

## Original Article

# Exercise training increases the stability of atherosclerotic plaques in apolipoprotein E (ApoE) gene-deficient mice

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**Abstract:** The aim of this study was to investigate the effect of exercise training on the stability of atherosclerotic plaques in apolipoprotein E (ApoE) gene-deficient mice. A total of 80 ApoE gene-deficient mice were randomly divided into exercise and control groups. The exercise group was subjected to 8 weeks of electric treadmill training, while the activity of animals in the control group was restricted for the same 8-week period. In the exercise group the thickness of the fibrous cap was significantly increased ( $P < 0.05$ ), and plaque stability was also increased. The plaque vulnerability index of the exercise group exhibited a significant difference compared to the control group ( $P < 0.05$ ). The expression of matrix metalloproteinase-9 (MMP-9) and MMP-14 was significantly lower in plaques from the exercise group ( $P < 0.05$ ), while blood lipid levels and plaque area showed no significant difference between the two groups ( $P > 0.05$ ). These data suggest that exercise could increase the stability of atherosclerotic plaques, and therefore delay the progression of atherosclerosis.

**Keywords:** Atherosclerosis, exercise, apolipoprotein E gene deficient mice

## Introduction

Atherosclerosis (AS) is a chronic multifactorial disease. The instability and rupture of AS plaques can lead to sudden clinical events, such as unstable angina, acute myocardial infarction, or stroke [1-5]. Regular exercise has been shown to slow the progression of human AS, and in animal experiments exercise training, such as treadmill running and swimming, was able to reduce AS lesions in mice [6-13]. Matrix reconstruction is a common occurrence in the development of AS plaques. Matrix metalloproteinases (MMPs) are a group of enzymes with common biochemical characteristics that degrade the extracellular matrix and participate in the reconstruction and rearrangement of the vascular matrix. Abnormal MMP secretion and activation is one of the main causes of the instability and progression of plaques [14]. Various MMPs have been shown to regulate the metabolism of collagen. They increase collagen catabolism, leading to collagen reduction, which in turn increases vulnerability to AS plaques and decreases plaque stability. MMP-9 and MMP-14 are important members of the

MMP family, and are thought to promote the rupture of AS plaques, thus promoting the occurrence and development of AS diseases [15, 16]. Several studies have demonstrated that exercise training could reduce damage due to AS by inhibiting inflammatory cytokines [17], promoting plaque stability [18], and improving antioxidant abilities [8] *in vivo*. However, to date there have been no reports regarding the effects of exercise on the vulnerability and stability of AS plaques and the relationship with MMPs.

The goal of this study was to explore the role of MMPs and physical activity in AS stability. We used ApoE-deficient mice as an AS model, and observed the effect of exercise on carotid AS plaques and MMP-9 and MMP-14 expression.

## Materials and methods

### Animals and grouping

Eighty 10-week-old male ApoE-deficient mice, weighing 22.6-26.3 g (purchased from the Peking University health science center), were

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**Figure 1.** Schematic diagram of catheterization surgery in right common carotid artery.

randomly divided into two groups: an exercise group ( $n = 40$ ) and a control group ( $n = 40$ ). All animals were fed a full fat diet (0.25% cholesterol + 15% fat) for the entire experimental procedure, with 10 g/day per mouse. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Qingdao University.

### *Establishment of a carotid AS model*

Carotid catheterization was performed as previously described [19]. Mice were anesthetized with an intraperitoneal injection of 0.08% sodium pentobarbital (40 mg/kg) (New Asia Pharmaceutical Co., Ltd). An incision was made into the skin at the middle of the neck and the glands and muscles were stripped to expose the right common carotid artery. The concomitant vagus nerve was carefully separated, the right common carotid artery was identified, and a silicone tube (length 3 mm, inner diameter 0.3 mm, mouse carotid artery diameter 0.5 mm) was placed around the vessel and secured in place (**Figure 1**). The skin was sutured and the mouse was returned to the cage.

