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Moxibustion at CV4 alleviates atherosclerotic lesions through activation of the LXRα/ABCA1 pathway in apolipoprotein-E-deficient mice

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Abstract

Objectives: To investigate the anti-atherogenic effect of moxibustion and whether it is mediated through the reverse cholesterol transport process.

Methods: 8-week-old male apolipoprotein E deficient (ApoE$^{-/-}$ knockout) mice were randomly divided into two groups (n=10 per group): atherosclerosis (AS) and AS plus moxibustion (AS+M). C57BL/6J mice of the same background (n=10) were selected as controls. Mice in the AS+M group received indirect moxibustion with an ignited moxa stick held over CV4. Mice of the AS and control groups were restrained in the same holder with an unlit moxa stick held over CV4. All treatments were performed for 20 min per day, 6 days per week for 12 weeks. After the treatment, the mice were euthanased and their serum lipids were measured. The aortic roots and thoracic aortas were collected for haematoxylin and eosin and red oil O staining, respectively, to analyse the atherosclerotic lesions. Expression of adenosine triphosphate binding cassette (ABCA)A1/G1 and liver X receptor α (LXRα) in the thoracic aorta were examined with Western blotting.

Results: The moxibustion-treated (AS+M) mice showed a significantly lower plaque area percentage in the aortic root and thoracic aorta, and higher expression of LXRα and ABCA1 in the thoracic aorta compared with the AS mice. No significant differences were found in average lipid area percentage in the thoracic aorta, or ABCG1 expression in the thoracic aorta, between mice in the AS+M and AS groups.

Conclusion: Moxibustion treatment at CV4 suppressed the progression of atherosclerotic lesions in ApoE$^{-/-}$ mice. The anti-atherogenic effect of moxibustion may be achieved by: (1) regulation of lipid metabolism, and thus prevention of lipid accumulation; and (2) upregulation of LXRα- and ABCA1-mediated cholesterol efflux in the lesion area.

Keywords

moxibustion, atherosclerosis, lipid metabolism, cholesterol efflux, reverse cholesterol transport

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Introduction

Atherosclerosis (AS), a disease characterised by deposition of excess lipids in the arterial vessels, has become the leading cause of death and disability world-wide. Dyslipidaemia is considered an important pathogenic factor for AS. Excess lipids in the serum enter the vascular wall, accumulate and are deposited in the endarterium, thereby initiating an early AS lesion.[1] High density lipoprotein (HDL) and apolipoprotein A-I (ApoA-I) play important roles in anti-atherogenesis. They transport excess cholesterol accumulated in the macrophages of atherosclerotic lesions back to the liver for subsequent

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conversion to bile in a process called reverse cholesterol transport (RCT).[2]

The adenosine triphosphate (ATP)-binding cassette transporters A1 and G1 (ABCA1, ABCG1) on the cell surface have a synergistic effect on RCT.[3] They play essential roles in mediating cholesterol efflux from macrophages; thus the excess cholesterol in cells could be transported by ApoA-I/HDL from the lesion area to the liver and other tissues.[4] Furthermore, ABCA1/G1 are transcriptionally regulated by the ligand-dependent nuclear receptor—that is, the liver X receptor α (LXRα) pathway.[5–7]

Moxibustion is an external therapy in Traditional Chinese Medicine. Heat is applied to the skin overlying traditional acupuncture points or certain locations on the body by burning moxa wool (the main material). Studies have suggested that it may be effective at reducing the risk of AS by regulating blood lipid levels.[8,9] However, these effects were mostly observed in the study of stroke, hyperlipidaemia and hypertension. Independent research on moxibustion for AS is yet to be conducted, and its underlying mechanism remains unclear. ApoE$^{-/-}$ (apolipoprotein E knockout) mice have serious lipid metabolism disorders. Composite AS plaques are spontaneously formed in their bodies,[10] so that they are used as a common animal model to study AS. In this study, we measured blood lipid levels and the area of arterial lesions to evaluate the anti-atherogenic potential of moxibustion. Furthermore, expression of ABCA1, ABCG1and LXRα in the thoracic aorta was observed to investigate whether this effect is mediated through the RCT process or not.

