

Original Article

The antiatherosclerotic and antianxiety-like effects of the *Cordyceps sobolifera* mycelium in apoE deficient mice

Cai-Xia Lou, Wei Huang, Yi Wang, Sheng-Lai Liu, Shao-Song Kuang, Zi-Liang Rao, Zhi-Yong Zhong, Xiao-Jiang Tang

Laboratory of Comparative Medicine, Guangdong Medical Laboratory Animal Center, Foshan, China

Received June 26, 2017; Accepted December 10, 2018; Epub April 15, 2019; Published April 30, 2019

Abstract: The present work evaluates the antiatherosclerotic and antianxiety-like effects of the *Cordyceps sobolifera* mycelium (CSM) in apoE deficient mice. The mice were fed a high fat and high cholesterol diet and CSM for 12 weeks. After that, an Open Field Test was carried out. Their aortas were dissected, Oil Red O stained, and photographed. The results showed that all the mice had lesions in the aorta. However, compared with the control mice, the atherosclerotic lesion areas in the whole aorta of the CSM-treated mice were decreased ($P<0.05$), the gene expressions of *Sptlc1* and *Sptlc2* in the CSM-treated bone marrow-derived macrophages and brain tissues were decreased ($P<0.05$), the *IL-6* protein levels of CSM-treated (CSM-MD or/and CSM-HD) mice in the macrophage culture supernatants and brain tissues were decreased ($P<0.05$), the *TNF- α* protein levels of the CSM-MD and CSM-HD groups in the serum and brain tissues were decreased ($P<0.05$), the *MCP-1* protein levels of the CSM-MD mice in brain tissues and the CSM-treated mice in the macrophage culture supernatants were decreased ($P<0.05$). During the Open Field Test, the durations in the outer ring of the CSM-HD treated mice decreased ($P<0.05$), and the durations and distances in the central area of the CSM-HD treated mice increased ($P<0.05$). Our study found that CSM can reduce murine atherosclerotic lesions and have antianxiety-like activities, and these effects may correlate with SPT inhibitory and impaired inflammatory responses.

Keywords: *Cordyceps sobolifera* mycelium, antiatherosclerotic effect, antianxiety-like behaviors, apoE deficient mice

Introduction

The *Cordyceps sobolifera* mycelium (CSM) is the fruiting body from *Cordyceps sobolifera*. As a traditional Chinese medicine, CSM is a kind of entomogenous medicinal fungi which contains amino acid, polysaccharide, cordyceptic acid, adenosine, ergosterol, etc. [1]. CSM is considered to have extensive pharmacological activities, such as improving renal function [2, 3], inducing hypoglycemia [4], having sedative properties [4], having anti-fatigue effects [5], etc. CSM is often regarded as an ideal substitute for *Cordyceps sinensis*, because they are basically similar in ingredients and function. Other studies have shown that CSM can decrease serine palmitoyltransferase (SPT) activity by 60.2% [6]. SPT is the first and rate-limiting enzyme of the de novo biosynthetic pathway of sphingomyelin. SPT has been implicated in the pathogenesis of atherosclerosis, a

disease in which macrophages are known to be a critical driving force in its development. In a previous study, we found that *Sptlc2*^{-/-} macrophages significantly lower sphingomyelin levels in plasma membranes and lipid rafts [7]. This reduction not only impairs inflammatory responses triggered by *TLR4* and its downstream NF- κ B pathways, but it also enhances reverse cholesterol transport mediated by ABC transporters [7]. Based on these results, CSM, as an SPT inhibitor, may be effective for atherosclerosis prevention and treatment. However, little research has been done in this area. The effects of CSM on macrophage-mediated atherogenesis are unknown. In this paper, we evaluated the antiatherosclerotic and antianxiety-like effects of the *Cordyceps sobolifera* mycelium in apoE^{-/-} mice.

The apoE^{-/-} mice were fed a high fat and high cholesterol diet and CSM for 12 weeks. Then,

their aortas were dissected, Oil Red O stained and photographed. The results showed that CSM could reduce the area of atherosclerotic lesions in mice. In order to understand the effects and possible mechanisms of CSM atherosclerotic plaque formation and antianxiety-like behaviors in *apoE*^{-/-} mice, we designed and conducted a series of experiments: 1) to ensure the successful establishment of the atherosclerosis model, we measured some plasma lipids at the end of the 12th week, 2) carried out the Open Field Test, 3) measured some biochemical levels in the plasma, 4) measured the gene expression of *Sptlc1* and *Sptlc2* in macrophage and brain tissues, 5) measured the inflammatory cytokines of the *IL-6*, *TNF-α* and *MCP-1* protein levels.

