Research Article

Received: 3 September 2019 Revised: 18 October 2019 Accepted article published: 31 October 2019 Published online in Wiley Online Library: 26 December 2019

(wileyonlinelibrary.com) DOI 10.1002/jsfa.10115

Regulation mechanism of silkworm pupa oil PUFAs on cholesterol metabolism in hepatic cell L-02

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Abstract

BACKGROUND: Silkworm pupa oil polyunsaturated fatty acid (SPO PUFA) has been confirmed to have a cholesterol-lowering function.

METHODS AND RESULTS: The effect of SPO PUFA and its main component, α-linolenic acid (ALA), on the metabolism of cholesterol and its regulation was investigated. The model of lipid denatured cells were constructed to carry out lipid accumulation, cholesterol metabolism and transformation. Real-time PCR and western blots were also used to analyze the expression levels of related genes and proteins to investigate the cholesterol efflux regulation mechanism. The data indicated that SPO PUFA and ALA dose-dependently decreased intracellular total cholesterol (TC) and enhanced total bile acid (TBA). They could also promote cholesterol removal by enhancing bile acid secretion and by upregulating genes LXRα, PPARγ, ABCA1, ABCG1, and CYP7A1, which were regulated by LXRα/PPARγ-ABCA1/ABCG1-CYP7A1 nuclear receptor signal pathways.

CONCLUSIONS: This study is of great significance in maintaining the balance of cholesterol and lipid metabolism, and in reducing the risk of steatohepatitis.

Keywords: Silkworm pupa oil (SPO); polyunsaturated fatty acids (PUFAs); α-linolenic acid (ALA); cholesterol metabolism; cell signaling pathway; hepatic cells.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are essential fatty acids that cannot be synthesized by the human body and must be obtained from the diet. Extracts from silkworm pupa can contain 90.7% unsaturated fatty acids (UFAs). Their chemical structure determines their cholesterol-lowering, anti-inflammation, and anti-tumor function, and their ability to prevent cardiovascular and cerebrovascular diseases.1–3 The main polyunsaturated fatty acids in silkworm pupa oil are oleic acid (OA), linoleic acid (LA), and α-linolenic acid (ALA). In particular, ALA, which can constitute up to 50% of silkworm pupa oil content, is a ω-3 polyunsaturated fatty acid (PUFA). It is a precursor to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which have vital physiological roles in human brain development, cardiovascular health, and inflammatory responses.4–7 α-Linolenic acid is widely used in the medical field for the prevention and treatment of multiple diseases, and in the food industry as a nutritious additive in foods and beverages.

Cholesterol not only plays an important role in the biofilm of animal cells but is also used as a crude material for the metabolic synthesis of bile acid. An excess or deficiency of cholesterol would affect normal life activities.[8] There are three ways for maintaining cholesterol metabolism balance: cholesterol synthesis, cholesterol absorption, and cholesterol efflux. Cholesterol metabolism is a particularly complex regulatory process, which is the result of multi-regulation. In the cholesterol efflux pathway, PPAR/LXR/RXR-ABCA1/ABCG1-CYP7A1 is one of the most important nuclear receptor signal pathways.[9,10] Cholesterol-lowering foods may affect the cholesterol metabolism by decomposing cholesterol into bile acid, regulating transporter factors such as the upstream genes, including liver X receptor (LXRα), peroxisome proliferator-activated receptor γ (PPARγ) and their downstream genes ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1) and cholesterol 7α hydroxylase (CYP7A1).[11] LXRα, a major transcription factor plays a key role in equilibrating decomposition and metabolism of cholesterol as well as lipid biosynthesis.[12,13] The transcription factor PPARγ and LXRα can stimulate cholesterol efflux by inducing the expression of ABCA1.[14–16] Transcription of membrane transporter ABCA1 is

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under the control of LXR. ABCG1 is another member of the ATP-binding cassette transporter superfamily, which reportedly contributes to HDL2- and HDL3-dependent cellular cholesterol efflux.[17,18] The activation of LXR could increase the expression of ABCG1 transcripts.[19] CYP7A1 controls the formation of bile acid. Research has found that rats and mice have the capacity to transform cholesterol to bile acids by the LXRα-mediated activation of CYP7A1 transcription.[20]

The study of SPO has mainly focused on its extraction and on the separation of its unsaturated fatty acids. Research into the effects of SPO on cholesterol metabolism at the cellular and molecular level was rarely reported. In this study, therefore, we investigated the effects of ALA- rich SPO PUFAs and pure ALA on lipid accumulation, cholesterol metabolism, and transformation for L02 cells under fatty change. Real-time PCR and western blot were also used to analyze the expression levels of LXRα, PPARγ, ABCA1, ABCG1, and CYP7A1 mRNA and their proteins, respectively, to explore the effects of SPO PUFAs and ALA on cholesterol efflux in L02 cells via the LXRα/PPARγ-ABCA1/ABCG1-CYP7A1 pathway.