Methods

Animals

Eight-week-old male ApoE$^{-/-}$ mice (n=20) and control C57BL/6J mice of the same background (n=10) were purchased from Peking University Health Science Centre. The ApoE$^{-/-}$ mice were fed a high-fat, cholesterol-rich/atherogenic diet (containing 15% fat, 2% cholesterol and 0.05% cholic acid) and the C57BL/6J mice were fed normal food. All animals were housed in individual cages and received ad libitum access to water and food in a temperature (20–24°C) and humidity (50–60%) controlled environment under a 12 hour light/dark schedule (lights on at 08:00).

Ethical approval of this study was obtained from the Medicine and Animal Ethics Committee (ref no. 1100000013479) at Beijing University of Chinese Medicine. All experiments were performed according to the National Guideline for the Care and Use of Laboratory Animals, Amendment 2 (State Council of China, 2013) and all efforts were made to minimise suffering.

Experimental design

Twenty ApoE$^{-/-}$ mice were randomly divided into two groups (n=10 per group): AS and AS plus moxibustion (AS+M). SPSS software (SPSS Inc., Chicago, IL, USA) was used for group allocation and a completely randomised block design was adopted taking weight as the block factor. Ten C57BL/6J mice were selected as the control group. Mice in the AS+M group received moxibustion using a moxa stick held over CV4 (*Guanyuan*) for 20 min while being restrained in a tubed-shape holder, with the central part of the abdomen and the four limbs exposed. The moxa stick (ϕ0.5 cm ×20 cm, Henan Nanyang Hanyi Moxa Co, Ltd) was ignited and held 2–3 cm above CV4, which was located on the midline of the lower abdomen, 10 mm inferior to the umbilicus according to a standard atlas of rat acupuncture points.[11] Mice in the AS and control groups were restrained in the holder with an unlit moxa stick over CV4. The study commenced 7 days after arrival of the animals to the laboratory to allow for acclimatisation. All treatments were performed 20 min per day, 6 days per week for 12 weeks. Twenty-four hours after the last treatment in the experiment, all mice were weighed and then anaesthetised using an intraperitoneal injection of pentobarbital (1%) at 50 mg/kg. Blood samples were obtained from each mouse through the common ophthalmic artery. Cervical dislocation was conducted shortly afterwards to ensure painless and ethical death of the mice.

Blood samples were collected for measurement of serum lipids. Aortic roots and thoracic aortas were collected for haematoxylin and eosin (HE) and red oil O staining, respectively. Expression of ABCA1, ABCG1 and LXRα in the thoracic aorta was examined by Western blotting. Statisticians and laboratory technicians were blind to the treatment allocation, while researchers who performed treatment on the mice were not.