Materials and methods

Mice and agents

All animal studies were performed in accordance with guidelines of the Institutional Animal Care and Use Committee at the Guangdong Medical Laboratory Animal Center. 40 female *apoE*^{-/-} mice and 8 female C57BL/6 mice (weighing 18~25 g, 10~12 weeks old, SPF level) were provided by the Guangdong Medical Laboratory Animal Center (Foshan, Guangdong, China). The quality certification numbers are 44007200020569 and 44007200020630. The experimental animals use license: SYXK (Guangdong) 2013-0002. The CSM powder (Lot: 20141010, a pure powder of cultivated *Cordyceps sobolifera* mycelium, through low temperature drying and ultramicro smash technology) was purchased from Zhejiang Fangya Biomedical Co., Ltd. Atorvastatin Calcium (Lot: 141220) was purchased from Beijing Jialin Pharmaceutical Co., Ltd. Oil Red O was purchased from Sigma Co., Ltd. Mouse Tumor necrosis factor α (*TNF-α*, Lot: CSB-E04741m), Mouse Interleukin 6 (*IL-6*, Lot: CSB-E04639m), and Mouse monocyte chemotactic protein 1 (*MCP-1*, Lot: CSB-E07430m) The ELISA kits were purchased from Cusabio Co., Ltd.

Model and diets

ApoE^{-/-} mice fed a high fat and high cholesterol diet are practical and effective for hyperlipidemia and atherosclerosis modeling. Hyperlipidemia is an important risk factor for atherosclerosis. This special feed formula is composed of sucrose (20%), lard (15%), cholesterol (1.2%), sodium cholate (0.2%), casein (10%), dicalci-

um phosphate (0.6%), powder (0.4%), premix (0.4%), and basic feed (52.2%). The quality ratio is composed of protein (19%), fat (18.5%), and carbohydrates (50.5%). The calorie ratio is composed of protein (17.5%), fat (37%), and carbohydrate (45.5%). The C57BL/6 mice as a blank control were given a normal diet during the experiment. During the modeling, all the treatment groups were orally administered by corresponding samples once a day of 0.1 mL/10 g bw, while the control group and blank control group were given normal saline.

Study design

The 8 female C57BL/6 mice were used as Normal (Nor) group directly. The 40 female *apoE*^{-/-} mice were randomly divided into 5 groups: the Control (Con) group, the Atorvastatin Calcium group (AC), the CSM with low (CSM-LD), the medium (CSM-MD), and the high dose (CSM-HD) treatment groups, with 8 mice in each group. The three CSM treatment groups were given doses of 33.3 mg/kg, 333 mg/kg, and 666 mg/kg bw respectively. The Atorvastatin Calcium group was given a dose of 5 mg/kg bw.

Plasma lipids measurement

Before the end of experiment, all the mice fasted for 12 hours, then we collected blood samples. We measured the total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels in the blood plasma.

Open Field Test

In order to evaluate the anxiety levels of the mice, an Open Field Test was conducted at the end of 12 weeks. The mice were placed in an Open Field arena (50 × 50 cm, Guangzhou Feidi Biological Technology Co., Ltd. Flyde-A) surrounded by a 35 cm high wall for 5 minutes. An automated smart system with a camera was positioned above the center of the open field. The total distance moved, the distance in the outer ring and the central area, the average speed, the duration of movement in the outer ring and the central area were assessed. Spending more time in the periphery was regarded as a sign of anxiety [8]. The box was cleaned first with ethanol and then with water before each mouse was introduced to the Open Field arena [8].

Table 1. Primer sequences used for real-time PCR

Gene	Sense	Sequence	Product size (bp)
<i>Sptlc1</i>	Forward	5'-AGTTGCAGGAGCGTTCTGATC-3'	106
	Reverse	5'-AGAGCAGGGTGGTCTTGGA-3'	
<i>Sptlc2</i>	Forward	5'-GAGAGATGCTGAAGCGGAACA-3'	98
	Reverse	5'-ATGAGCTGCTGACAGGCAAA-3'	

Mouse atherosclerotic lesion measurement

The whole aortas of all the mice were separated under a stereo microscope at the end of the 12th week. The hearts and aortas of mice were perfused with PBS before the aorta separation. The whole aorta includes the aortic arch, the thoracic and abdominal aorta, and the iliac aorta. Then, the aortas were dissected, Oil Red O stained, and photographed. The proportions of the lesion area to the whole area were analyzed by Image J software.

Bone marrow-derived macrophage culture

We separated the femurs of mice and used a needle and syringe to wash out the bone marrow cells. The bone marrow cells were cultured in a conditioned medium (DMEM + 10% FBS + 1% P/S + 1% Glu + 20% L₉₂₉-cell culture supernatant) and maintained at 37°C with 5% CO₂ for 5~7 days in vitro for inducing into purified bone marrow derived macrophages.