### *Exercise training*

Four weeks after surgery, mice in the exercise group were subjected to training on a 0°-gradient electric rodent treadmill (15 m/min)

(wi5635 12-track mouse treadmill, DXY (Beijing) Tech Co., Ltd) for 30 min a day, six days a week (the mice rested on Sunday), for a total of eight weeks. For the control group, exercise was restricted by keeping the animals a cage that limited their activity to one hour a day for a total of eight weeks.

### *Blood tests*

Before the mice were euthanized, they were anesthetized and a blood sample was collected from the abdominal aorta. Serum was isolated from part of the blood sample and sodium citrate was added to the remainder for plasma isolation to test levels of total cholesterol (TC), HDL-C, triglycerides (TG), and LDL-C.

### *Pathological assay*

Twelve weeks after catheterization, mice were euthanized by injecting an overdose of pentobarbital sodium. For the histopathological assay of plaques, vessels were irrigated with PBS and perfused with 4% paraformaldehyde [20]. The right carotid artery was isolated, and a sample piece was embedded in OCT. Frozen sections were cut (20 consecutive slices of 6  $\mu\text{m}$  each, with a 50  $\mu\text{m}$  interval), and stained with hematoxylin-eosin (HE), picosirius red (Sigma, USA), or oil red O (Sigma, USA). Adjacent sections were used for immunohistochemical staining to detect the presence and distribution of smooth muscle cells, macrophages, MMP-9 and MMP-14 within the plaques.

### *Histopathological assay*

The section containing the largest plaque was selected for morphological analysis. Total plaque area was measured using image analysis software; the measurement was taken three times in order to obtain a mean value. All measurements were carried out under the same conditions. Parameters were calculated as follows: (1) vascular area at the plaque site; (2) plaque area; (3) fibrous cap thickness; (4) lipid core area; (5) cap/core ratio; (6) collagen content: positive picosirius red staining area and ratio of positively stained area to plaque area; (7) lipid content: positive oil red O staining area and ratio of positively stained area to plaque area; (8) macrophage content: positive immunohistochemical staining area and ratio of positively stained area to plaque area; (9) plaque rupture rate: plaque rupture was defined as fibrous cap rupture, or plaque rupture plus

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**Table 1.** Body weight and blood lipids between the two groups ( $\bar{x} \pm s$ )

	Exercise group	Control group	P
Original body weight (g)	26.71 $\pm$ 1.29	26.34 $\pm$ 1.21	0.473 <sup>a</sup>
Final body weight (g)	28.43 $\pm$ 1.52	28.91 $\pm$ 1.71	0.325 <sup>a</sup>
TC (mmol/L)	15.13 $\pm$ 2.86	16.37 $\pm$ 3.21	0.435 <sup>a</sup>
LDL-C (mmol/L)	5.01 $\pm$ 1.02	5.13 $\pm$ 1.17	0.485 <sup>a</sup>
HDL-C (mmol/L)	1.42 $\pm$ 0.24	1.44 $\pm$ 0.26	0.414 <sup>a</sup>
TG (mmol/L)	0.39 $\pm$ 0.11	0.41 $\pm$ 0.12	0.369 <sup>a</sup>

Note: Compared between the two groups, <sup>a</sup>P > 0.05.

thrombosis, or a fibrous cap embedded inside a plaque [21]; (10) vulnerability index: vulnerability index = (macrophage content + lipid content)/(collagen content + smooth muscle cell content); each variable was expressed as the ratio of positively stained area to plaque area; (11) MMP-9 and MMP-14: area of positive MMP-9 and MMP-14 immunohistochemical staining and ratio of positively stained area to plaque area.