MATERIALS AND METHODS

Chemicals and cell preparation

Silkworm pupa oil was obtained from Nantong Fulier Biological Company (Nantong, China). Oleic acid (OA), α-linolenic acid (ALA), oil red O stain, trypsin, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Methyl thiazol tetrazolium bromide (MTT) was obtained from Amresco (Amresco, Solon, USA). RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Grand Island, USA). Penicillin and streptomycin were purchased from Hyclone. Anti-LXRα (14351-1-AP) and Anti-ABCG1 (13578-1-AP) were purchased from Proteintech (Proteintech Group, Inc., Rosemont, USA), Anti-PPARγ (ab27649), Anti-ABCA1 (ab18180), and Anti-CYP7A1 (ab65596) were obtained from Abcam (Abcam Group, Inc., Cambridge, UK).

The L-02 human hepatic cell line was provided by the China Center Type Culture Collection (CCTCC). L-02 cells were cultured in RPMI-1640 medium containing 10% (v/v) FBS, 100 μg mL^{-1} penicillin and streptomycin. Cells were incubated at 37 °C with 5% CO_2, and the medium was changed every 3 days.

SPO PUFAs components analyze

Silkworm pupa oil (100 mg) was mixed with 0.05 mL Tween-80, and diluted with 70 °C ultrapure water to 10 mL, and 10 mg mL^{-1} SPO PUFA liquid was obtained after homogeneous mixing and filtration.[21] Gas chromatography (GC) connected with a FID detector was used for fatty acid content quantitative analysis of silkworm pupa oil capsules. The procedures were as follows: PEG-20 M capillary column (30 m × 0.25 mm × 0.25 μm); column temperature and holding time: 195 °C, 50 min; 1 μL of the sample was injected; the carrier gas was Ne; H_2 pressure was 50.0 psi; air pressure was 60.0 psi, with a flow rate of 30 mL min^{-1}; injection port temperature was 225 °C; column pressure was 20.0 psi; the FID detector temperature was 300 °C.

The effects of SPO PUFAs and ALA on L-02 cells

The effects of SPO PUFAs and ALA on L-02 cells were examined with a standard MTT assay. L-02 cells (1×10^4) were inoculated into a 96-well plate and cultured for 12 h. Subsequently, the cells were treated with different concentrations of SPO PUFAs and ALA, ranging from 100 to 1600 μg mL^{-1}, and from 2.5 to 40 μg mL^{-1}, respectively (the ALA solution was prepared as described by Hu *et al.*).[22] Cells without SPO PUFAs or ALA addition were set as a blank group. The medium was removed and the cells were washed three times after they were cultured for 24 h. Then, cells were cultured for 4 h with 200 μL MTT solution (10% 5 mg mL^{-1} MTT with 90% medium). Finally, cells were dissolved with 150 μL DMSO after the MTT solution was removed. An iMark microplate reader was used to express cell viability at a wavelength of 490 nm after being shaken for 10 min. The cell viability was calculated as follows: the proliferation rate (%) = OD490 (sample)/OD490 (control) × 100%.

Steatosis hepatic cell model establishment

RPMI-1640 medium with oleic acid was used for a steatosis hepatic cell model establishment assay. Oleic acid was conjugated to fatty acid-free bovine serum albumin (BSA) based on a method described previously.[21–23] Briefly, oleic acid was dissolved in absolute ethanol, then mixed with 0.1 mol L^{-1} NaOH solution and 10% BSA solution (final concentration in the medium was 1%). It was ensured that the final concentration of ethanol did not exceed 0.5% (v/v). Human hepatic L02 cells were seeded into six-well cell culture plates (Costar, Solarbio, Beijing, China) with a cell density of 2×10^5 in a 1000 μL/well and cultured in RPMI-1640 medium with 10% (v/v) FBS for 12 h. After that, cells were treated overnight with serum-free RPMI-1640 when the cell density reached 70%. Finally, they were changed into RPMI-1640 medium containing 0.5 mmol L^{-1} oleic acid for 24 h to build a steatosis hepatic cell model.[24]