Quantitative PCR analysis

The macrophages from all the groups were cultured and harvested for the *Sptlc1* and *Sptlc2* mRNA analyses. Total RNA for quantitative PCR was isolated from the macrophages. The isolated RNA underwent DNase I (Promega) treatment to remove genomic DNA and was then converted into cDNA with Oligo dT and Prime Script Reverse Transcriptase (TAKARA, RR047A) reagents. Quantitative PCR was done with a SYBR Premix Ex TaqII kit (TAKARA, RR820A). The thermocycling conditions were as follows: 95°C for 3 min, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C, then, 15 s at 95°C, 30 s at 60°C and 15 s at 95°C, in 20 mL volume. The samples were analyzed with a real-time PCR system (ABI 7300), with GAPDH as the internal standard. Relative expression was analyzed using the comparative Ct method (2- $\Delta\Delta C_t$). The primer sequences used for the real-time PCR are shown in **Table 1**.

Inflammatory factors analysis

Murine bone marrow-derived macrophages from all groups were treated with 10 ng/mL of lipopolysaccharide (LPS) (Sigma) overnight. Then the cells were washed twice with PBS, and centrifugated.

We measured the inflammatory cytokines of IL-6, and the TNF- α and MCP-1 protein levels using enzyme-linked immunosorbent assay (ELISA) kits in the serum, macrophage culture supernatants, and brain tissues. We prepared the reagents, samples and standards as instructed. We added 100 μ L of the standard or sample per well and incubated them for 2 hours at 37°C. Next, we removed the liquid of each well, without washing them. We then added 100 μ L Biotin-antibody (1x) to each well and incubated them for 1 hour at 37°C. Then we aspirated and washed them 3 times. Then we added 100 μ L of horseradish peroxidase (HRP)-avidin (1x) to each well and incubated them for 1 hour at 37°C. Then we aspirated and washed them 5 times. Then we added 90 μ L of tetramethylbenzidine (TMB) substrate to each well. We incubated them for 15~30 minutes at 37°C and protected them from light. Then we added 50 μ L of stop solution to each well. Then we read them at 450 nm within 5 minutes.

Statistical analysis

Results are reported as the mean \pm SD. Comparisons between two groups were performed using Student's *t* test and ANOVA followed by an LSD (least significant difference) analysis. A *P* value <0.05 was considered statistically significant.

Results

Hyperlipidemia occurred in the apoE^{-/-} mice after they were fed a high fat and high cholesterol diet for 12 weeks

The total cholesterol (TC, **Figure 1A**) and low-density lipoprotein cholesterol (LDL-C, **Figure 1D**) levels showed a significantly wider gap between the normal mice and the apoE^{-/-} mice (*P*<0.01). There were no significant differences in total triglyceride (TG, **Figure 1B**) and high-density lipoprotein cholesterol (HDL-C, **Figure 1C**) between the normal mice and the apoE^{-/-} mice. There were no significant differences in blood triglyceride and cholesterol levels bet-