### RT-PCR

Three fresh mouse carotid plaques samples were combined and 1 mL Trizol/50-100 mg tissue was added to extract total RNA. A UV spectrophotometer was used to determine RNA purity and yield. Reverse transcription reactions were performed using a TaKaRa RNA PCR kit (Shenzhen Jingmei Biotech Co., Ltd). Primer sequences: MMP-9: upstream sequence: 5'-GACAGGCACTTACCGGCTA-3', downstream sequence: 5'-CCCGACACACAGTAAGCATTCTG, fragment length 130 bp; MMP-14: upstream sequence: 5'-ACGTGCAGCAGCATTGGA-3', downstream sequence: 5'-CAACAGGAGCAAGTGTCCTTC-3', fragment length: 140 bp;  $\beta$ -actin (internal reference): upstream sequence: 5'-CACTGTGCCATCTACGA-3', downstream sequence: 5'-GTAGTCTGTCCAGTCCCG-3', fragment length: 82 bp. Reaction conditions: denaturation at 95°C for 10 s, annealing at different temperatures for 30 s, extension at 72°C for 30 s, with 40-50 cycles. PCR products (10  $\mu$ L) were then run on a 1.2% agarose gel, and a gel imaging analysis system was used to semi-quantitatively analyze the absorbance (OD) of the electrophoretic bands. Results were expressed as the ratio of the OD values for the target genes to  $\beta$ -actin.

### Western blot

AS plaques were sampled from three right common carotid arteries, and cut into pieces using

fiber surgical scissors to extract total cellular protein. Each specimen, comprising 50  $\mu$ g of microparticle proteins and a protein marker, was subjected to electrophoresis on a 10% SDS-polyacrylamide gel at 18 V/cm. Proteins were then transferred onto a nitrocellulose membrane, and incubated overnight with 1:500 MMP-9, 1:500 MMP-14, and 1:3000  $\beta$ -actin antibodies in TBS-T at 4°C. After several washes in TBS-T, membranes were incubated with an HRP-conjugated secondary antibody at

room temperature for 1 h. Detection was performed by adding a chromogenic agent drop wise onto the membranes. A JD801 image analysis software system (Nanjing University) was used to determine band intensities, and therefore expression levels of MMP-9 and MMP-14 protein in AS plaques.

### Statistical analysis

All data were expressed as  $\bar{x} \pm s$ . Measurement data were subjected to the t test, and counting data were analyzed using the  $\chi^2$  test, with P < 0.05 considered statistically significant. All data were statistically analyzed using SPSS13.0 software.

## Results

### General conditions

There was no statistically significant difference between the body weights of the animals in the exercise and control groups at the beginning of the experiment (P = 0.473), or at the end of the experiment (weight before animal was euthanized and samples were collected) (P = 0.325, **Table 1**).

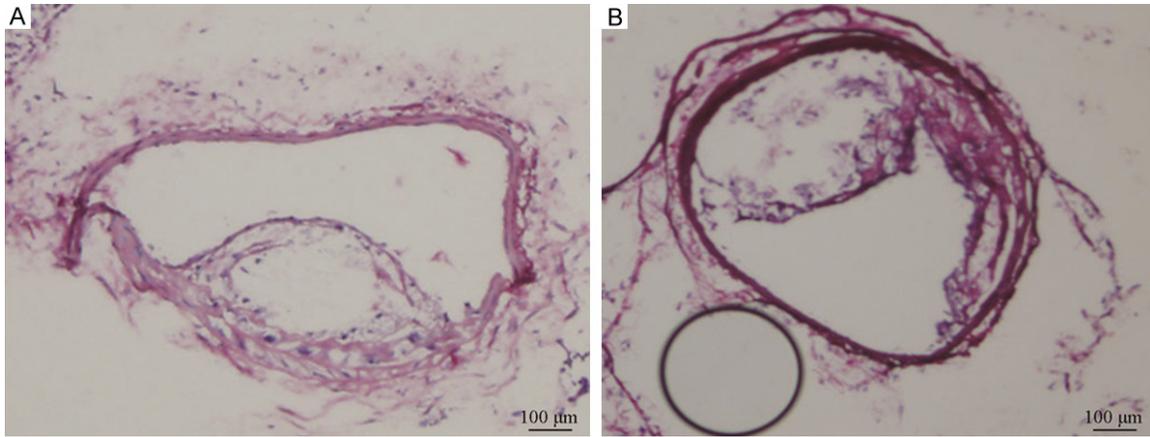
### Blood tests

**Table 1** shows the comparison of lipid levels between the two groups. There was no significant difference in serum concentrations of TC, LDL-C, HDL-C, and TG between the exercise and control groups (P > 0.05).

