Original Article
Aerobic exercise combined with samara oil can improve hyperlipidemia by reducing PCSK9 and increasing LDLR

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Abstract: Hyperlipidemia is a risk factor for stroke, coronary heart disease, myocardial infarction, sudden death, etc. Aerobic exercise and reasonable diet are effective ways to regulate lipid metabolism and the combination of exercise and diet can improve hyperlipidemia more effectively. However, the mechanism is not entirely clear. PCSK9 is a gene involved in cholesterol metabolism, it causes a decrease in LDL receptors on the surface of hepatocytes, which in turn reduces the ability of hepatocytes to clear LDL-C particles, leading to elevated cholesterol. In this study, aerobic exercise combined with samara oil reduced the levels of TC, TG and LDL-C ($p<0.05$) and the degree of liver pathological in hyperlipidemic mice. Mechanistically, exercise and feeding of samara oil down-regulated PCSK9 mRNA and protein level and elevated the expression of LDLR ($p<0.05$). In terms of intervention effect, exercise alone was slightly better than samara oil alone, but there was no significant difference ($p>0.05$). Exercise combined with samara oil was significantly better than the single intervention and almost returned to normal level ($p>0.05$). These findings show that aerobic exercise combined with samara oil can improve hyperlipidemia by down-regulating the expression of PCSK9 and up-regulating the expression of LDLR.

Keywords: Aerobic exercise, samara oil, hyperlipidemia, PCSK9, LDLR

Introduction

Hyperlipidemia (HL) refers to the lipid concentration in plasma exceeding the normal range, which was also called dyslipidemia. Hyperlipidemia is a potential pathogenic factor for major diseases such as atherosclerosis and coronary heart disease [1]. Prevention and treatment of hyperlipidemia has very important practical significance for controlling the incidence and mortality of cardiovascular and cerebrovascular diseases [2]. In recent years, studies have found that an imbalance of expression of a certain genes causes abnormal changes in the receptors, apolipoproteins, or enzymes involved in mediating the blood lipid balance and synthesis, transport, and metabolism of lipoproteins become abnormal, increasing systemic blood lipid levels [3].

Since Seidah [4] discovered pro-protein convertase subtilisin/kexin type 9 (PCSK9) in 2003, researchers discovered that PCSK9 binds epidermal growth factor (EGF) domain of low-density lipoprotein receptor (LDLR) to promote LDLR to co-internalize and transport to lysosomes for degradation. Thus, LDLR can no longer return to the cell membrane to function. PCSK9 is considered to play a key role in the lipid metabolism [5-10], and has received increased attention [11].

For hyperlipidemia, regular aerobic exercise and adjustment of diet are currently the most popular prevention methods [12, 13]. Samara oil is a pale yellow transparent oily liquid obtained from the processing of the seeds of the oil palm tree. It is a unique treasure resource in China after the Quaternary glacial action. Samara oil is composed of 17 amino acids, of which 7 are essential for human [14]. The function is to improve human immunity, enhance vitality, anti-oxidation, and lower blood fat [15-17]. In 2011, it was approved by the Ministry of Health as a new resource food [18]. Aerobic exercise refers to the physical exercise in an environment with sufficient oxygen supply, so that the respiratory and circulatory system can supply sufficient oxygen, enhance the heart and lung function, and effectively increase the nutrient and oxygen of the whole body, such as swimming, jogging, etc. [19]. Studies have sh-

own that aerobic exercise [22] and samara oil [15] have positive effects in reducing blood lipids, respectively, but the study on the effect of aerobic exercise combined with samara oil on important genes of cholesterol metabolism PCSK9 and LDLR has not been reported in detail. Therefore, this study designed experiments to induce hyperlipidemia in C57BL/6J mice by high-fat diet, and observe the effect of aerobic exercise combined with samara oil on PCSK9 and LDLR expression in hyperlipidemia mice.