Lipid accumulation assay

To investigate the effects of SPO PUFAs and ALA on lipid accumulation in L02 cells, L02 cells were divided into four groups: a control group (C, normal group) was treated with a serum-free culture medium including 1% BSA; a model group (M, steatosis cells model group) was treated with serum-free 1640 medium including 0.5 mmol L^{-1} oleic acid and 1% BSA; SPO PUFA treatment groups were treated with SPO PUFA concentrations of 200, 400, and 800 μg mL^{-1}, the culture condition was the same as the model group; ALA treatment groups were treated the same as the model group, including 5, 10, and 20 μg mL^{-1} ALA. All the cell groups were treated for 24 h. After 24 h, hepatic L02 cells were washed three times with phosphate buffer saline (PBS) before 4% (m/v) formaldehyde added. After standing for 5 min at 4 °C, cells were washed three times with PBS again and then stained with 0.5% (m/v) oil red O for 10 min. Subsequently, cells were washed several times with 60% (v/v) isopropyl alcohol. Finally, cells were observed and imaged using inverted fluorescence microscope (DMi8, Leica DMIRB, Wetzlar and Mannheim, Germany) after washing three times with PBS.

Cholesterol efflux assay

Total cholesterol (TC) in cells and total bile acid (TBA) in the supernatant were determined to reflect cholesterol efflux. Cells were washed twice with PBS. Cholesterol was extracted with cell lysis buffer, and determined following the cholesterol assay kit (Applygen, Beijing, China), and a BCA protein assay kit (Applygen). Cell supernatant was collected to detect TBA content following the TBA assay kit's instructions (R&D, USA).

The effect of SPO PUFAs and ALA on related gene expression in L02 cells

Total cellular RNA was lysed and harvested using the total RNA kit (Omega Bio Tek Inc., Norcross, GA, USA) according to the

Table 1. Oligonucleotides used in quantitative real-time PCR

	Forward (5′ → 3′)	Reverse (5′ → 3′)
LXRα	GGACCAGCTCCAGGTAGAGA	ACATCAGTCGGTCATGGGGA
PPARγ	TCAAGAAGACGGAGACAGACA	TGGAAGAAGGGAAATGTTGG
ABCA1	GGGAGAGCACAGGCTTTGAC	CACTCACTCTCGCTCGCAA
ABCG1	ATGGCTTAGACCGGGAAGAT	CCAGGTACAGCTTGGCATTT
CYP7A1	CTTCTGCGAAGGCATTTGGG	TCCGTGAGGGAATTCAAGGC
GAPDH	CAGGAGGCATTGCTGATGAT	GAAGGCTGGGGCTCATTT

manufacturer's instructions, and the previous experimental preparation was based on the previous research methods.[10] A Revert Aid™ First Strand cDNA Synthesis Kit (Thermo Scientific, Shanghai, China) was used for reverse transcription. A SYBR Premix Ex Taq™ II (Thermo Scientic, Shanghai, China) was used for the real-time PCR detection system. Primer sequences were listed in Table 1.

Cholesterol-metabolism-related proteins in L02 cells were further examined with a western-blot assay. Cells were washed with PBS at room temperature before 0.4 mL of cold radio immunoprecipitation assay (RIPA) buffers (Beyotime, Shanghai, China) with fresh protease inhibitor PMSF (Beyotime) were added. Subsequently, cells were scraped and the lysates were centrifuged at 10 000×*g* for 10 min. An equal volume of RIPA buffer was added into cell lysates, and boiled for 10 min before SDS-PAGE separation. Afterwards, the protein was transferred to the nitrocellulose membrane (Millipore, Bedford, MA, USA) using an electroblotting apparatus. The membrane was further incubated with the indicated primary antibody, followed by a secondary antibody conjugated with horseradish peroxidase. Finally, the proteins were supplemented with ECL western-blotting detection reagents (Thermo), and analyzed using the Bio-Rad Chemi Doc MP imaging system.

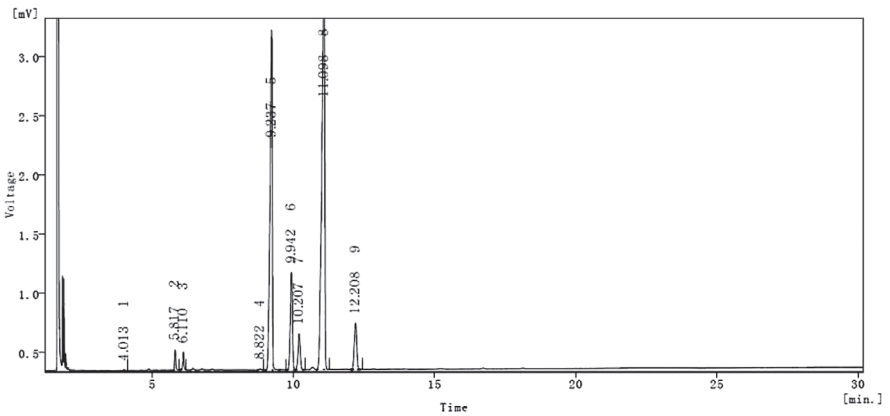
Statistical analysis

All statistical analyses were calculated using SPSS 11.5 software. Statistical evaluation was determined by one-way analysis of variance (ANOVA) followed by a *post hoc* Turkey's test. Differences where $P < 0.05$ were considered statistically significant.