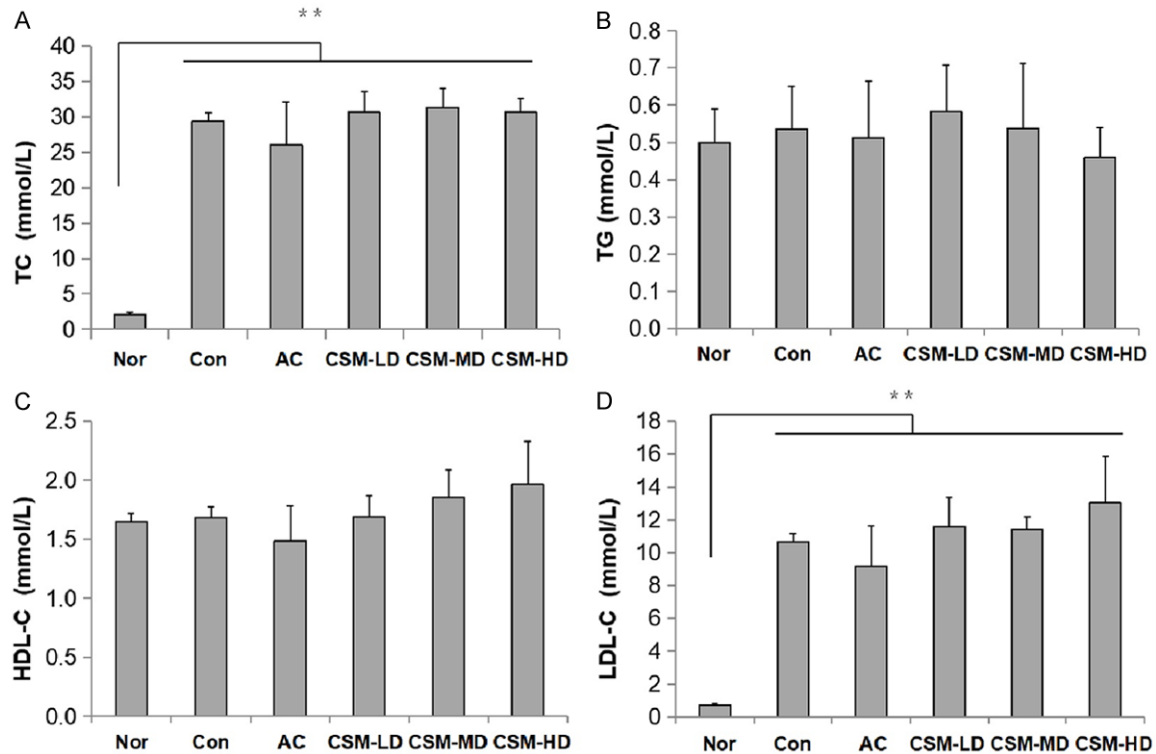


Figure 1. Hyperlipidemia occurred after the mice were fed a high fat and high cholesterol diet for 12 weeks. The CSM has no apparent effects on blood triglyceride or cholesterol levels. A. TC (mmol/L); B. TG (mmol/L); C. HDL-C (mmol/L); D. LDL-C (mmol/L). ** $P < 0.01$ Compared with the control group.

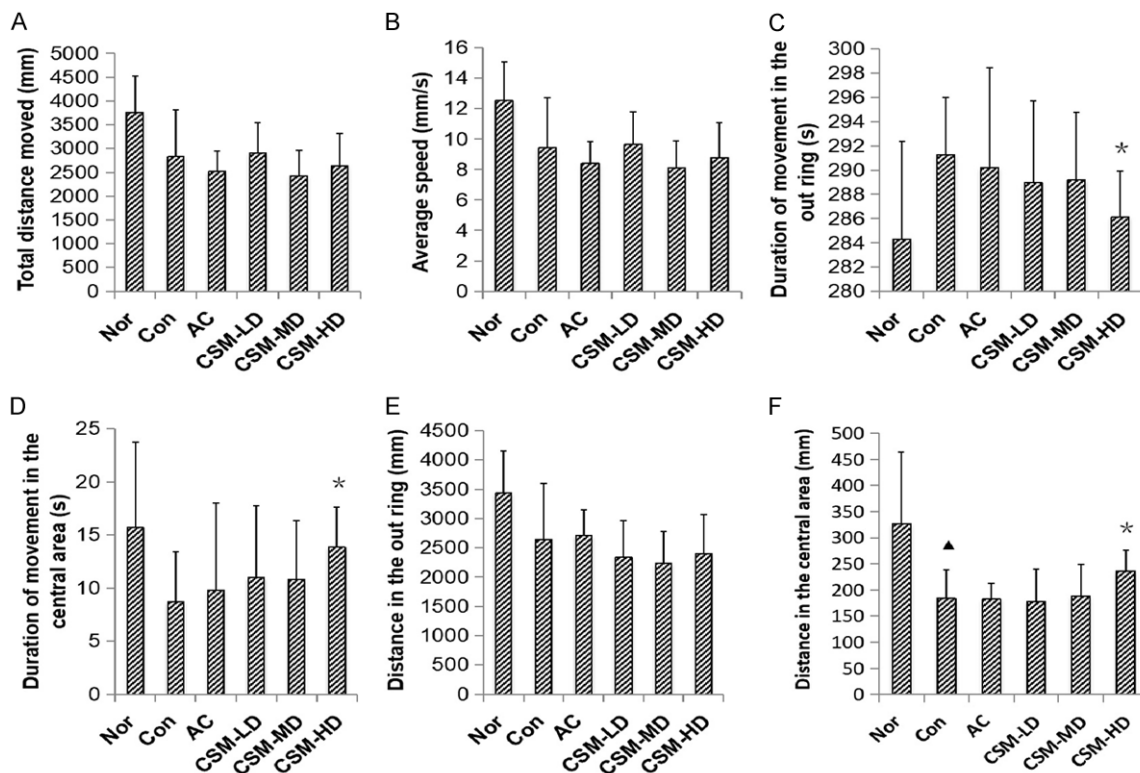


Figure 2. The results of the Open Field Test. CSM may have an antianxiety-like effect. A. Total distance moved (mm); B. Average speed (mm/s); C. Duration of movement in the outer ring (s); D. Duration of movement in the central area

(s); E. Distance in the outer ring (mm); F. Distance in the central area (mm). $\Delta P < 0.05$ Compared with normal group; $*P < 0.05$ Compared with control group.