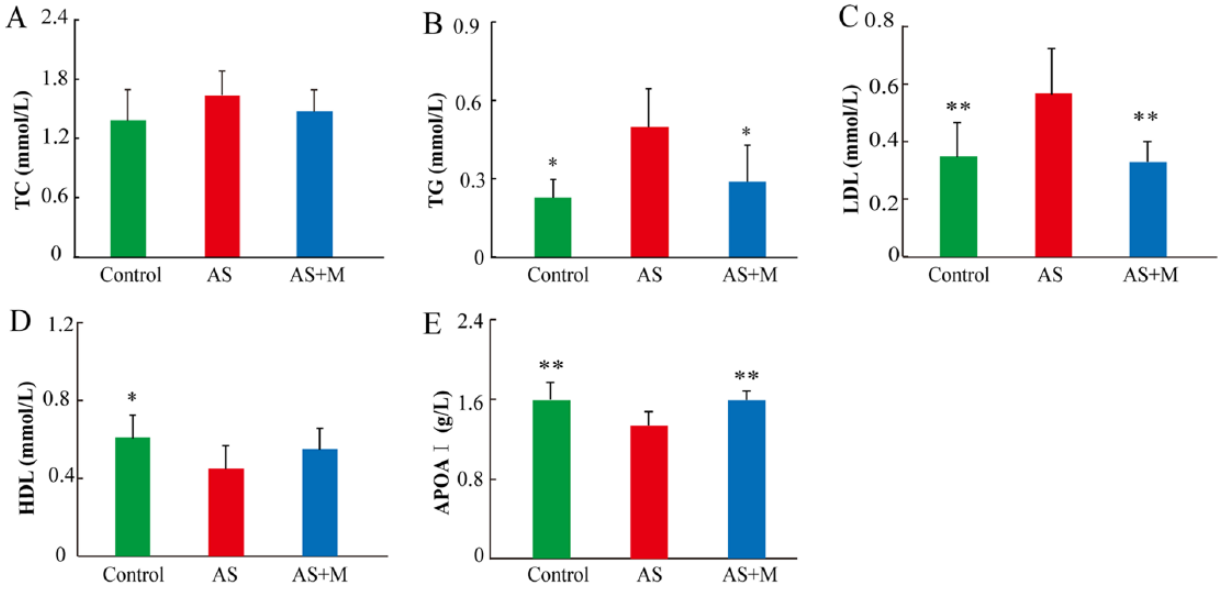
Measurement of serum lipid

Mice were anaesthetised with 1% pentobarbital sodium (50 mg/kg intraperitoneally) and 1–1.5 mL blood samples were then collected from the abdominal aorta. After centrifugation for 15 min (4°C, 1500 rpm), the serum was stored at −20°C until use. Levels of total cholesterol (TC), triglyceride (TG), low density lipoprotein-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and ApoA-I were measured using kits from Sigma Diagnostics (TC: 26745MSDS, TG: TRI19-1KT MSDS, LDL: SRP6477 MSDS, HDL: PZ0226 MSDS, ApoA-I: A0722MSDS).

Analysis of lesions in the aortic root and thoracic aorta

The aortic root of each animal was dissected and fixed overnight in 4% paraformaldehyde, and then paraffin-embedded and sectioned at 5 μm thickness. The sections were stained with HE to evaluate the lesions and plaque area.

Figure 1. Effect of moxibustion on levels of serum TC (A), TG (B), LDL (C), HDL (D) and ApoA-I (E). Data are expressed as mean±SD, n=10/group. *P<0.05 versus AS, **P<0.01 versus AS. ApoA-I, apolipoprotein A-I; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TC, total cholesterol; TG, triglyceride.



The thoracic aorta was dissected and fixed overnight in 4% paraformaldehyde, dehydrated with 20% and 30% sucrose, embedded in OCT compound and frozen immediately in liquid nitrogen, then stored in a fridge at −80°C. Each thoracic aorta was sectioned (10 µm) using a freezing microtome (Leica CM1850) and stained with oil red O to visualise the extent of the lipid deposition.

The aortic images were captured by a Canon 450D (three sections per animal). The cross sectional area of the aorta, plaque and lipid were analysed using Image-Pro Plus 6 software. The plaque and lipid area percentage were used for data analysis and were calculated as follows:

Plaque area percentage=plaque area/cross sectional area of aorta ×100%

Lipid area percentage=lipid area/cross sectional area of aorta ×100%.

Western blotting for ABCA1, ABCG1 and LXRα

The thoracic aortic tissue was put into cryogenic vials, which were cryopreserved immediately in liquid nitrogen and stored at −80°C for use after completion of all sample collections.

The thoracic arteries (length 1.5 cm) were dissected and used to analyse the protein levels using Western blotting. Lysates (10–30 µg protein) were loaded onto 10% SDS-PAGE gels and blotted onto a polyvinylidene difluoride membrane. After being blocked with 5% powdered skim milk for 2 hours in phosphate-buffered saline containing 0.1% Tween 20 (PBST), the membranes were incubated with ABCA1 antibody (ab18180, Abcam, UK, 1:500), ABCG1 antibody (ab52617, Abcam, UK, 1:500) and LXRα antibody (ab41902, Abcam, UK, 1:500) overnight at 4°C, and then incubated with secondary antibody anti-rabbit/mouse-HRP (Santa Cruz Biotech, Santa Cruz, CA, 1:2000) for 1 hour at 37°C Image-Pro Plus 6.