RESULTS

SPO PUFAs components analyze

A typical GC chromatogram analysis result is shown in Fig. 1. The peaks were identified according to the retention times of standards. The essential components of peaks 2, 3, 4, 5, 6, and 8 were



Peak number	Fatty acids	Retention time (s)	Content (%)
2	C16:0 (Palmitic acid)	5.817	1.1
3	C16:1 (Palmitoleic acid)	6.110	1.0
4	C18:0 (Stearic acid)	8.822	0.1
5	C18:1 (Oleic acid)	9.237	32.0
6	C18:2 (Linoleic acid)	9.942	8.8
8	C18:3 (α-Linolenic acid)	11.098	48.9

Figure 1. Gas chromatography map and components analysis of SPO fatty acids.

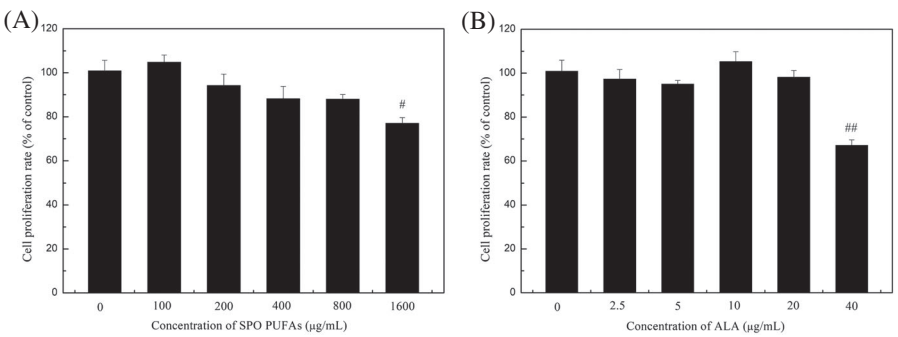


Figure 2. Effect of SPO PUFAs and ALA on L02 cell proliferation (## means $P < 0.01$, # means $P < 0.05$ compared with control groups).

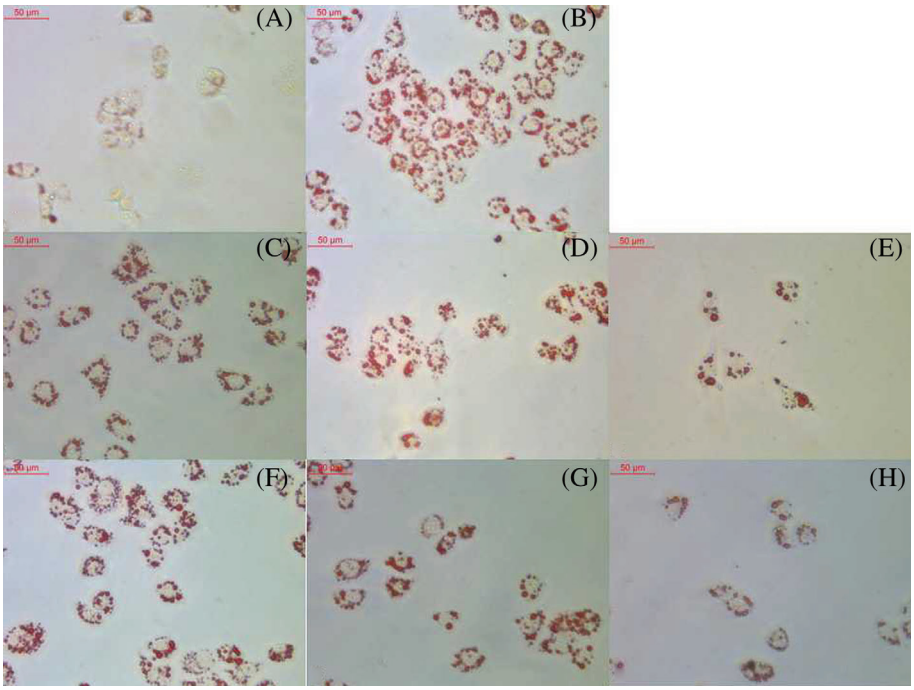


Figure 3. The accumulation of lipid droplets in different groups observed by oil red O staining. (A): control group; (B): model group; (C–E) represented SPO PUFAs-200, 400, 800 μg mL^{-1}, respectively; F, G, H represented ALA-5, 10, 20 μg mL^{-1}, respectively.

