, Haijun Zhao1,2, Yuan Wang1,2, Dongfang Kan1, Yimin Zhang1,3, Liangqing Guo5, Jiajian Lv1, Qian Hao1, Xu Tian1, Changhong Liu4*, ShiJun Wang1,2,3*, and Xiaochun Han1,2*

1 Shandong University of Traditional Chinese Medicine, Jinan, CHINA
2 Shandong Co-Innovation Center of Classic TCM formula, Shandong University of Traditional Chinese Medicine, Jinan, CHINA
3 Shandong Provincial Chinese Medicine Classical Prescription Demonstration Engineering Technology Research Center, Jinan, CHINA
4 Department of Gastroenterology, Shandong Provincial Qianfoshan Hospital, the First Hospital Affiliated with Shandong First Medical University, Jinan, CHINA
5 Department of Endocrinology, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, CHINA

Abstract: The lipid metabolism disorder is the key role of Nonalcoholic fatty liver disease (NAFLD). Selenoprotein P plays an important role in the pathological process of lipid accumulation. Coix lacryma-jobi seed oil (CLSO) is an active component extracted from Coix lacryma-jobi seed (CLS) which has been found to be effective of reducing blood fat and antioxidative. But the effect and mechanism of CLSO on NAFLD are not clear. The aim of this study was to explore the therapeutic effect and mechanism of CLSO in the treatment of NAFLD. Our result showed that CLSO decreased the liver/body weight ratio, lowered the total cholesterol (TC) and triacylglycerol (TG), and elevated the high density lipoprotein (HDL) in serum. CLSO reduced the lipid deposition in the liver of NAFLD rats. In addition, CLSO could bring down the abnormal expression of superoxide dismutase (SOD) and malondialdehyde (MDA). Moreover, CLSO significantly declined the liver apolipoprotein E (apoE), apolipoprotein E receptor (apoER) and selenoprotein P I (SePP1) expression. In vivo, CLSO decreased the lipid droplets and TG level, reduced the protein expression of SePP1, apoER, phosphor-adenosine 5′-monophosphate (AMP)-activated protein kinase (p-AMPK) in the cytoplasm of HepG2 cells induced by oleic acid and palmitic acid (OP). At the same time, lipid accumulation was observed in the Sepp1 high expression cells induced by endoplasmic reticulum (ER) activator tunicamycin (Tm). CLSO could identically reduce the protein expression of SePP1, apoER, p-AMPK in the cytoplasm of HepG2 cells induced by Tm. This result not only proved the CLSO had therapeutic effect on NAFLD, but also confirmed its mechanism associated with degrading the phosphorylation of adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) which led to the decrease of the expression SePP1/apoER2 in order to reduce lipid accumulation. The study suggests CLSO has great medicinal value in treating NAFLD besides its edibility.

Key words: nonalcoholic fatty liver disease (NAFLD), Coix lacryma-jobi seed oil (CLSO), hepatokine, selenoprotein P, antioxidant, high-fat diet

1 Introduction
NAFLD is a clinical syndrome characterized by diffuse hepatic parenchymal cell steatosis and fat accumulation1. The global prevalence of NAFLD is up to 20% to 30% in the adult population and 70% in the obese population2. NAFLD has become the first cause of chronic liver disease in the Western countries and the second cause in China3. However, there are few ideal medications for NAFLD, and

* Correspondence to: Xiaochun Han, Shandong University of Traditional Chinese Medicine, Jinan, CHINA. ShiJun Wang and Changhong Liu have equal contributions.
E-mail: hanxch002@163.com
Accepted February 8, 2021 (received for review September 12, 2020)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs
7%-30% patients with NAFLD will progress to non-alcoholic steatohepatitis (NASH). Half of NASH may progress to fibrosis or cryptogenic cirrhosis. The prevention and therapy of NAFLD has been a worldwide issue.