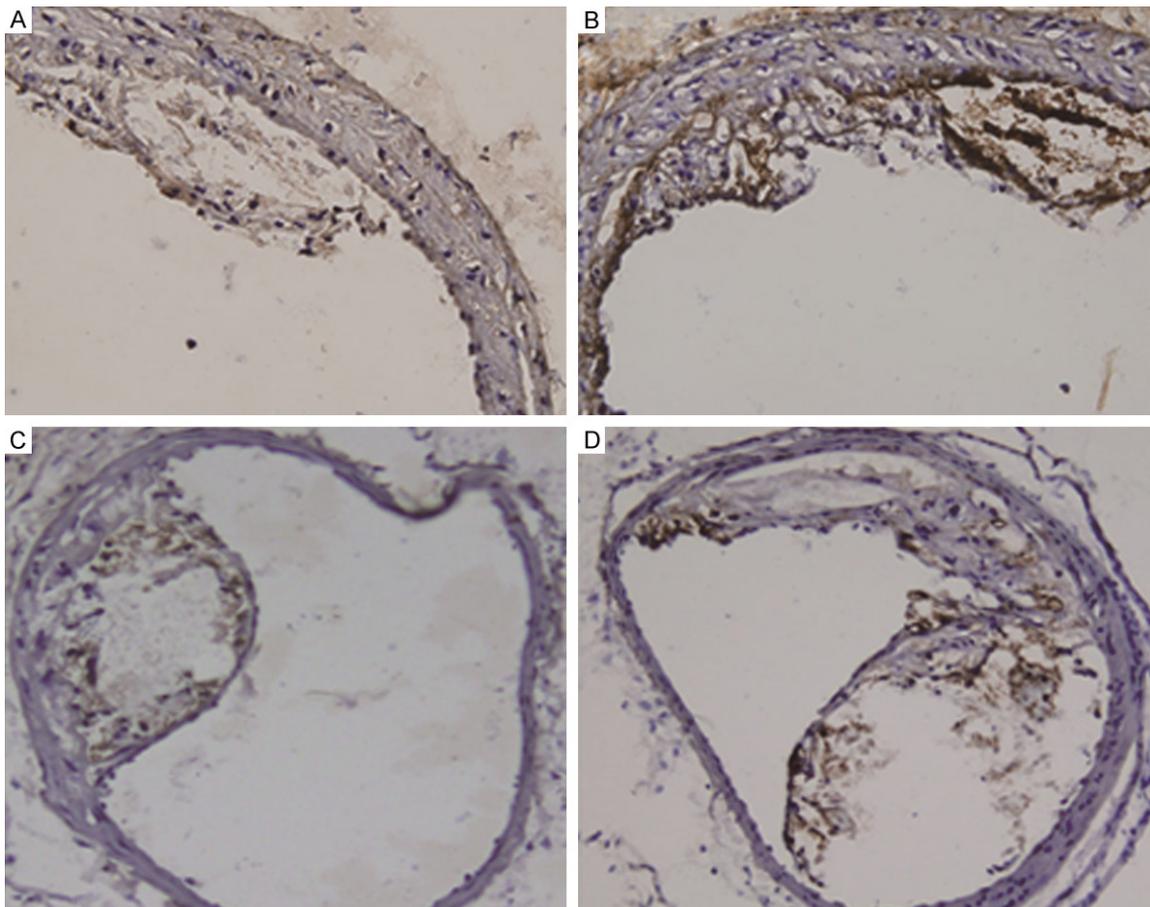
### Histopathological inspection of the plaques