Statistical analysis

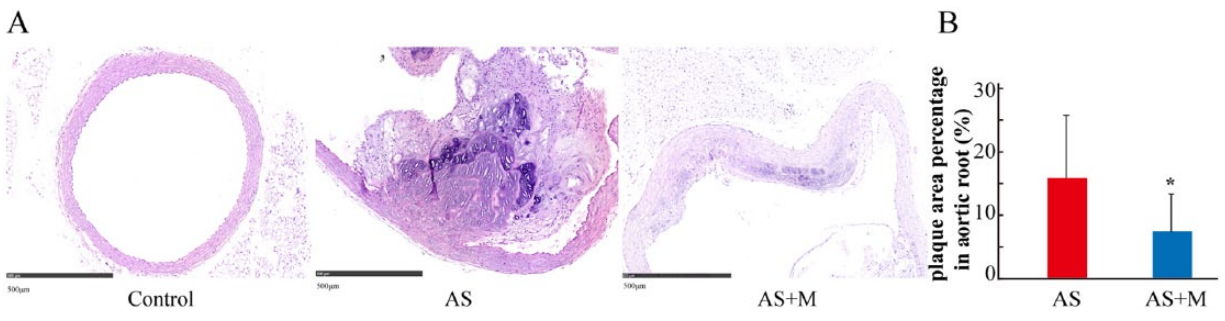
Data were expressed as mean±SD. Groups were compared by one-way analysis of variance (ANOVA) followed by post-hoc test of least significant difference (LSD) using SPSS17.0 software. A probability level of $P<0.05$ was set as the threshold of statistical significance.

Results

Moxibustion regulated blood lipid levels in ApoE$^{-/-}$mice

Compared with the control group, the ApoE$^{-/-}$ mice showed a significantly higher level of serum TG (AS vs Control: 0.50±0.25 vs 0.23±0.07 mmol/L, P=0.003, power=0.92, Figure 1B) and LDL (AS vs Control: 0.57±0.16 vs 0.35±0.12 mmol/L, P=0.001, power=0.95, Figure 1C), and a significantly lower level of serum HDL (AS vs Control: 0.45±0.12 vs 0.61±0.12 mmol/L, P=0.02, power=0.89, Figure 1D) and ApoA-I (AS vs Control: 1.33±0.14 vs 1.58±0.17 g/L, P=0.001, power=0.96, Figure 1E). No significant difference in serum TC level was found between the ApoE$^{-/-}$ mice and control group (AS vs Control: 1.63±0.24 vs 1.38±0.31 mmol/L, P>0.05, power=0.62, Figure 1A).

Figure 2. (A) Representative histomorphological (HE stained) images of the aortic root (100×). Bar=500 μm. (B) Comparison of plaque area percentage between AS and AS+M groups. Data are expressed as mean±SD, n=10/group. *P<0.05, AS+M versus AS. AS, atherosclerosis; M, moxibustion.



AS mice treated with moxibustion showed significantly lower levels of serum TG (AS+M vs AS: 0.29±0.16 vs 0.50±0.25mmol/L, P=0.03, power=0.69, Figure 1B) and LDL (AS+M vs AS: 0.33±0.07 vs 0.57±0.16 mmol/L, P=0.001, power=0.99, Figure 1C) and a significantly higher level of serum ApoA-I (AS+M vs AS: 1.58±0.09 vs 1.33±0.14 g/L, P=0.001, power=0.99, Figure 1E) than untreated AS mice. No significant difference was found in serum HDL level between ApoE$^{-/-}$ mice treated with moxibustion or not (AS+M vs AS: 1.63±0.24 vs 1.38±0.31 mmol/L, P>0.05, power=0.64, Figure 1D).

Moxibustion alleviated atherosclerosis in ApoE$^{-/-}$mice

Through HE staining of the aortic root, no atherosclerosis lesions were found in C57BL/6 mice, while arterial wall damage and atherosclerosis plaque were apparent in ApoE$^{-/-}$ mice. The ApoE$^{-/-}$ mice treated with moxibustion exhibited a lower plaque area percentage (AS+M vs AS: 7.34±6.02% vs 15.76±9.87%, P=0.04, power=0.71, Figure 2).

Through red oil O staining of the thoracic aorta, no atherosclerosis lesions were found in the C57BL/6 mice. The plaque area percentage in moxibustion treated ApoE$^{-/-}$ mice was significantly lower than those in the untreated AS group (AS+M vs AS: 2.47±1.77% vs 9.87±7.27%, P=0.01, power=0.9, Figure 3A,B). The average lipid area percentage in the moxibustion treated ApoE$^{-/-}$ mice did not significantly differ from than that in the untreated AS group (AS+M vs AS: 1.04±0.94% vs 1.64±1.35%, P>0.05, power=0.3, Figure 3A,C).