At present the pathogeneses of NAFLD have not been fully elucidated. The generally accepted theory is "two-hit theory" or "multiple-hit theory". The first "hit" refers to the lipid accumulation in the liver caused by insulin resistance, which increases the sensitivity of the liver to injury events. The second "hit" refers to lipid peroxidation, oxidative stress, inflammation, mitochondrial dysfunction and other factors which further lead to NASH. In this procedure, the lipid metabolism disorder plays the key role.

CLS is a traditional plant which has been cultivated in China for 4,000 years. Chinese people used CLS as both food and medicine. In traditional China medicine (TCM), CLS is frequently used in the treatment of obesity, Metabolic Syndrome (MetS), hypertension, tumor, NAFLD, digestive problems, etc. CLSO is an active component extracted from CLS. It has been found to be effective against cancer. We have observed that CLSO had the effect of reducing blood fat and antioxidative in high-fat (HF)/diet rats. It is well known that hyperlipidemia and lipid peroxidation are major pathogenic factors for NAFLD, which suggests that CLSO may have a certain therapeutic effect on NAFLD.

Hepatocyte in the pathogenesis of lipid accumulation has been paid more and more attention. Hepatokine refers to a class of cytokine or active polypeptides which are liver-derived secretory that include fibroblast growth factor-21 (FGF-21), insulin-like growth factors (IGF), SePP, fetuin-A (fet-A) and leukocyte cell-derived chemotaxin 2 (LECT2). There are literature reports that SePP up-regulation has been correlated with insulin resistance. But the relationship between SePP1 imbalance or accumulation of lipid and NAFLD is still unknown.

Considering the abnormal expression of hepatokine in NAFLD, we hypothesize that the effects of CLSO might be associated with the suppression of hepatokine up-regulation. We choose the SePP1, which is closely related to antioxidation and insulin resistance, as the index to determine the effects of CLSO in vitro. At the same time, we use the endoplasmic reticulum (ER)/activator tunicamycin (Tm) to induce Sepp1 high expression. The results will clarify the pathogenesis of NAFLD and lay a foundation for the pharmacological action of CLSO.

2 Materials and Methods
2.1 In vivo NAFLD rats induced by HF diet
2.1.1 Animals and drugs
In this study, 50 male wistar rats weighing 180-200 g were obtained from Weitong Lihua Experiment Animals Inc. Ltd. (SCXK2016-0006, Beijing, China). The animals were housed in individually ventilated cages (IVCs) at 25°C, a 12-hr dark-light cycle and free diet and water. All experimental procedures were approved by the Committee of Animal Research, Shandong University of Traditional Chinese Medicine. The rats were kept in the lab for 7 days to adapt to the environment. The CLSO was purchased from Zhejiang Kanglaite Pharmaceutical Co. Ltd. (Kanglaite, China, Product lot number: 20180406).

2.1.2 Protocol of the study
Animals were randomly divided into 5 groups (n = 10 for each group). The control group (C) received normal diet: AIN-93M standard diet for 16 weeks. The model group (M) received HF diet: diet containing 55.6% of calories from fat for 16 weeks. Group LC, MC and HC received the HF diet same to model group and received CLSO intrastragastric administration (0.28, 0.56, 1.12 g kg⁻¹ d⁻¹, respectively) for additional 14 days (total 16 weeks). The volume of gavage was calculated based on the weight. The normal rats and the model rats were intrastragastric administration with normal saline. The animals were weighed every two weeks.

2.1.3 Ethics
The study protocol was approved by Shandong University of TCM Ethical Committee (NO. SDUTCM2018090101). The experiment had taken place in the laboratory of Shandong University of TCM.

2.1.4 Measurements of biochemical parameters in serum
The rats were anesthetized with 10 mg/mL pentobarbital sodium (40 mg/kg, i.p.) based on weight after 14 days treatment of CLSO. Blood was collected by anesthetizing of rats with the 4% isoflurane via the respiratory route by exposing them to R500 anesthesia apparatus for small animals (RWD, China). The serum was obtained after centrifugation and the tissue supernatant was taken after homogenate. TC, TG, low density lipoprotein (LDL), HDL, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were evaluated by an automatic biochemical analyzer (Hitachi, Japan).