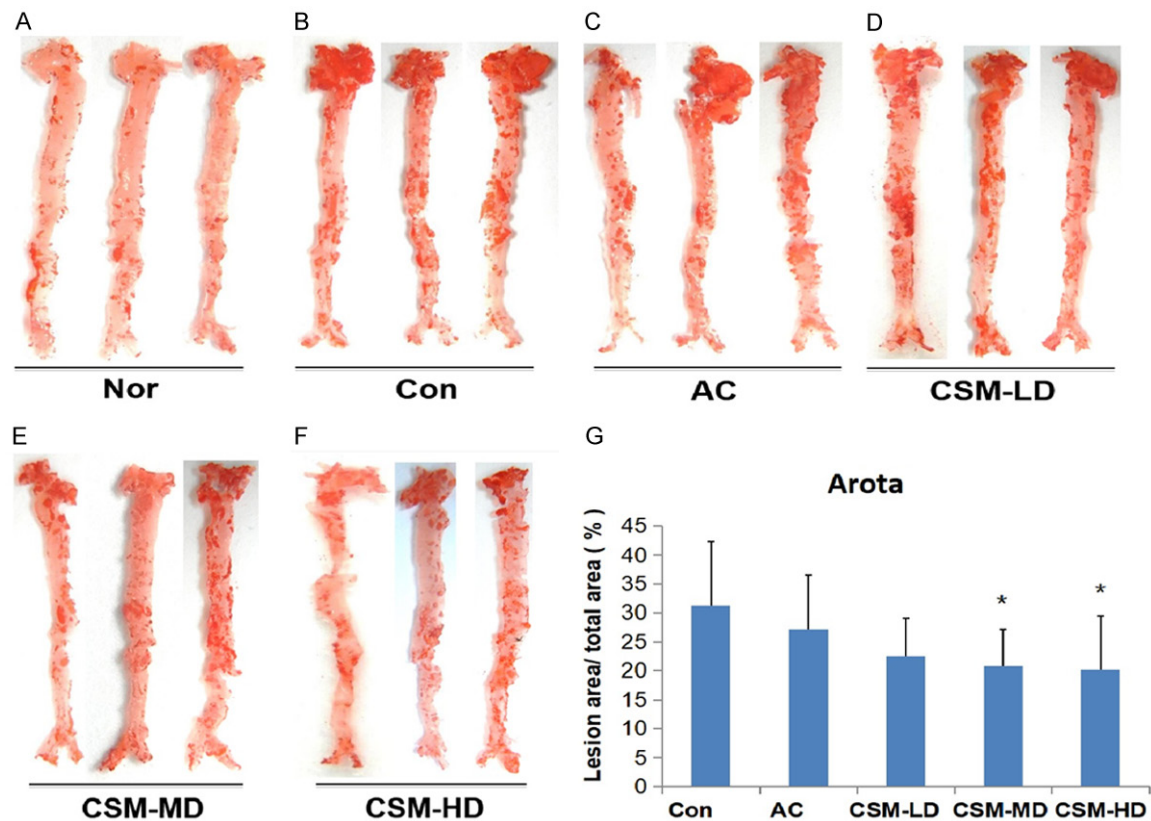


Figure 3. The results of aorta lesion test. A. Normal group; B. Control group; C. Atorvastatin calcium group; D-F. CSM with low, medium and high dose treatment groups; G. The proportion of the lesion area to the whole area. $*P < 0.05$ Compared with the control group.

ween the control mice and the CSM-treated mice (**Figure 1A-D**). These data indicated that hyperlipidemia occurred in the *apoE*^{-/-} mice after being on high cholesterol diets for 12 weeks. The CSM has no apparent effects on blood triglyceride and cholesterol levels in the *apoE*^{-/-} mice.

CSM may have antianxiety-like effect

Compared with control mice, the duration in the outer ring (**Figure 2C**) of the CSM-HD treated mice significantly decreased ($P < 0.05$), and the duration (**Figure 2D**) and distance (**Figure 2F**) in the central area of the CSM-HD treated mice significantly increased ($P < 0.05$). There were no significant differences in total distance moved (**Figure 2A**), average speed (**Figure 2B**), or distance in the outer ring (**Figure 2E**) between the control mice and the CSM-treated mice. These data indicated that CSM may have an antianxiety-like effect.