Moxibustion up-regulated LXRα and ABCA1 expression in the thoracic aorta of ApoE$^{-/-}$ mice

Compared with the C57BL/6 mice, the ApoE$^{-/-}$ mice showed significantly lower LXRα, ABCA1 and ABCG1 expression in the thoracic aorta (AS vs Control: 0.56±0.06 vs 0.71±0.15, P=0.004, power=0.87, Figure 4A; 0.52±0.17 vs 0.82±0.06, P<0.001, power=0.99, Figure 4B; 0.64±0.09 vs 0.75±0.12, P=0.04, power=0.72, Figure 4C).

Compared with those in the AS group, the ApoE$^{-/-}$ mice treated with moxibustion showed significantly higher LXRα and ABCA1 expression (AS+M vs AS: 0.67±0.07 vs 0.56±0.06, P=0.03, power=0.99, Figure 4A; 0.64±0.22 vs 0.52±0.17, P=0.05, power=0.37, Figure 4B). No significant difference was found in ABCG1 expression between ApoE$^{-/-}$ mice in the AS+M group and the AS group (0.72±0.14 vs 0.64±0.09, P=0.14, power=0.61, Figure 4C).

Discussion

Studies have found that acupuncture/moxibustion treatment is beneficial to the cardiovascular system through regulation of blood lipids and blood glucose, and attenuation of hepatic lipid accumulation.[12–15] The clinical effectiveness of moxibustion for the regulation of blood lipids has been reported in previous studies.[8,9] In a randomised control trial of 160 patients with hyperlipidaemia, moxibustion treatment showed a better therapeutic effect than diet therapy.[8] In a review of 25 studies of moxibustion treatment on hyperlipidaemia and atherosclerosis, including clinical trials and animal studies, the authors came to the conclusion that moxibustion suppresses the development of atherosclerosis by regulating lipid metabolism, thrombogenesis and inflammatory reactions.[9] In this study, we showed experimental evidence that moxibustion could suppress the progression of atherosclerotic lesions in ApoE$^{-/-}$ mice. Furthermore, this effect may be achieved by enhancing cholesterol efflux from macrophages, which is mediated by the LXR-ABCA1 pathway.

To create an animal model of atherosclerosis, the ApoE$^{-/-}$ mice (8 weeks old) were fed a high-fat, cholesterol-rich diet for 13 weeks. We found the formation of early plaques in both the aortic root and the thoracic aorta. Furthermore, the ApoE$^{-/-}$ mice treated with moxibustion showed less formation of AS plaque and less lipid content

Figure 3. (A) Representative histomorphological (red oil O stained) images of the thoracic aorta (100×). Bar=500 μm. (B) Comparison of plaque area percentage between AS and AS+M groups. (C) Comparison of lipid area percentage between AS and AS+M groups. Data are expressed as mean±SD, n=10/group. *P<0.05, AS+M versus AS. AS, atherosclerosis; M, moxibustion.