2.1.5 Ratio of liver weight to body weight
After the rats were executed, the liver was taken out and weighed on the analytical balance (BSS 124S, Germany). The ratio of liver weight to body weight was liver weight/body weight.

2.1.6 Measurements of antioxidant profile in serum
MDA was assayed by MDA Assay Kit (Shanghai Chuangxiang Biological Technology Co., Ltd. no. CK-E60367R), while SOD by SOD assay kit (Shanghai Chuangxiang Biological Technology Co., Ltd. no. CK-E60367R). The method of evaluating MDA and SOD was enzyme linked immunosorbent assay (ELISA) (MS 352, Labsystems Multiskan, Finland).

2.1.7 Histological examination
Histological examination of the same lobe of liver in each
animal was performed on paraformaldehyde. The fixed tissues were embedded in paraffin and cut into 4 μm thick sections. After hematoxylin-eosin(HE) and oil red O staining, the stained sections were viewed with the whole slide Pannoramic MIDI scanner(3D histech).

2.1.8 Measurements of apoE, apoER2 and SePP1 level in hepatic tissue

The hepatic tissues from same lobe were homogenized in cold saline solution. The homogenates were centrifuged at 4500 r/min for 15 min at 4°C to remove the nuclear fraction, and the supernatants were re-centrifuged at 10000 r/min for 10 min at 4°C to obtain the supernatants for assessing apoE, apoER and SePP1 by ELISA. apoE, apoER and SePP1 assay kit were purchased from Shanghai Chuangxiang Biological Technology Co., Ltd (no. CK-E30129R, CK-E95966R, CK-E95968R).

2.1.9 Western blot analysis

After treatment, the hepatic tissues were washed with ice-cold PBS and collected in RIPA buffer containing proteinase inhibitor and phosphatase inhibitor. Protein concentrations were measured by the bicinchoninic acid (BCA) method. Protein solution was added into 5 × protein sample buffer at a ratio of 4:1, denatured in boiling water for 15 minutes, separated by SDS-PAGE electrophoresis, and transferred to PVDF membrane at 25 V constant pressure, minutes, separated by SDS-PAGE electrophoresis, and transferred to PVDF membrane at 25 V constant pressure, and probed with antibodies to AMPK transcribed with antibodies to AMPK and p-AMPK. The images were scanned. After finishing and decolouring, the optical density of target blots was analyzed by densitometry using scientific imaging software (Image J).

2.2 In vitro NAFLD model of HepG2 cell

2.2.1 Cell culture

Human hepatocellular carcinoma cell line HepG2 (TCHu72, the Chinese Academy of Sciences cell bank) was grown at 37°C in 5% CO₂ in Dulbecco’s modified Eagle medium (DMEM) (Kaiji Company, China) containing 10% fetal calf serum (Gibco, American), 10 ml/L penicillin and streptomycin (Magen, China). All cellular experiments were approved by the Committee for Cellular Study at our institute.

2.2.2 Intervention

Cells were divided into 5 groups: control group (Con group), hepatic steatosis group induced by oleic acid and palmitic acid (OP group), Sepp1 high expression group induced by endoplasmic reticulum (ER) activator tunicamycin (Tm group), OP + CLSO (10 μM) group, Tm + CLSO (10 μM) group. The dosage of CLSO was based on the maximum inactive dosage (Supporting Information 1).

2.2.2.1 Exposure of HepG2 cells to oleic acid and palmitic acid

Oleic acid and palmitic acid (OP) were purchased from Jiancheng Corporation (Nanjing, China). For the experiments, the cells were seeded in at a density of 4 × 10⁶ cells per well and cultivated for 24 h. The cells were cultivated with fresh DMEM media for 12 h. And, the mixture of oleic acid and palmitic acid (0.5 mM, 2:1 ratio) was used to induce lipid droplet accumulation in the HepG2 cells. The cells were harvested for subsequent assays at specific time points.