There was no significant difference in plaque areas between the two groups (exercise group: 71000  $\pm$  8000  $\mu$ m<sup>2</sup>, control group: 82000  $\pm$  11000  $\mu$ m<sup>2</sup>, P = 0.073). However, the thickness of the fibrous cap was significantly increased in the exercise group compared to the control

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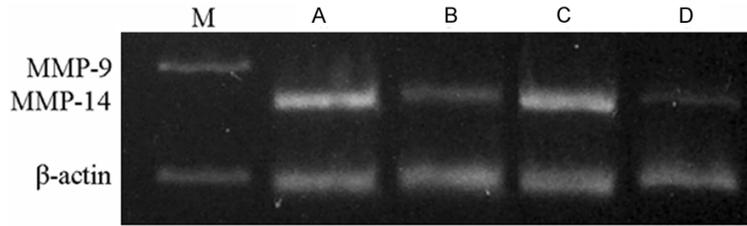


**Figure 2.** Carotid artery of ApoE-defected mice (HE staining,  $\times 100$ ). A: Carotid artery of the exercise group, the fibrous cap was thicker, and the cap/core ratio was big; B: Carotid artery of the control group, the fibrous cap was thinner, and the cap/core ratio was small.

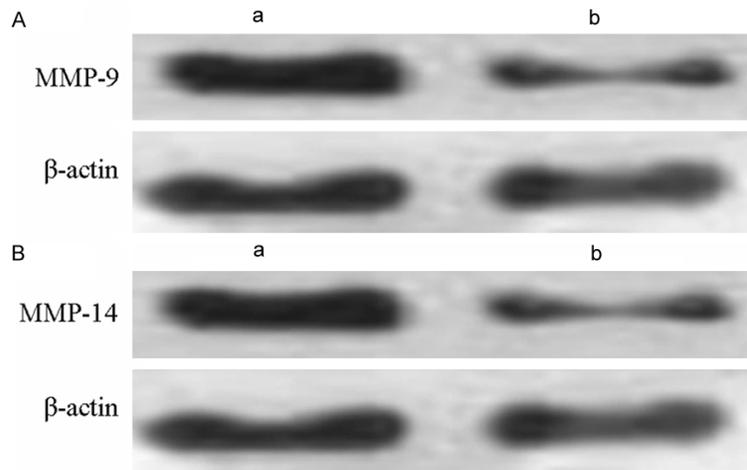


**Figure 3.** Expression detection of MMP-9 and MMP-14 in the carotid artery plaques of ApoE-defected mice (immunohistochemical staining,  $\times 200$ ). A: Expression of MMP-9, Carotid artery of the exercise group, the staining proportion of the plaques was low; B: Expression of MMP-9, Carotid artery of the control group, the staining proportion of the plaques was high; C: Expression of MMP-14, carotid artery of the exercise group, the staining proportion of the plaques was low; D: Expression of MMP-14, carotid artery of the control group, the staining proportion of the plaques was high.

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**Figure 4.** Expressions of MMP-9 and MMP-14 mRNA in the two groups by RT-PCR. A: MMP-9 expression in the control group; B: MMP-9 expression in the (AE) exercise group; C: MMP-14 expression in the control group; D: MMP-14 expression in the (AE) exercise group.