Materials and methods

Animals and exercise protocol

A total of 50 male C57BL/6J mice, weighing (18-20) g, were provided by the Experimental Animal Center of China National Food and Drug Administration, and the license number was SCXK (Beijing) 2014-0013. Mice divided into 5 cages in a room with a 12:12 hour light-dark cycle, the temperature was 18°C-23°C and the humidity was 50%-60%. The mice had free access to both food and water.

After 2 weeks of adaptive feeding, mice were randomly divided into a normal control group (NC group, n=10), a high fat group (M group, n=10), a high fat with exercise group (ME group, n=10), a high fat with samara oil group (MS group, n=10) and a high fat with exercise and samara oil group (MES group, n=10). The high-fat diet was composed 22.4% of protein, 45.1% of carbohydrate, 16.4% of fat, 5.8% of crude fiber, 1.7% of calcium, 1.1% of phosphorus. After successful modeling in hyperlipidemia mice, the MS groups were fed with samara oil (0.5 mg/g) daily while feeding high-fat diet, NC and ME group fed with saline. The ME group were used for treadmill exercise after completing the familiar training. The MES group were used for the intervention of aerobic exercise combined with samara oil. Aerobic exercise intensity was determined to be 15 m/min × 45 min, 6% slope, six days of exercise per week (starting at 7 pm), and each exercise had a 5-minute warm-up, total 8 weeks.

Tissue collection

After the last running of the experiment, fasting for 12 hours, weighed, abdominal anesthesia was performed with sodium pentobarbital at a dose of 80 mg/Kg, and 2 mL abdominal aortic

blood was taken. The mice were euthanized, and all the livers were quickly taken out, and washed repeatedly with ice-cold physiological saline until no blood, and the water was blotted with a filter paper, photographed and stored. After the tissue collection was completed, the mouse carcass was treated according to relevant requirements.

Determination of plasma marker of cholesterol metabolism

The collected mouse blood was centrifuged at 12000 rpm for 2 minutes at 4°C, and the upper serum was taken and stored at -20°C until analyzed. Plasma total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were measured by microplate reader using assay Kit (Nanjing Institute of Bioengineering).

Determination of mice liver tissue lesion size

Part of the liver tissue of mice was fixed with 10% formalin for 24 hours. The gradient ethanol was dehydrated and transparent, embedded in paraffin, and the sections were dewaxed to hydration by gradient ethanol, stained with hematoxylin and eosin (H&E). The pathological changes of the liver were observed under the microscope by the size of vacuoles and lipid droplets.

RNA isolation and PCR procedures

Liver tissue was ground to a powder form under the condition of continuously adding liquid nitrogen, and total RNA of the liver was extracted according to the operation requirements of the column animal tissue total RNA extraction kit (Shanghai Shenggong Bioengineering Co., Ltd.), and the RNA concentration was measured using a fluorescence spectrophotometer.

The mouse liver tissue RNA was reverse-transcribed into an amplified cDNA template in a metal bath at 4°C according to the TaKaRa reverse transcription kit instructions, and added to the subsequent RT-PCR reaction system. The primers used in RT-PCR are shown in **Table 1**.

Western blot

The liver tissue was homogenized with cell lysate to no obvious tissue granules, centri-

Table 1. qPCR assays

Transcript	Forward Primer	Reverse Primer
β-Actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA
PCSK9	GTCACACAGCCTAAGAAGTCGTCG	CTGGTAGCTGATGCGGTGAC
LDLR	ACTCACGGGTTCAGATG	AGGTACTGGCAACCACCATT