2.2.2.2 Exposure of HepG2 cells to ER stress inducers

According to the literature, the high express of SePP1 can be induced by endoplasmic reticulum (ER) stress inducers tunicamycin (Tm). So we co-cultured the HepG2 cells with 4 mg/L Tm (Solarbio, Beijing, China) and DMED media.

2.2.3 Oil Red O staining

The cells were washed with ice-cold PBS to remove unbound stain, and then fixed in 1 ml of 4% paraformaldehyde for 30 min. After fixation, the cells were washed with distilled water and stained with Oil Red O solution (in 60 % isopropanol) for 20 min. The stained cells were repeated dyed by Harris’s hematoxylin for 15 seconds after washed with 75% alcohol to remove excess dyes. The cells were captured by optical microscope (Olympus IX71, Japan). Fat droplets in the HepG2 cells were stained red.

2.2.4 Western blot analysis

After treatment, the cells were washed with ice-cold PBS

![Fig. 1](image-url)
and collected in RIPA buffer containing protease inhibitor and phosphatase inhibitor. Protein concentrations were measured by the bicinchoninic acid (BCA) method. Protein solution was added into 5 × protein sample buffer at a ratio of 4:1, denatured in boiling water for 15 minutes, separated by SDS-PAGE electrophoresis, and transferred to PVDF membrane at 25 V constant pressure, and probed with antibodies to SePP1 (1:1000; Rabbit Polyclonal, lot number: ab109514, abcam); apoER2 (1:1000; Rabbit Polyclonal, lot number: ab204112, abcam); GAPDH (1:1000; Rabbit Polyclonal, lot number: GB12001, Servicebio). The images were scanned. After finishing and decoloring, the optical density of target blots was analyzed by densitometry using scientific imaging software (Image J).

2.3 Statistical analysis
Data was expressed as X ± S. One-way analysis of variance (ANOVA) and Dunnett’s t test were used for the difference comparison. All the data were analyzed by IBM SPSS21.0 software. P < 0.05 was considered as statistical significance.

3 Results
3.1 Effect of CLSO in NAFLD rats
3.1.1 Effect of CLSO on body weight and liver/body weight ratio in NAFLD rats
The increase of liver mass is thought to be associated with the development of NAFLD. To determine the effect of CLSO on the ratio of liver/body weight, all animals and their livers were weighed after the remedy of 14 days. As shown in Fig. 1, liver/body weight ratio significantly increased in the HF diet rats (p < 0.05 vs. control group), while body weight did not increase significantly (p > 0.05 vs. control group). Furthermore, rats on CLSO treatment group demonstrate a statistically significant decrease in liver/body weight ratio (p < 0.05 vs. model group) (Fig. 1).

3.1.2 Effect of CLSO on serum levels of TC, TG, LDL, HDL in NAFLD rats
As shown in Fig. 2, the TC, TG of model rats increased while the HDL decreased significantly (p < 0.05 vs. control group); different doses of CLSO could lower the serum TC and TG, elevated the HDL. At high doses (1.12 g mg kg⁻¹ d⁻¹), the TC, TG, LDL and HDL levels of the NAFLD rats reached or were close to the level of control rats.

3.1.3 Effect of CLSO on fat accumulation of liver in NAFLD rats.
HE and Oil Red O stain were used to evaluate the effects of CLSO on fat accumulation in NAFLD rats. The structure of hepatic lobules and nuclei was clear and the hepatic
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3.1.4 Effect of CLSO on serum level of ALT, AST, SOD and MDA in NAFLD rats

As shown in Fig. 4, the ALT in model group was abnormal \((p<0.05\ \text{vs.}\ \text{control group})\), and it could return to normal after high doses CLSO \((1.12 \text{ g mg kg}^{-1} \text{ d}^{-1})\) treatment \((p<0.01\ \text{vs.}\ \text{model group})\); MDA of model rats increased while the SOD decreased significantly \((p<0.05\ \text{vs.}\ \text{control group})\); medium dose and high dose \((0.56, 1.12 \text{ g mg kg}^{-1} \text{ d}^{-1})\) of CLSO could also reduce the abnormal expression of SOD and MDA \((p<0.05\ \text{vs.}\ \text{model group})\).