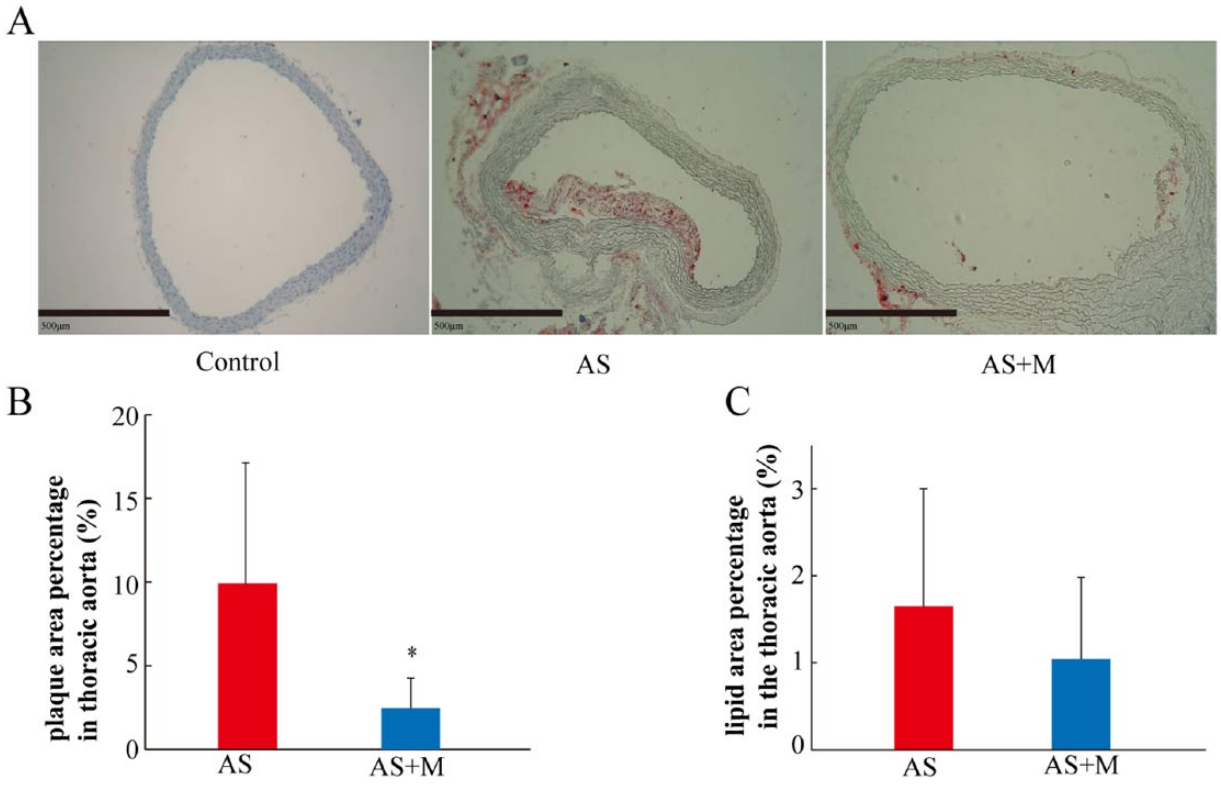
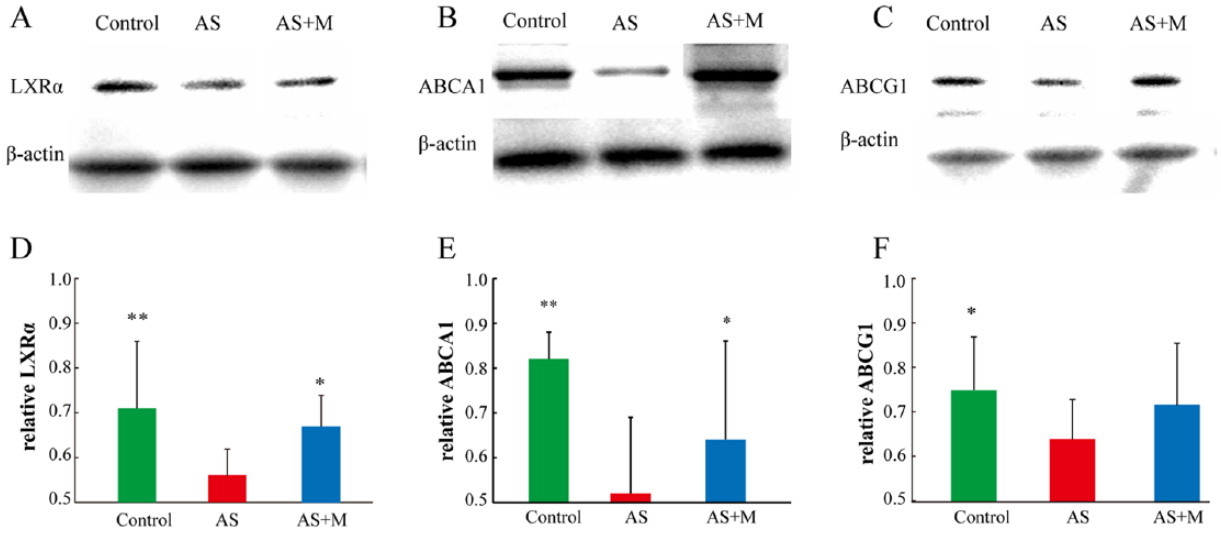


Figure 4. Effects of moxibustion on LXRα, ABCA1 and ABCG1 expression. (A–C) Representative Western blots using Odyssey software. (D–E) Comparison of LXRα, ABCA1, ABCG1 expression between the different groups. Protein expression levels were normalised to β-actin. Data are expressed as mean±SD, n=10/group. *P<0.05 versus AS, **P<0.01 versus AS. AS, atherosclerosis; M, moxibustion; ABC, adenosine triphosphate binding cassette; LXR, liver X receptor.



in the aorta, which demonstrates the anti-atherogenic effect of moxibustion.