β-Actin was used as an internal reference, and the expression levels of the target genes PCSK9 and LDLR were calculated according to the Ct value measured by a Roche Light Cycler 96 real-time PCR instrument, and the relative expression amount of each target gene mRNA was calculated according to the formula $2^{-\Delta\Delta Ct}$.
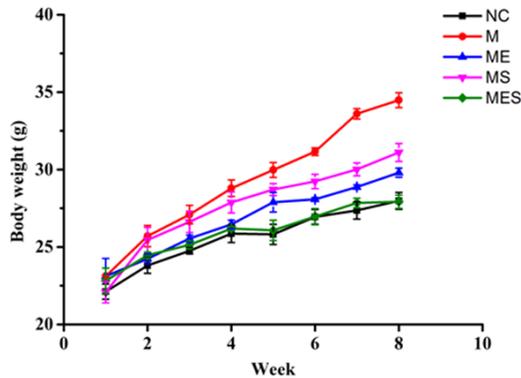

Figure 1. Body weight change of each group during exercise intervention, NC: normal control group, M: high-fat model group, ME: high-fat with aerobic exercise group, MS: high-fat with samara oil group; MES: high-fat with aerobic exercise and samara oil group.
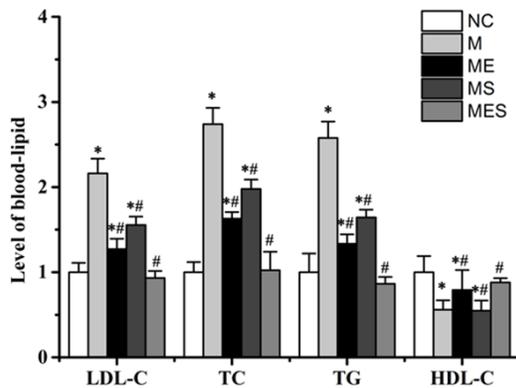

Figure 2. Aerobic exercise combined with samara oil reduced the level of LDL-C, TC, TG, increased the level of HDL-C in hyper-lipidemic mice. *$p<0.05$ vs. NC group, #$p<0.05$ vs. M group.

fuged and supernatant was kept for protein analyses. A BCA colorimetric method was used to determine the protein concentration. The protein was quantified by 120 μg, boiled for 5 minutes, and loaded onto a 10% SDS-PAGE gel, 1.5 hour, full wet electro-transfer transferred to the PVDF membrane. Block with 5% skim milk powder for 1 hour at room temperature. Primary anti-(rabbit anti-mouse PCSK9, LDLR polyclonal antibody, 1:1000) and secondary antibody (1:5000) were added, and the target band was detected by chemiluminescence, and exposed on a gel imaging system. The ratio of the gray value of the target protein to the internal reference protein was determined by Image J software to obtain the relative expression level of the target protein.

Statistics

All experimental data are expressed as mean ± standard deviation (mean ± SD), and statistical analysis was performed using SPSS 17.0 software. After normality and homogeneity test of variance, one-way analysis of variance was performed, and SNK-q was used for comparison between groups. $p<0.05$ indicates that the difference was statistically significant.

Results

Aerobic exercise combined with samara oil reduced body weight of hyper-lipidemic mice

The weight changes of the each group during the intervention period are shown in **Figure 1**. The body weight of the M group was significantly higher than NC group ($p<0.05$). Compared with the M group, the body weight of the ME, MS, MES group was significantly decreased ($p<0.05$). Basically, the weight of MES group was equivalent to the normal level, and there was no statistically significant difference compared with the NC group ($p>0.05$).

Aerobic exercise combined with samara oil decreased blood lipid level in hyper-lipidemic mice