3.1.5 Effect of CLSO on apoE, apoER and SePP1 in liver tissues of NAFLD rats

The decrease of liver apoE is thought to be associated with the development of NAFLD. As shown in Fig. 5, the apoE and apoER of model rats increased while the SePP1 decreased significantly \((p<0.05\ \text{vs.}\ \text{control group})\); medium dose \((0.56 \text{ mg kg}^{-1} \text{ d}^{-1})\) of CLSO could increase the liver apoE and apoER; high dose \((1.12 \text{ g mg kg}^{-1} \text{ d}^{-1})\) of CLSO could increase apoER, low dose \((0.28 \text{ mg kg}^{-1} \text{ d}^{-1})\), medium dose \((0.56 \text{ mg kg}^{-1} \text{ d}^{-1})\) and high dose \((1.12 \text{ g mg kg}^{-1} \text{ d}^{-1})\) of CLSO could lower the abnormal expression of SePP1 \((p<0.01\ \text{vs.}\ \text{model group})\).

3.1.6 Effect of CLSO on AMPK and p-AMPK in liver tissues of NAFLD rats

As shown in Fig. 6, high-fat diet increased the expression of AMPK in the liver tissue of rats, low \((1.12 \text{ g mg kg}^{-1} \text{ d}^{-1})\) and high doses \((1.12 \text{ g mg kg}^{-1} \text{ d}^{-1})\) of CLSO have little effect on the expression of AMPK; Compared with the model group, low dose \((0.28 \text{ mg kg}^{-1} \text{ d}^{-1})\) and high dose \((1.12 \text{ g mg kg}^{-1} \text{ d}^{-1})\) of CLSO \((p<0.01)\) could lower the abnormal expression of p-AMPK \((p<0.01\ \text{vs.}\ \text{model group})\).

3.2 Effect of CLSO in HepG2 cells

3.2.1 Effect of CLSO on lipid accumulation in HepG2 cells

The effect of CLSO on lipid accumulation in HepG2 cell was evaluated by Oil Red O staining and the TG concentra-
There was no lipid accumulated in the control cells. There were evident lipid droplets in the cytoplasm of OP and Tm intervene cells. This indicated that the lipid accumulation in the HepG2 cells was related to Sepp1. After CLSO treatment, the lipid droplets decreased significantly which indicated that CLSO could reduce the lipid accumulation (Fig. 7A). TG level also showed the same trend. TG increased in OP and Tm groups which indicated that both OP and Tm could induce lipid accumulation. And CLSO decreased the lipid accumulation (Fig. 7B).

3.2.2 Effect of CLSO on the expression of SePP1, apoER, AMPK and p-AMPK in HepG2 cells

In order to explore the mechanism of CLSO on ameliorating lipid accumulation in HepG2 cells, we examined the protein expression of SePP1, apoER, AMPK, p-AMPK and GAPDH. In this study, compared with control group, the protein expression of SePP1, apoER, p-AMPK increased significantly ($p<0.01$ vs. control group). These data suggested that the possible mechanisms of lipid accumulation in HepG2 cells were associated with the activation of the p-AMPK/SePP1/apoER2 pathway. The phosphorylation of AMPK could induce the increase of SePP1 expression, which led to the increase of lipid accumulation in HepG2. For further verification, the expression of SePP1/apoER2/p-AMPK of OP + CLSO group decreased significantly compared with the OP group ($p<0.01$ vs. OP group). The same time, the expression of SePP1/apoER2/p-AMPK of Tm + CLSO group decreased significantly compared with the Tm group ($p<0.01$ vs. Tm group). This result further proves that the effect of CLSO on ameliorating lipid accumulation is associated with degrading the phosphorylation of AMPK which leads to the decrease of the expression SePP1/apoER2 in order to reduce lipid accumulation (Fig. 8).
4 Discussions