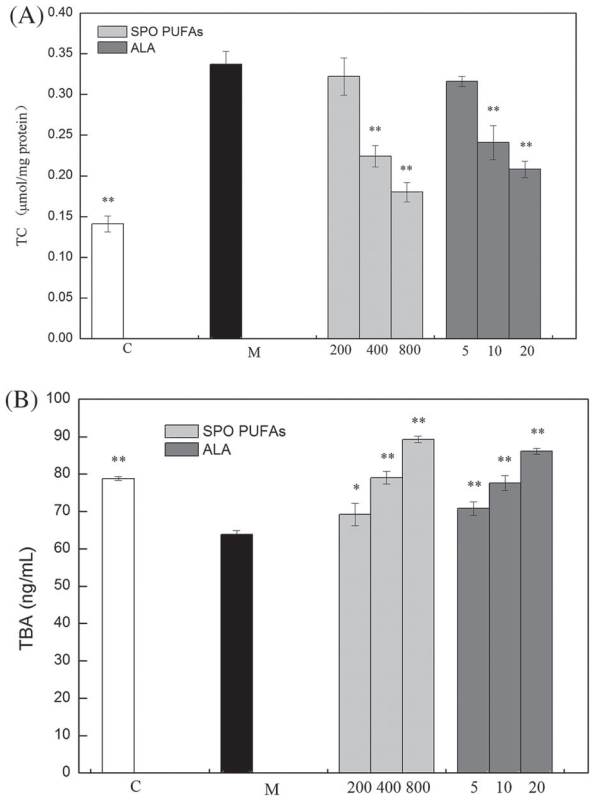


Figure 4. TC (A) and TBA (B) levels in different groups. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with model groups.

palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, and α-linolenic acid, respectively. Their relative content was 1.1%, 1.0%, 0.1%, 32.0%, 8.8%, and 48.9% on the basis of peak areas. It can be seen from the table that the unsaturated fatty acid content reached 90.7%, while that of ALA being the highest, at nearly 50%. This indicated that ALA was the main unsaturated fatty acid in SPO. Therefore, ALA was selected to conduct further experiments compared with SPO PUFAs.

Growth inhibitory effect of SPO PUFAs and ALA on L02 cells

The viability of L02 cells after they were treated with different concentrations of SPO PUFAs and ALA is shown in Fig. 2. As the figure shows, when the concentration of SPO PUFAs (Fig. 2(A)) and ALA (Fig. 2(B)) was below 800 and 20 μg mL^{-1} respectively, L02 cell proliferation rates were greater than 88% and 95%. The cell proliferation rates were 77.23% and 69.99% respectively, when the concentration of SPO PUFAs and ALA increased to 1600 and 40 μg mL^{-1}. Their cytotoxicity was evaluated as grade 1 and 2 according to the US Pharmacopeia toxicity grading method. In truth, any substance, even the common nutrients, can be harmful for cells if it is present beyond a certain dose. The purpose of this study is to investigate the functional effect of SPO PUFAs and ALA in a safe dose range, and the actual nutrient intake would be much lower than this experimental concentration. The concentrations of SPO PUFAs and ALA selected in the following assays were therefore under 1600 μg mL^{-1} (SPO PUFAs) and 40 μg mL^{-1} (ALA).

SPO PUFAs and ALA suppressed lipid accumulation of L02 cells

The accumulation of lipids in L02 cells of SPO PUFAs and ALA treatment groups after an oil red O staining assay are shown in Fig. 3. The results indicated that cells in the model group were observed to have more stained lipid droplets compared to the control group. Interestingly, the number of intracellular lipid droplets showed a dose-dependent decrease in the SPO PUFAs and ALA treatment groups. Furthermore, the high-dose treatment group showed the minimum quantity and the smallest of lipid droplets, indicating the obvious inhibition effects of SPO PUFAs and ALA.