Elevated lipid levels are key factors in the development of hyperlipidemia. The serum lipid levels of the mice after the intervention are shown in **Figure 2**. The levels of LDL-C, TC and TG in the M group were significantly higher than normal group, which were 2.16 times, 2.74 times, 2.58 times respectively, in contrast, the HDL-C level was significantly lower, which was about 0.65 times. Compared with the M group, the level of LDL-C, TC and TG in MS and ME group
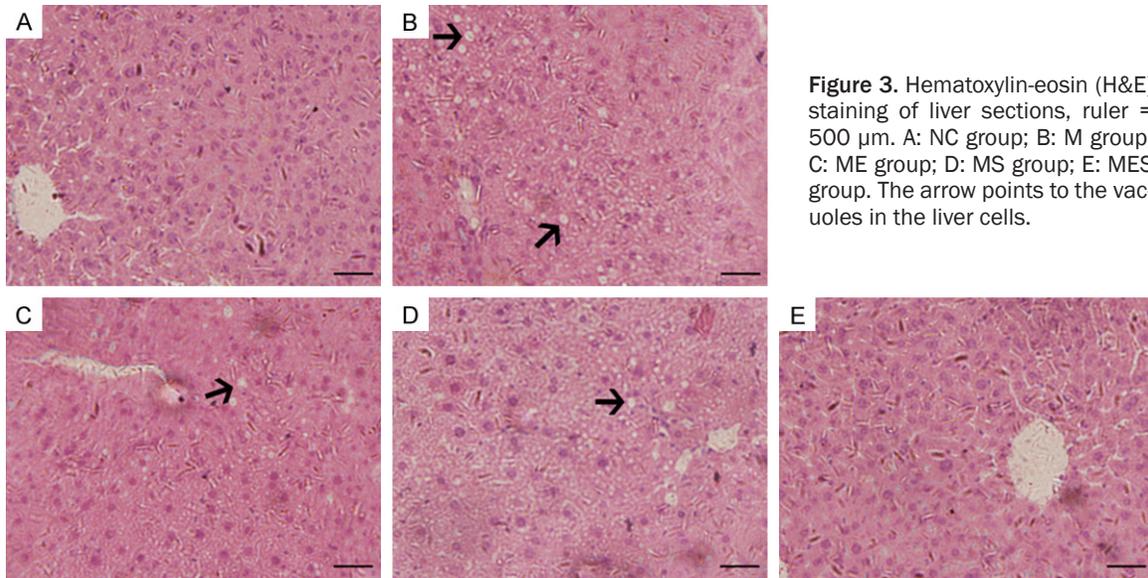
Figure 3. Hematoxylin-eosin (H&E) staining of liver sections, ruler = 500 μm. A: NC group; B: M group; C: ME group; D: MS group; E: MES group. The arrow points to the vacuoles in the liver cells.
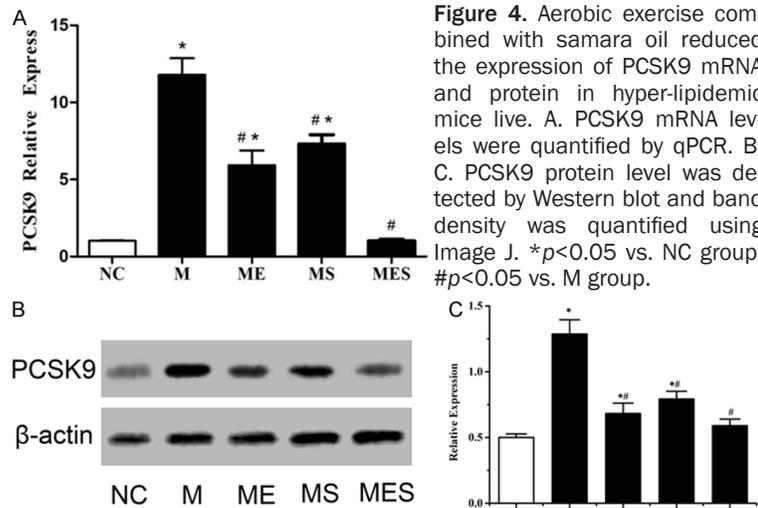

Figure 4. Aerobic exercise combined with samara oil reduced the expression of PCSK9 mRNA and protein in hyper-lipidemic mice live. A. PCSK9 mRNA levels were quantified by qPCR. B, C. PCSK9 protein level was detected by Western blot and band density was quantified using Image J. *$p<0.05$ vs. NC group, #$p<0.05$ vs. M group.

were significantly decreased, the level of HDL-C was significantly increased ($p<0.05$), and the effect on ME group was better than the MS group. Serum LDL-C, TC and TG level exhibited a significant decrease, and the decreased HDL-C cholesterol level recovered to the normal level in response to aerobic exercise combined with samara oil in MES group, and there was no statistically significant difference between the MES vs. the NC group ($p>0.05$).