The “second strike” doctrine is a well-recognized theory of fatty liver. “The First Strike” was the accumulation of intrahepatic lipids caused by insulin resistance, which increased the sensitivity of the liver to injury events. “The Second Strike” is a series of processes in which the liver further develops inflammation, necrosis and fibrosis under the combined effects of inflammation, oxidative stress, lipid peroxidation and mitochondrial dysfunction. In fact, the occurrence of NAFLD is the result of a multi-factor injury, and the accumulation of fat in liver is the first step in its onset. At this stage, liver factors also play an important role.

Hepatokine is a protein with similar hormonal properties which is secreted by liver cells and is involved in autocrine, paracrine and endocrine signaling. The liver has sensitive metabolic activities. During fasting, liver cells can exhibit high expression of up to several thousand genes, especially genes involved in fatty acid metabolism and ketone body formation. This process depends on the transcription of the nuclear receptor peroxisome proliferator-activated receptor (PPAR-α). After feeding, insulin stimulates the synthesis of hepatic glycogen and fatty acids. This process also involves the participation of a large number of tran-
scription factors such as fork head box protein O1 (FoxO1) and sterol regulatory element binding protein (Srebp)-1c17-19. Some scholars believe that the liver is similar to adipose tissue and muscle tissue, and has the function of endocrine, which can regulate insulin resistance and the progression of NAFLD by secreting specific hormones20. Abnormal liver factors can lead to metabolic disorders in the body, and metabolic diseases such as T2DM and NAFLD21.

The earliest discovered liver factor was the fibroblast growth factor (FGF21), which is regulated by PPAR-α. In addition to FGF21, adropin, angiopoietin-like protein 4 (ANGPTL4), fetuin-A, fetuin-B, hepassocin, leucocyte cell-derived chemotaxin 2 (LECT2), follistatin, growth differentiation factor 15 (GDF15), retinol-binding Protein 4 (RBP4) and selenoprotein P (SeP) are also important liver factors22-25.

SeP is a protein which is built by the peptide chain with Sec in form of Se, and is a main carrier for the biological action of Se. Se is an essential nutrient for the body, with the potential functions to enhance immunity, anti-aging, prevent cardiovascular diseases and certain tumors. However, studies have also reported that mice overexpressing selenoprotein glutathione peroxidase (GPX1) can perform like T2DM symptoms26. In a large community study supplemented with selenium to prevent prostate cancer, there has also been an increase in the incidence of T2DM, which led to the early termination of the project27. People began to realize that the relationship between Se and the body might be more complicated. The Sep formed by selenium in the body is a research hotspot for its biological effects.

The processes of selenoproteins synthesis in humans and mammals are similar. SeH is converted to selenophosphate (H2PO3SeH) by selenophosphate synthetase (SPS2 or SPS1) in food or in vivo. H2PO3SeH reacts with PSer-tRNA[Sec] under the action of SecS to generate Sec-tRNA[Sec]. Next, Sec-tRNA[Sec], with the assistance of other factors, finally incorporates Sec into the peptide chain of the selenoprotein mRNA. Among these cofactors, SECIS is a special nucleotide sequence on the 3’ untranslated region (3’UTR) of selenoprotein mRNA itself. The SECIS sequences of various selenoprotein genes are quite conservative, and it takes effect with its special secondary stem-loop structure.