The proportions of the lesion area to the whole area in the aortas were decreased in the treatment groups

All the *apoE*^{-/-} mice had obvious atherosclerotic lesions after being fed a high fat and high cholesterol diet for 12 weeks. After Oil Red O staining, the atherosclerotic lesion areas shown in the whole aortas of the CSM-MD and CSM-HD treated mice decreased compared with the control mice ($P < 0.05$, **Figure 3**). These results indicated that CSM could reduce murine atherosclerotic lesions.

The gene expressions of Sptlc1 and Sptlc2 in the macrophages and brain tissues were decreased in the treatment group

Compared with the control mice, the gene expressions of *Sptlc1* and *Sptlc2* in 'the CSM-treated groups' bone marrow-derived macrophages (**Figure 4A**) and brain tissues (**Figure 4B**) were significantly decreased ($P < 0.05$).

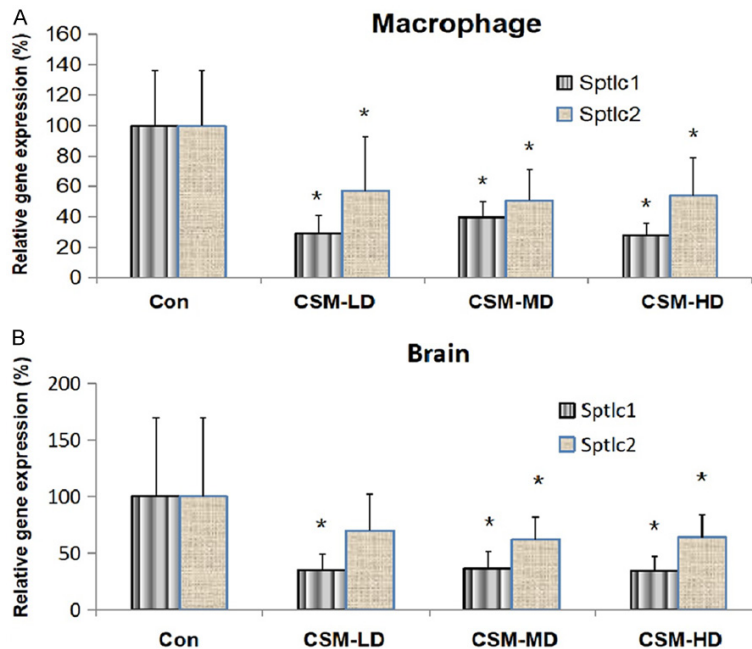


Figure 4. The gene expressions of *Sptlc1* and *Sptlc2* in the macrophages were decreased in the treatment group. A. Macrophage; B. Brain tissue. * $P < 0.05$ Compared with the control group.

These results indicated that CSM could potentially inhibit the macrophage and brain SPT activity by decreasing the gene expression of *Sptlc1* and *Sptlc2* in the corresponding tissues, which may influence the antiatherosclerotic and antianxiety-like effects.

The expressions of the inflammatory signal factors in macrophages and brain tissues were decreased in the treatment groups

Compared with the control mice, the *IL-6* protein levels of CSM-treated (CSM-MD or/and CSM-HD) mice in the macrophage culture supernatants and brain tissues were decreased ($P < 0.05$, **Figure 5A**), the *TNF- α* protein levels of CSM-MD and CSM-HD mice in the serum (**Figure 5B**) and brain tissues (**Figure 5C**) were decreased ($P < 0.05$), the *MCP-1* protein levels of the CSM-MD mice in the brain tissues (**Figure 5D**) and the CSM-treated mice in the macrophage culture supernatants (**Figure 5E**) were decreased ($P < 0.05$).

Discussion

In this study, we have demonstrated a novel, essential role for CSM in reducing murine atherosclerotic lesions and antianxiety-like effects.

Our data suggest that CSM can decrease the atherosclerotic lesion areas in murine aortas, decrease the gene expressions of *Sptlc1* and *Sptlc2* in bone marrow-derived macrophages and brain tissues, decrease the *IL-6* and *MCP-1* protein levels in macrophage culture supernatants and brain tissues, and decrease the *TNF- α* protein levels in serum and brain tissues. During the Open Field Test, CSM decreased the duration in the outer ring and the distance in the central area and increased the duration in the central area.