Aerobic exercise combined with samara oil decreased liver pathological structure in hyper-lipidemic mice

In the NC group, the morphology of the liver cells was normal, arranged regular and dense, and the nucleus was clearly visible. (**Figure 3A**). The liver cells in the M group were obviously swollen and arranged loosely. There are a large number of vacuola cells and lipid droplets of different sizes can be seen, occasionally spotted necrosis, and the field of view is covered with steatosis cells (**Figure 3B**). The degree of hepatocyte lesions in ME group was significantly better than M group, less lipid droplets and swelling degree reduced (**Figure 3C**). Liver cell steatosis was improved in the MS group, the normal liver cells were observed, lipid vesicles have significantly lessened and become smaller (**Figure 3D**). The liver tissue degeneration in the MES group was largely restored, and the liver cells were arranged neatly. The nucleus is located in the center of the cell, occasionally a few lipid droplets, no vacuoles (**Figure 3E**).

Aerobic exercise combined with samara oil reduced PCSK9 expression in liver of hyper-lipidemic mice

qPCR and Western blot was used to detect the relative expression of PCSK9 mRNA (**Figure 4A**) and protein (**Figure 4B** and **4C**) in the liver. The results show that compared with the NC group, expression of PCSK9 in the liver of M group was
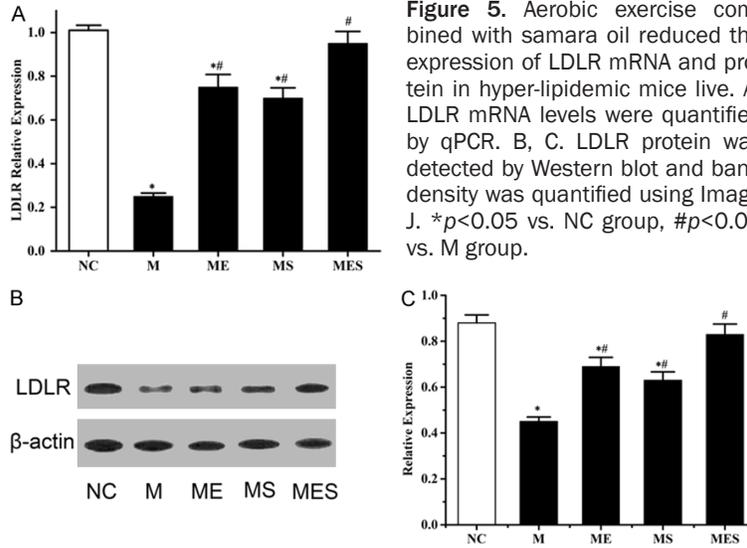
Figure 5. Aerobic exercise combined with samara oil reduced the expression of LDLR mRNA and protein in hyper-lipidemic mice live. A. LDLR mRNA levels were quantified by qPCR. B, C. LDLR protein was detected by Western blot and band density was quantified using Image J. *$p<0.05$ vs. NC group, #$p<0.05$ vs. M group.

Discussion

Hyperlipidemia is an important influencing factor for many serious diseases. It is the main pathogenic factor of atherosclerosis. It often causes serious consequences such as fatty liver, atherosclerosis, coronary heart disease, etc [3]. A large number of studies have shown that long-term high-fat diet feeding can cause weight gain, blood lipid levels, and liver pathological changes [20, 21]. In this study, a hyperlipidemia mouse model was established through a high-fat diet. The results show that compared with the NC group, the levels of TC, TG, and LDL-C in the M group were significantly increased, the level of HDL-C was decreased. Pathology examination showed that the liver of the M group had obvious fat lesions, which proved that the mouse model of hyperlipidemia was successfully constructed in this study.