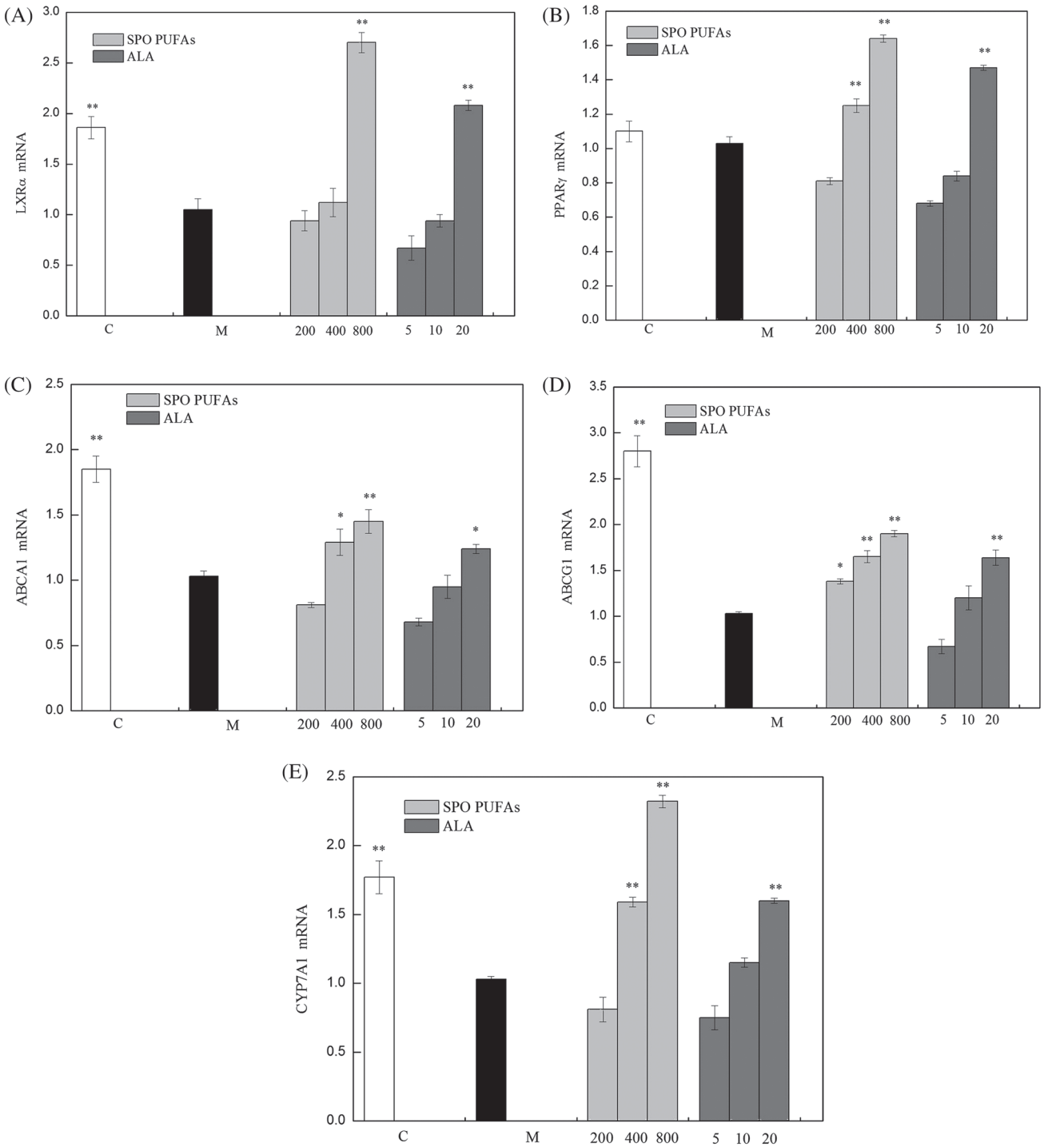


Figure 5. Effects of SPO PUFAs and ALA on LXR/PPAR-ABCA1/ABCG1-CYP7A1 nuclear receptor signaling pathways of cholesterol metabolism from real-time PCR. (a–e) are the expressions of nuclear receptors LXRα, PPARγ, ABCA1, ABCG1, and CYP7A1, respectively. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with model groups.

The effects of SPO PUFAs and ALA on TC and TBA content in L02 cells

The effects of SPO PUFAs and ALA on lipid accumulation in L02 cells were further quantitatively analyzed by intracellular TC and extracellular cholesterol metabolite TBA content. Figure 4(A) showed that intracellular TC in model group was significantly increased compared with the control group. After SPO PUFAs and ALA intervention, cholesterol level in cells dramatically decreased dose dependently. Compared with the model group, the TC content was reduced by 2.96%, 31.04%, and 43.27% in the SPO PUFAs treatment groups (200, 400, and 800 μg/mL, respectively), and by 3.43%, 26.07%, and 35.89% in the ALA treatment groups (5, 10, and 20 μg/mL, respectively), with the concentration increased. Significant differences were also found between dose groups in the SPO PUFAs and ALA treatment groups.