A total of 25 Sep have been discovered, and there are 24 species in rodents28. The effect of some Sep is clear, such as GPXs, TrxRs, DioTs. But SePP1 was a newly defined liver factor in recent years. Its function is still unclear29. Sepp1 full-length expression product in human plasma contains 10 Sec residues. SePP1 in rat plasma contains 4 subtypes30. There are two SECIS structures on the Sepp1 mRNA 3’UTR, and the second SECIS (SECIS 2) appears to be involved in directing the first Sec into the N-terminus of the peptide chain, while the remaining UGA is involved in recognition by SECIS1. Studies31 have shown that SePP1 released by liver synthesis can transport Se to the peripheral target tissues, bind heavy metals, resist oxidation, regulate and respond to the body’s Se nutrition level, which seems to be related to the occurrence of metabolic diseases.

Coix lacryma-jobi seed (CLS) is regarded as a dual-use food in China. In the traditional Chinese medicine theory, CLS can invigorate spleen to remove phlegm and has a good effect on hepatic stasis. Its various extracts have also been found to have many health and medical effects. Coix seed oil (CLSO) is an extract of CLS and is sometimes used clinically as an anti-cancer adjuvant.
medicine also holds that NAFLD is due to the deficiency of spleen, which in turn causes Taning blood, phlegm, blood stasis and resistance of hepatic vein.

Many vegetable oils such as olive oil, palm oil, Nigella sativa oil and Patchouli Oil have been reported to improve the symptoms of NAFLD. But so far there has not been any study of CLSO in the treatment of this disease. The main ingredients in CLSO are fatty acids such as palmitic, stearic, oleic, and linoleic acids. CLSO has been used in the treatment of non-small cell lung cancer, gastrointestinal malignancy, primary liver cancer and other malignant tumors in the past 20 years, with high clinical efficacy and safety. We tried to use CLSO to treat NAFLD, and the results proved that it also had a good effect. However, the specific chemical composition of the therapeutic effect is not clear, which is the focus of our next research. CLSO is extracted from coix seed, which is easy to obtain and cheap. Therefore, we try to treat NAFLD with CLSO. The results showed that CLSO could significantly relieve the symptoms of NAFLD in rats induced by high-fat diet, and also have a significant effect on the oleic acid-induced HepG2 NAFLD cell model.

The use of CLSO on treating NAFLD in vitro and in vivo has also confirmed that the liver factor SePP1 played an important role. Both in vivo and in vitro, CLSO has an obvious effect of reducing hepatocyte lipid accumulation and lowering blood lipids. Vivo studies showed that the effect of CLSO on lowering blood fat was related to improving SOD expression and decreasing MDA. This process is accompanied by changes in SePP1. This result is basically consistent with other scholars’ researches on the relationship between SeP and metabolic diseases. Bi M et al. found that SeP is associated with indicators of hepatic oxidative damage. Misu et al. considered that SeP can promote insulin resistance in liver and skeletal muscle. A study of SeP-deficient mice found that mice had elevated glucose tolerance and increased insulin sensitivity in liver and muscle tissue compared to normal mice, but intraperitoneal injection of SeP purified protein significantly reduced insulin sensitivity and glucose tolerance in wild-type C57BL/6 mice. However, 71 patients with metabolic syndrome (MetS) had elevated levels of plasma SePP compared with 74 patients without metabolic syndrome. The physiological concentration of SeP inhibits vascular endothelial growth factor-stimulated cell proliferation, tubule formation and migration. SeP inhibits vascular endothelial growth factor-induced production of reactive oxygen species (ROS) and phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2) and extracellular signal-regulated kinase 1/2 (ERK1/2). Mice overexpressing SePP1 and SEPP1(−/−) mice were damaged by wound closure. However, SEPP1(+/-) mice have reduced blood flow and increased vascular endothelial cells after hind limb ischemia. Mita used anti-human SeP monoclonal antibody AE2 to counteract the activity of SeP in mice, which could significantly improve glucose intolerance and insulin resistance caused by human SeP administration. It is considered that hepatic factor SeP would be a new molecular target for the treatment of type 2 diabetes.