Currently, the effective way to prevent abnormal blood lipid metabolism is good living habits and long-term aerobic exercise. Aerobic exercise can improve the body's lipid metabolism by increasing energy consumption, improving lipid oxidation and reducing body fat accumulation [22]. The study found that long-term effective aerobic exercise can effectively reduce the body weight and serum levels of TC, TG, and LDL-C. Samara oil contains very rich unsaturated fatty acids, plant sterols, multivitamins, especially vitamin E, etc. According to research, samara oil can antioxidant, regulate lipid metabolism, prevent cardiovascular diseases, etc. [14-17]. Experimental studies have compared the effects of samara oil and common edible peanut oil on the antioxidant capacity and lipid metabolism of common diet mice. It was found that compared with common edible peanut oil, samara oil can reduce the levels of TC, TG and LDL-C in serum and improve the antioxidant capacity more effectively [15]. In other clinical studies, levels of serum TC, TG, and LDL-C of patients with hyperlipidemia who eat samara oil were significantly lower and the level of HDL-C is increased than those of hyperlipid-

significantly increased (p<0.05). Compared with the M group, the expression of PCSK9 in the liver of the ME and MS groups was significantly reduced (p<0.05), but it was different from the normal mice in the NC group (p<0.05). In the MES group, the expression of PCSK9 was significantly reduced, and there was no statistically significant difference compared with NC group (p>0.05). It has been proven that aerobic exercise combined with samara oil can significantly down-regulate the expression level of PCSK9.

Aerobic exercise combined with samara oil increased LDLR expression in hyper-lipidemic mice

PCSK9 mediates the degradation of LDLR and negatively regulates LDLR. qPCR and Western blot were used to test whether the expression level of LDLR mRNA (**Figure 5A**) and protein (**Figure 5B** and **5C**) after exercise and samara oil was related to the decrease of PCSK9 level. The results show that compared with the NC group, expression of LDLR in the M group was significantly decreased compared to the NC group (p<0.05). Compared with the M group, expression of LDLR in the liver of the ME and MS groups was significantly increased (p<0.05), but it was different from the normal mice in the NC group (p<0.05). In the MES group, expression of LDLR in the liver of mice was significantly increased, and there was no statistically significant difference compared with the NC group (p>0.05).

emia without samara oil [17]. The ability of samara oil to regulate lipid metabolism is unquestionable, however the effect of samara oil on cholesterol regulation genes PCSK and LDLR has not been reported in detail.

In this experimental study, C57BL/6J mice fed a high-cholesterol diet were given aerobic exercise intervention, samara oil intervention, aerobic exercise combined with samara oil intervention, and the results show that all three intervention methods played an improvement role in body weight, lipid levels, liver pathological and PCSK9, LDLR expression levels. But the best effect was from aerobic exercise combined with samara oil intervention, where the weight, blood lipid level and liver pathological of the aerobic exercise combined with samara oil group were not statistically different from normal mice, and the expression levels of PCSK9 and LDLR were also similar to normal mice. The second best effect was aerobic exercise intervention, samara oil intervention, which indicates that long-term effective aerobic exercise combined with samara oil can more effectively improve the abnormal lipid metabolism caused by high-fat diet. These data provide insights and evidence for mechanisms that regular exercise combination dietary can effectively improve blood lipid levels.

Conclusion

Aerobic exercise combined with samara oil can effectively reduce the body weight, serum lipid levels, improve the degree of liver tissue fat lesions, decrease the expression level of liver PCSK9, and increase the expression level of LDLR. Aerobic exercise combined with samara oil intervention was significantly better than aerobic exercise intervention or samara oil intervention alone.

Acknowledgements

Disclosure of conflict of interest

None.

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