**Figure 5.** Expressions of MMP-9 and MMP-14 protein in the two groups by Western Blot. A: Control group; B: (AE) exercise group.

group ( $P = 0.029$ ) (exercise group:  $7.25 \pm 1.35 \mu\text{m}$ , control group:  $6.73 \pm 0.96 \mu\text{m}$ ) (**Figure 2**). The cap/core ratio was also increased in the exercise group (exercise group:  $0.09 \pm 0.02$ , control group:  $0.06 \pm 0.02$ ,  $P = 0.045$ ) (**Figure 2**), as was the collagen content (exercise group:  $16.34\% \pm 4.72\%$ , control group:  $11.23\% \pm 3.57\%$ ,  $P = 0.005$ ). The lipid contents were significantly reduced in the exercise group compared to the control group (exercise group:  $12.25\% \pm 2.01\%$ , control group:  $21.23\% \pm 3.56\%$ ,  $P = 0.002$ ). In the exercise group, four plaque fibrous caps were broken, and four were buried inside plaques. No thrombosis or bleeding occurred, and the plaque rupture rate was 20%. For the control group, four plaques were ruptured and formed a thrombus and four exhibited inner hemorrhage. Four fibrous caps were buried inside plaques and eight were broken. The plaque rupture rate was 50% for the control group. The difference in plaque rupture rate between the two groups was statistically significant ( $P = 0.005$ ). The plaque vulnerability

index between the two groups showed a statistically significant difference (exercise group:  $0.81\% \pm 0.43\%$ , control group:  $2.83\% \pm 1.02\%$ ,  $P = 0.009$ ).

### Immunohistochemical staining

Immunohistochemical staining showed that intraplaque expression of MMP-9 was lower in the exercise group than in the control group (exercise group:  $4.29\% \pm 0.31\%$ , control group:  $9.78\% \pm 0.98\%$ ,  $P = 0.019$ ) (**Figure 3**). Intraplaque expression of MMP-14 was also lower in the exercise group than in the control group (exercise group:  $6.89\% \pm 0.57\%$ , control group:  $12.32\% \pm 1.44\%$ ,  $P = 0.021$ ) (**Figure 3**).

### RT-PCR

Consistent with this, expression of MMP-9 mRNA in the carotid artery plaques of the exercise group was reduced by 69.3% compared to the control group, and the difference between the two groups was statistically significant ( $P = 0.028$ ) (**Figure 4**). Expression of MMP-14 mRNA in carotid artery plaques of the exercise group was reduced by 77.2%, and the difference between the two groups was statistically significant ( $P = 0.028$ ) (**Figure 4**).

### Western blot

MMP-9 protein expression in the carotid artery plaques of the exercise group was reduced by 61.9% compared to the control group ( $P < 0.05$ ) (**Figure 5**). Expression of MMP-14 protein was reduced by 56.3% in the exercise group compared to the control group ( $P < 0.05$ ) (**Figure 5**). The differences between the two groups were statistically significant.

### Discussion

AS plaques are composed of a fibrous cap and a lipid core, whose stability is mainly determined by the thickness of the fibrous cap

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Vulnerable plaques are at high risk for thromboembolic complications, and display characteristics such as thin fibrous caps, large lipid cores, reduced collagen content, and a decrease in the density of smooth muscle cells [22]. AS plaques were found in the carotid artery of about 80% of patients with cerebral infarction. Among these, vulnerable plaques would often rupture and fall off, causing artery-to-artery embolization. These vulnerable plaques can also lead to in situ thrombosis formation, which is the major pathological mechanism observed in patients with cerebral infarction. It is difficult to identify vulnerable plaques via imaging. Even in cases where blood vessel imaging displayed arterial lumen parts that were completely normal, hidden AS plaques could still be present. Furthermore, these hidden plaques may not produce any symptoms or evidence of ischemia before they trigger thrombosis [23].

MMPs are an important family of enzymes that degrade the extracellular matrix, which could result in the instability or rupture of fibrous caps [24]. MMP-9 is secreted by neutrophils, macrophages, and smooth muscle cells. It is also overexpressed in vulnerable plaque regions where it destroys the basement membrane and extracellular components of the vessel wall, leading to the collapse of fibrous caps and affecting plaque stability [25]. Loftus [26] analyzed different MMP-9 expression levels in clinical unstable plaques, and found a positive correlation between intraplaque MMP-9 expression levels and the presence of microemboli. MMP-9 is a major factor in the stability of AS plaques. MMP-14 is a special protease member of the MMP family. It is involved in the proteolysis of a variety of extracellular and membrane associated substances, and therefore is closely related to the pathophysiology of vascular diseases [27]. In recent years, several studies have shown that a variety of pro-AS factors may promote MMP-14 expression, and collagen content in MMP-14-deficient mice was significantly higher than in wild-type controls [28]. The current studies showed that MMP-9 and MMP-14 are important factors for plaque stability, and abnormal expression of MMP-9 and MMP-14 are a strong indication of plaque vulnerability.