In our study, dyslipidaemia was present in ApoE$^{-/-}$ mice after 13 weeks on an atherogenic diet. Dyslipidaemia is an important risk factor for the development of atherosclerosis and is characterised by increased levels of TC, TG and LDL and decreased levels of HDL and ApoA-I. Lipids are carried by lipoprotein in plasma; most (60–70%) are carried by LDL and can enter the arterial wall and accumulate inside, thereby causing lipid deposition and initiating early atherosclerosis.[16] LDL is prone to oxidative modification into oxidised LDL (ox-LDL) that can induce endothelial cell activation in the vessel wall. Ox-LDL can also be taken up by macrophages, turning them into foam cells and thereby contributing to the formation of so-called 'foamy atherosclerotic plaques'.[12] HDL and APoA-I are considered to be atheroprotective by increasing RCT, whereby they accept cholesterol and transport it from atherosclerotic lesions back to the liver for excretion into the bile.[17,18] We found that moxibustion at CV4 down-regulated the increased TG and LDL and up-regulated decreased APoA-I in ApoE$^{-/-}$ mice. This result is in line with previous studies of moxibustion. This function may play an important role in the anti-atherogenic effect of moxibustion.

Excess accumulation of cholesterol within macrophages at sites of atherosclerotic lesions converts them into foam cells and accounts for the lesion-deposited cholesterol.[19] The function of LXRα, a nuclear receptor, has attracted increasing attention in AS treatment. LXRα is a cholesterol-sensing nuclear receptor and is regarded as one of the key regulators of lipid metabolism and transport. Studies have shown that LXR agonists increase RCT from macrophages by increasing the expression of cholesterol efflux transporters ABCA1 and ABCG1.[5,6] Excessive cell cholesterol concentration leads to LXRα activation (by combination with the retinoid X receptor) and then up-regulation of the expression of ABCA1 and ABCG1 at the cell surface.[5,6] ABCA1 and ABCG1 have complementary roles in mediating cholesterol efflux.[4,20] ABCA1 mediates cholesterol efflux to lipid-free apolipoproteins such as ApoA-I and ApoE, but not to large HDL particles.[21] ABCG1 has been shown to mediate cholesterol efflux from macrophages to HDL particles, but not to lipid-free apolipoproteins.[22,23] Furthermore, they act in a sequential manner: ABCA1 generates nascent HDL particles from lipid-poor ApoA-I,[24] which then facilitate cholesterol efflux via ABCG1, followed by formation of mature HDL particles.[25] This experiment showed decreased LXRα, ABCA1 and ABCG1 expression in the thoracic aorta in the ApoE$^{-/-}$ mice, and moxibustion at CV4 up-regulated the decreased LXRα and ABCA1 expression, indicating an effect promoting cholesterol efflux. Through promoting the RCT mediated by LXRα-ABCA1-ApoA-I, moxibustion may prevent foam cell formation and cholesterol accumulation, thus relieving the progression of the AS lesion.

There are several limitations to the present study. First, based on clinical observations, the treatment period was set at 12 weeks and no evaluation was made between 4 and 8 weeks of follow-up; thus the therapeutic effect of moxibustion for AS was not fully elucidated. We plan to extend the follow-up evaluation to include weeks 4 and 8 in future studies. Second, moxibustion at a single point is unlikely to fully explore the mechanism of action, since traditional acupuncture points are mostly targetted in combination to treat diseases in a clinical setting. Future research should include evaluation of the effects and underlying mechanism of different traditional acupuncture point combinations incorporating different body surface areas.

In conclusion, our study provides experimental evidence that moxibustion treatment at CV4 suppresses the progression of atherosclerotic lesions in ApoE$^{-/-}$ mice. The anti-atherogenic effect of moxibustion may be achieved by: (1) regulation of lipid metabolism, and thus prevention of early lipid accumulation; and (2) up-regulation of LXRα- and ABCA1-mediated cholesterol efflux in the lesion area.

Contributors

YC and BZ conceived and designed the study. YC and JL performed the experiments. CH performed the data analyses. All authors wrote, read and approved the final version of the manuscript accepted for publication.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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