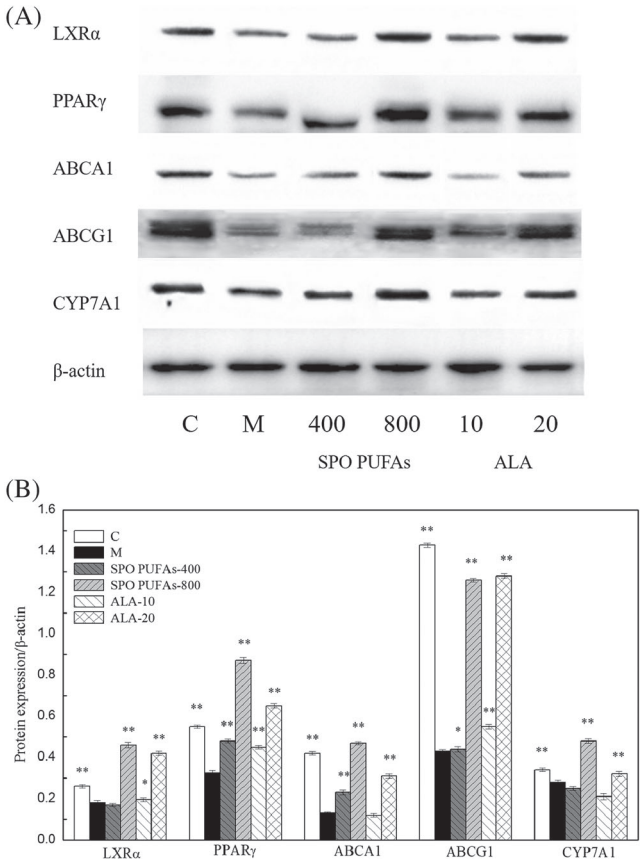


Figure 6. Effects of SPO PUFAs and ALA on protein expression from western blot analysis. $^{**}P < 0.01$, $^{*}P < 0.05$, compared with model groups.

Extracellular cholesterol metabolite TBA content is shown in Fig. 4(B): the TBA content in the model group was lower than that in the control group. Compared with the model group, the TBA content was increased by 7.71%, 23.59%, and 40.64% in the SPO PUFAs treatment group, and by 11.14%, 18.60%, and 2.34% in the ALA treatment group with their concentration increased. That was, SPO PUFAs and ALA promoted the cholesterol the process of intracellular cholesterol metabolized into bile acids and excreted extracellular in L02 cells were promoted by the effects of SPO PUFAs and ALA.

The effects of SPO PUFAs and ALA on expression of related genes and proteins in L02 cells

The effects of SPO PUFAs and ALA on the expression of related genes are shown in Fig. 5. Compared with the model group, the SPO PUFAs and ALA treatment groups rapidly upregulated mRNA levels of *LXRα*, *PPARγ*, *ABCA1*, *ABCG1*, and *CYP7A1* in a dose-dependent manner. The effect was most significant in the high dose group. In Fig. 5(a), gene *LXRα* expression was 2.70 and 2.08 times higher than in the model group at the highest concentrations of SPO PUFAs and ALA, while no significant difference was found in other concentrations, whether in SPO PUFAs or ALA treatment groups. Figure 5(b) showed that *PPARγ* mRNA levels were higher than in the model group from a concentration of 400 μg mL^{-1} in the SPO PUFAs treatment group. The expressions of gene *ABCA1* in SPO PUFAs (400 and 800 μg mL^{-1}) and ALA (20 μg mL^{-1}) were increased by 1.25, 1.40 and 1.20 times in the model group, but all were lower than in the control group (Fig. 5(c)). The expression of *ABCG1* was similar to gene *ABCA1*, most of the treatment groups were higher than model group (Fig. 5(d)). The expressions of gene *CYP7A1* mRNA in SPO PUFAs under 400 and 800 μg mL^{-1} were 1.55 and 2.25 times higher than model group. Similarly, *CYP7A1* gene expression in ALA under 20 μg mL^{-1} was 1.56 times higher than model group (Fig. 5(e)). The results indicated that the expression of related genes *LXRα*, *PPARγ*, *ABCA1*, *ABCG1*, and *CYP7A1* in L02 could be significantly upregulated by SPO PUFAs and ALA. The upregulation effects were also obvious in the high-dose group, and the upregulation effect of SPO PUFAs was better than that of ALA.

The LXRα, PPARγ, ABCA1, ABCG1, and CYP7A1 proteins were further analyzed in this study. A western blot analysis is shown in Fig. 6. It shows that SPO PUFAs and ALA treatment groups could upregulate protein levels dose-dependently compared with model groups, and the most significant result was in the high-dose group. The result of the western blot analysis (Fig. 6(a)) was consistent with mRNA levels (Fig. 6(b)). The expression of protein LXRα was upregulated by SPO PUFAs and ALA in their highest concentration groups, and the elevated levels were higher in SPO PUFAs than in the ALA treatment group. For the PPARγ protein, all of the treatment groups could up-regulate its expression. Especially the group of 800 μg mL^{-1} in SPO PUFAs, it presented the most obviously upregulation effect. The successive order of upregulation effects on protein ABCA1 were 800 μg mL^{-1} of SPO PUFAs, 20 μg mL^{-1} of ALA, and 400 μg mL^{-1} SPO PUFAs, respectively. All the treatment groups upregulated the ABCG1 protein, with the most significant effect for the highest concentration. The upregulation effects on protein CYP7A1 were obvious at higher dosages of SPO PUFAs and ALA.