Most scholars believe that the regulation of SeP is related to the phosphorylation levels of AMPK and ACC. Compared with wild-type mice, the phosphorylation of IGF-1, AKT and ERK after I/R increases significantly in SeP-KO mice. In H4IIEC hepatocytes, glucose treatment can increase the gene expression and transcriptional activity of SEPP1. However, the phosphorylation levels of AMPK and ACC decreased after purification of SeP protein, and the expression of related genes of β-oxidation of fatty acids in cells was inhibited. This change was consistent with the expression of SeP. After transfecting rat cells with adenovirus-mediated dominant-negative-(DN) and constitutively active (CA) AMPK, DN-AMPK can aggravate the down-regulation of SeP-mediated protein kinase B phosphorylation, while overexpression of CA-AMPK inhibits the damage of SeP in the insulin pathway, and it is certain that AMPK is involved in the regulation of insulin pathway by SeP. Our studies in vivo also show therapeutic effect of CLSO on NAFLD is related to SePP/apoER2/AMPK pathway. In vitro studies, we used OP to induce lipid accumulation in HepG2 cells, and endoplasmic reticulum activator Tm to induce high expression of SePP1. It was confirmed that SePP1 was also highly expressed in OP-induced hepatocytes, and lipid accumulation was also observed in SePP1 high-expressing cells. This also proved our hypothesis that hepatocyte lipid accumulation in NAFLD was associated with SePP1 and the mechanism was related to p-AMPK. After CLSO treatment, and the decrease of SePP1 and p-AMPK was found in each group, which further indicated that the therapeutic effect of CLSO on the phosphorylation of AMPK was related to the regulation of SePP1.

In vitro experiments, we induced high expression of SePP1 after using the ER activator Tm, while the effect was not particularly ideal. Some scholars have also induced high expression of SePP1 by lentiviral transfection. Due to limited funding and experimental conditions, it was temporarily impossible to construct a highly expressed cell or animal with SePP1 in a more rational way. From the current results, lipid accumulation in hepatocytes was related to the regulation of SePP1 by p-AMPK. CLSO could act on this process and play a role in the treatment of NAFLD.

Our researches show that CLSO could improve the oxidative stress, reduce the phosphorylation level of AMPK in hepatocytes, and thus reduce the lipid accumulation caused by SePP1. The result of this research displays instructive experience for clinical treatment of NAFLD.
5 Conclusions
The experimental results showed that CLSO could significantly decrease the hepatic steatosis of NAFLD rats for therapeutic purposes; could significantly reduce the lipid accumulation in liver tissue; decrease the level of TC, TG, LDL and increase the level of HDL in serum; could significantly decrease the level of ALT, MDA and increase the level of SOD in serum; and could significantly decrease the expression of SePP1 in liver. The effect of CLSO on ameliorating lipid accumulation is associated with degrading the phosphorylation of AMPK which leads to the decrease of the expression SePP1/apoER2 in order to reduce lipid accumulation. The results of this study provide a scientific basis for the good clinical efficacy of CLSO, and provide evidence for further research of related NAFLD.

Acknowledgements
This work was supported by grants from Shandong Provincial Key Research and Development Program (No.2019GSF108210), National Natural Science Foundation of China (No.81703838, No.81874411), Natural Science Foundation of Shandong province (No. ZR2019ZD23, No.ZR2019MH064), College Youth Creative Talents Introduction Program of Shandong Province (No. Lujiaorenzi2019-9-201), Shandong Province Higher Education Youth Innovation Science and Technology Program (No.2019KJK013).

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of Interests
The authors have declared that they have no competing interests.

Authors’ Contribution
Xiaochun Han, Changhong Liu and Shijun Wang are fully responsible for the study designing, experiment adjustment, drafting, and finalizing the paper. Liangzhen Gu and Yanan Zhang wrote the manuscript. Haijun Zhao, Yuan Liu and Rui Ren are fully responsible for the study designing, experiment adjustment, drafting, and finalizing the paper.

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