The ApoE-deficient mice used in this study are a good animal model of AS and are the most widely used genetically engineered animals in

AS research. They mimic the effects of a high fat diet in humans, and can form wide-range AS lesions after only a short breeding time [29]. The mouse carotid AS plaque model using silicone catheterization has advantages such as a relatively fixed artery stenosis area and a shorter formation time. It reproduces the pathological process of human carotid artery stenosis, and does not disrupt the endothelial cell layer of arteries [19]. In our study we showed that the AS plaque lesions formed using this method were significant, with extensive arterial intimal lipid deposition, deeply stained plaques, and an accumulation of large red lumps. The vascular intima was seriously injured and the original waveform was lost; the fiberboard split into multiple layers and exhibited large gaps and fractures. Animals in the exercise group exhibited lighter arterial plaque staining, superficial scattered lipid distribution, lighter vascular intimal injury, and densely arranged elastic fibers (with occasional faults), indicating that exercise significantly inhibited AS plaque progression in ApoE-deficient mice. In the exercise group, fibrous cap thickness ( $P < 0.05$ ), the cap/core ratio ( $P < 0.05$ ), and intraplaque collagen content were all significantly increased ( $P < 0.01$ ), whereas lipid content ( $P < 0.01$ ), and the plaque vulnerability index ( $P < 0.01$ ) were significantly reduced. Taken together, these data suggest that exercise may reduce arterial plaque vulnerability and increase plaque stability. Our experiments did not reveal a statistically significant difference in AS plaque area between the exercise group and the control group. This could be related to the intensity and time of exercise; an eight-week exercise regime may not be sufficient to cause differences in atherosclerotic plaque area. Body weight and blood lipid levels were also not significantly different between the exercise and control groups ( $P > 0.05$ ). This prompts the possibility that exercise could have some other anti-AS mechanism independent from lipid regulation, and thus could have anti-AS roles as well as delaying the development of AS plaques.

Results from an immunohistochemical assay, RT-PCR and western blot all revealed that intraplaque expression of MMP-9 and MMP-14 mRNA and protein was lower in the exercise group than in the control group. MMP-9 and MMP-14 play important roles in the rupture and vulnerability of AS plaques and therefore the exercise-induced reduction in their expression

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could enhance AS stability and, reduce plaque vulnerability. This would ultimately prevent further development of AS and reduce acute thrombosis or embolism events caused by plaque instability. These results suggested that exercise could ameliorate atherosclerotic lesion formation by reducing the intraplaque expression of MMP-9 and MMP-14.

Previous studies showed that exercise increased the body's antioxidant capacity, improved the concentration of biological nitric oxide (NO) and the activity of superoxide dismutase (SOD), thus improving endothelial function and delaying the occurrence and development of AS plaques [11]. It was also reported that exercise reduced levels of the pro-inflammatory cytokines monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in AS mice, leading to the proposal that exercise may reduce AS lesions via an anti-inflammatory mechanism [30]. AS is a multifactorial pathological process, and by focusing our research on the aspect of enhancing plaque stability and reducing plaque vulnerability, we further confirmed the role of exercise in preventing AS. Finally, it is possible that exercise not only prevents the occurrence of AS, but could also be used to treat AS that has already occurred. This has important clinical implications and could provide new ideas for clinicians to prevent and treat the occurrence and development of atherosclerosis in the heart, brain, and kidney.

### Disclosure of conflict of interest

None.

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