DISCUSSION

Silkworm pupa are the main by-products of the silkworm industry. Silkworm pupa oil contains a large amount of unsaturated fatty acid and is especially abundant with ALA, which is beneficial for our health. Some studies have found that SPO could regulate serum lipid, inhibit the oxidized modification of serum lipid, decrease TC and TG, and increase serum EPA and DHA.[25] The results of our previous studies indicated that SPO could reduce the serum TC and TG, increase high-density lipoprotein cholesterol (HDL-C) and apolipoprotein A-I (Apo A-I), and enhance the activity of lipoprotein lipase (LPL) and hepatic lipase (HL) in hyperlipidemic rats.[26] However, studies of the effects of SPO PUFAs on regulating cholesterol metabolism, and their mechanism in hepatic cells, was rarely reported. The SPO PUFAs used in this study could remove most of the saturated fatty acid while maintaining other beneficial unsaturated fatty acids.

Hepatocytes could better reflect the metabolic status of cholesterol. The HepG2 human liver cancer cell and the L02 liver normal cell are often used for the study of cholesterol metabolism.[27–30] The results of this study demonstrated clearly that SPO PUFAs and ALA could significantly decrease the TC level in steatosis L02 hepatic cells induced by OA and could promote cholesterol metabolism. The effect of SPO PUFAs was better than single ALA. This may be explained as that SPO PUFAs is mainly composed of ALA but also contains a certain proportion of other unsaturated fatty acids, which may have certain synergistic effects. Our study also showed that SPO PUFAs and ALA could promote intracellular cholesterol metabolized into bile acids and excreted extracellular obviously. These results are consistent with other reports.[31,32]

After a comprehensive analysis of the experimental results, we found that SPO PUFAs and ALA decreased TC concentration by increasing the expressions of gene *LXRα*, *PPARγ*, *ABCA1*, *ABCG1*, and promoted TBA concentration by enhancing expression of the *CYP7A1* gene. This implied that *LXRα/PPARγ-ABCA1/ABCG1-CYP7A1* may be the signal pathway for cholesterol metabolism in hepatic cells. Our previous research also found that pomegranate ellagic acid regulated cholesterol metabolism through the *LXR/PPAR-ABCA1* pathway.[33]

There are other studies that are in some ways consistent with our current findings. Desvergne and Wahli[34] reported that fatty acids played a critical role in the activation of *PPARγ*, which indirectly upregulated the expression of the *ABCA1* and *ABCG1* genes by elevating *LXR* gene expression. Kamisako reported that an n-3 fatty acid-rich diet was able to upregulate bile acid and cholesterol excretion, and increase hepatic expression of *CYP7A1*, *CYP8B1*, and *CYP27A1* in mice.[31] Tang[35] showed that the n-3 polyunsaturated fatty acids EPA and DHA could reduce cholesterol levels significantly, and elevate CYP7A1 and ABCG8 protein expression in HepG2 cells. Chiang[36] proved that CYP7A1 can regulate CYP8B1, which mediate bile acid synthesis through cholic acid synthesis.

In conclusion, this study systematically investigated the effects of SPO PUFAs and ALA on relevant genes and proteins in the signal pathway of cholesterol efflux. The results indicated that SPO PUFAs and ALA could reduce intracellular TC and lipid droplets, enhance bile acid in the medium, and therefore increase cholesterol efflux in L02 hepatic cells. Its mechanism may be regulated by the *LXRα/PPARγ-ABCA1/ABCG1-CYP7A1* cell signal pathway: SPO PUFAs and ALA were mainly used to regulate the relative expressions of the upstream genes *LXRa* and *PPARγ* to upregulate the expression of the downstream genes *ABCA1*, *ABCG1*, and *CYP7A1*. These transcription factors are therefore of great significance in maintaining the balance of cholesterol and lipid metabolism, and reducing the risk of atherosclerosis.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (grant no. 31871801), the Fundamental Research Funds for the Central Universities (no. GK201803068; GK201703066), and the Science and Technology Research of Shaanxi Province (no. 2018SF-384; 2019NY-136), and was also supported by Shaanxi Provincial Science and Technology Department project no. 2018TD-020.

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