According to earlier studies, the inhibition of SPT activity could be an alternative treatment for atherosclerosis [9, 10]. Both *LCB1* and *LCB2* are

myriocin-binding proteins and are responsible for SPT activity in vivo [11, 12]. *Sptlc1*^{-/-} and *Sptlc2*^{-/-} mice can decrease liver *Sptlc1* and *Sptlc2* mRNA by 44% and 57% and can decrease liver SPT activity by 45% and 60% [13]. CSM can decrease serine palmitoyltransferase (SPT) activity by 60.2% [6]. Myriocin as a specific SPT inhibitor from *Isaria sinclairii* can decrease the atherosclerotic lesion areas in *apoE*-deficient mice [11]. Atherosclerosis is an inflammatory disease [14]. Attenuation of the anti-inflammatory macrophage activation may lead to an early resolution of inflammation [15]. Cytokine signaling in the brain could regulate neurotransmitter metabolism, neuroendocrine function and the neural circuitry of mood disorders [16]. Psychological stress is reported to modulate cytokine production [16]. In general, our findings are consistent with the results from previous studies. We hypothesize that the mechanisms for CSM on antiatherosclerotic and antianxiety-like effects are related to SPT inhibitory and impaired inflammatory responses.

Overall, in this study, we described the antiatherosclerotic and antianxiety-like effects of CSM in *apoE*^{-/-} mice. Next, our research will aim to investigate the further mechanisms of these

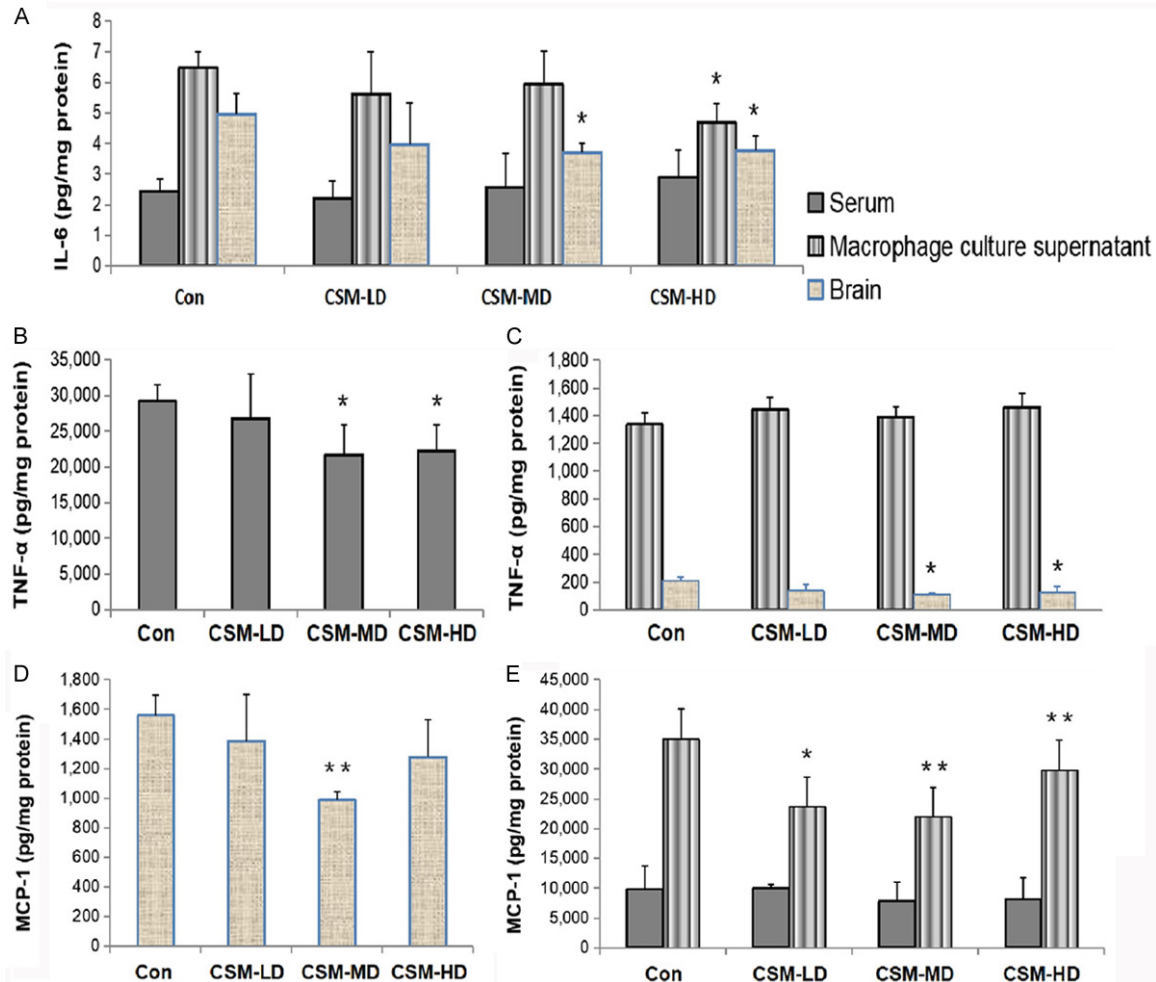


Figure 5. The expression of the inflammatory signal factors in the macrophages and brain tissues were decreased in the treatment groups. A. *IL-6*; B, C. *TNF-α*; D, E. *MCP-1*. * $P < 0.05$ Compared with the control group.

phenomena. From a therapeutic standpoint, our findings could pose a new strategy for enhancing the treatment of atherosclerosis complicated by anxiety-like behaviors.

Acknowledgements

This work was financially supported by grants to C.L. (Guangdong Science and Technology Projects, no. 2014A020221017, 2016A02022-2044; the National Natural Science Foundation of China, no. 81403212; Chinese Medicine Research Projects of Guangdong Province, nos. 20171034, 20142019; Guangdong Medical Research Project, no. B2014062), S.K. (Guangdong Science and Technology Project, no. 2013B060300033) and X.T. (Guangdong Science and Technology Project, no. 2016A0-30303026).

Disclosure of conflict of interest

None.

Address correspondence to: Cai-Xia Lou, Laboratory of Comparative Medicine, Guangdong Medical Laboratory Animal Center, 119 Poyang Road, Huang Qi Town, Nanhai District, Foshan 528248, Guangdong, China. Tel: +86-20-81111465; Fax: +86-20-81111465; E-mail: alou_1218@163.com

References

- [1] Xu S, Ye S and Chen J. The development of new resource: Cordyceps sobolifera mycelium in food. *Edible and Medicinal Mushrooms* 2011; 19: 34-38.
- [2] Xie W, Guo Y and Sheng Y. Effect of cordyceps sobolifera mycelium on chronic renal failure in rats. *Chinese Journal of Pharmaceuticals* 2011; 42: 770-772.

- [3] Liu Y, Chen Y, Wang L, Zhao Z, Liu W, Wang Z, Deng Y and Ma L. Effect of Cordyceps sobolifera mycelium on some protein and mRNA expressions such as α -SMA in tubular epithelial cells exposed to TGF- β 1. Chinese Journal of Integrated Traditional and Western Nephrology 2012; 13: 1046-1049.
- [4] Wen L, Tang Y and Zhang P. Active ingredients in Cordyceps sobolifera. Jiangsu Journal of Traditional Chinese Medicine 2006; 27: 45-46.
- [5] Jin L, Lv J, Yang J, Li D and Li A. Effects of paecilomyces cicadidae total polysaccharides on macrophages of old rats. Chinese Journal of Pathophysiology 2006; 22: 116-119.
- [6] Lin N. Study of established SPT inhibitor screening model by HPLC. The master degree dissertation of Beijing University of Traditional Chinese Medicine 2011.
- [7] Chakraborty M, Lou CX, Huan C, Kuo MS, Park TS, Cao G and Jiang XC. Myeloid cell specific serine palmitoyl transferase subunit 2 haploinsufficiency reduces murine atherosclerosis. J Clin Invest 2013; 123: 1784-1797.
- [8] Svensson M, Rosvall P, Boza-Serrano A, Andersson E, Lexell J and Deierborg T. Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia. Neurobiol Stress 2016; 5: 8-18.
- [9] Park TS, Panek RL, Mueller SB, Hanselman JC, Rosebury WS, Robertson AW, Kindt EK, Homan R, Karathanasis SK and Rekhter MD. Inhibition of sphingomyelin synthesis reduces atherogenesis in apolipoprotein E knockout mice. Circulation 2004; 110: 3465-3471.
- [10] Hojjati MR, Li Z, Zhou H, Tang S, Huan C, Ooi E, Lu S and Jiang XC. Effect of myriocin on plasma sphingolipid metabolism and atherosclerosis in apoE-deficient mice. J Biol Chem 2005; 280: 10284-10289.
- [11] Yasuda S, Nishijima M and Hanada K. Localization, topology, and function of the LCB1 subunit of serine palmitoyltransferase in mammalian cells. J Biol Chem 2003; 278: 4176-83.
- [12] Chen JK, Lane WS and Schreiber SL. The identification of myriocin-binding proteins. Chem Biol 1999; 6: 221-235.
- [13] Hojjati MR, Li Z and Jiang XC. Serine palmitoyl-CoA transferase (SPT) deficiency and sphingolipid levels in mice. Biochim Biophys Acta 2005; 1737: 44-51.
- [14] Taleb S. Inflammation in atherosclerosis. Arch Cardiovasc Dis 2016; 109: 708-715.
- [15] Decano JL, Mattson PC and Aikawa M. Macrophages in vascular inflammation: origins and functions. Curr Atheroscler Rep 2016; 18: 34.
- [16] Salim S, Chugh G and Asghar M. Inflammation in anxiety. Adv Protein Chem Struct Biol 2012; 88: 1-25.