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Original Article

Effects of swimming on the development of atherosclerosis in mice

Longpu Zhang[1], Jian Chi[1], Junxia Chen[2], Shiqin Jiang[3], Di Wu[1]

[1]China Basketball College, Beijing Sport University, Beijing 100084, China; [2]Art and Sports Teaching and Research Office, School of Humanities and Economic Management, China Agricultural University (Yantai), Yantai 264670, Shandong Province, China; [3]Competitive Sports Division, Shandong Shooting Bicycle Sport Management Center, Jinan 250100, Shandong Province, China

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Abstract: Objective: To investigate the effects of swimming on the formation of atherosclerotic lesions and the corresponding mechanism. Methods: 20 ApoE-deficient young male mice of SFP grade were assigned equally into two groups: atherosclerosis group and swimming group. Atherosclerosis models were established by feeding with high cholesterol diet. Swimming exercise was performed at a frequency of 90 min per day, 6 days per weeks for 10 weeks. The weight index, histologic changes of aorta area, blood lipid levels, expression levels of eNOS, tNOS and iNOS, expression levels of MMP-9 and MMP-14, inflammatory factor levels, and oxidative stress status were compared between the two groups. Results: Compared to the atherosclerosis group, the plaque area, plaque rupture rates, and vulnerable index in the aorta of the swimming group were significantly less and the fibrous cap thickness was greater. The weight of mice and serum lipid levels in the swimming group were superior. In addition, in contrast to atherosclerosis group, mRNA expression levels of eNOS, tNOS, iNOS, and SOD in the swimming group were significantly elevated, while the levels of MMP-9, MMP-14 and MDA, and serum levels of IL-6, Lp-PLA2, and TNF-α were significantly decreased. Conclusion: Swimming exercise significantly decreases the development of atherosclerotic plaque in ApoE-deficient mice, possibly due to a reduction in the expression of blood lipid, MMP-9, MMP-14, MDA, IL-6, Lp-PLA2, and TNF-α and elevation in the expression of eNOS, tNOS, iNOS, and SOD.

Keywords: Swimming, atherosclerosis, ApoE-deficient, mechanism

Introduction

Atherosclerosis is currently recognized as a common pathologic basis of stroke, coronary heart disease, and other thrombotic diseases. It is a process during which excess triglyceride, cholesterol, and other lipids accumulate in the blood vessel wall to form a plaque, and ultimately result in sclerosis, stenosis, or occlusion of arteries and restricted blood flow [1, 2]. This disease is initiated with accumulation of cholesterol-rich lipoproteins in the arterial intima, resulting in local oxidative stress, dysfunction of endothelial cells, recruitment and activation of inflammatory cells, matrix reconstruction, and establishment of inflammation [3, 4]. Despite major advances in pharmacologic and procedural intervention for atherosclerosis, it is still the leading cause of death and disability in the world.

In recent years, as the costs of healthcare have increased, a standard therapy needs to be prescribed. It was reported that compared to procedures and pharmacologic treatment, aerobic exercise was beneficial to patients with atherosclerotic diseases and much more cost-effective [5, 6]. Moreover, aerobic exercise has been widely popularized as a powerful strategy to reduce the risks of developing atherosclerosis and alleviate the symptoms [7, 8]. Many trials on human reported that different kinds of aerobic exercise training can significantly reduce risk of cardiovascular events [9, 10]. Ongoing studies been conducted to investigate the underlying mechanisms. However, data on the anti-atherogenic roles of exercise are still scarce [11]. Therefore, this study tried to explore the mechanisms of swimming in reducing atherosclerosis development. ApoE-deficient mice were used to establish athero-

sclerosis model by taking cholesterol diet, and swimming practice was carried out to observe its effect. Subsequently, we evaluated the blood lipid levels, inflammatory factors, oxidative stress markers, matrix metalloenzyme expression and endothelial nitric oxide production in atherosclerotic plaques. The results of this study provided experimental evidence for treatment of atherosclerosis.

Materials and methods

Laboratory animals

A total of 20 ApoE-deficient young male mice of SFP grade were enrolled in this study and the mice were obtained from Experimental Animal Center in Beijing Sport University (Beijing, China). The age was from 7 to 8 weeks old. The housing, breeding and animal experiments were approved by the Institutional Animal Care and Use Committee of Beijing Sport University (approval No. 2019-161) and were in accordance with the rules and regulations on use of laboratory animals. These ApoE-deficient mice were divided into an atherosclerosis group and a swimming group. The atherosclerosis model was constructed according to instructions reported by a previous study [12]. All the ApoE-deficient mice were fed a high cholesterol diet. The mice from the swimming group were forced to swim for 10 weeks at a frequency of 90 min per day and 6 days per week. The mice from atherosclerosis group did not take swimming exercise. The mice were placed in cages for 10 weeks. All the mice were sacrificed under deep anesthesia with overdose pentobarbital (Sigma, 130 mg/kg intraperitoneal injection). Blood samples were collected, and serum was obtained by 3000 r/min centrifugation for 10 min. These serum samples were stored at -80°C. The aorta tissues were harvested and immediately stored in liquid nitrogen for protein analysis and histologic examination.

Hematoxylin-eosin (H&E) staining

The aorta tissues were fixed in 4% neutral formalin for 24 h and then embedded in paraffin. 3-μm sections were obtained from each paraffin block. The sections were stained with hematoxylin and eosin as in a previous study [13]. First, the sections were immersed in xylene and alcohol, respectively. Next, the sections were stained with hematoxylin for 3 min and eosin for 1 min. Then, they were immersed in alcohol and xylene again. Finally, a synthetic resin was applied for mounting slides. Slides were observed in ten high power fields under a light microscope and the plaque area, fibrous cap thickness, plaque rupture rates, and vulnerable index were calculated.

Detection of indexes

Blood lipid levels were detected using an automatic biochemical analyzer (Olympus, Japan), and the superoxide dismutase (SOD), and malonialdehyde (MDA) levels were examined by the biochemical enzyme assay kits (Sigma, USA). Assays were completed strictly following the instructions in the kits. The inflammatory cytokine levels, including interleukine-6 (IL-6), lipoprotein-associated phospholipase A2 (Lp-PLA2), and tumor necrosis factor-α (TNF-α), were determined by the enzyme-linked immunosorbent assay (ELISA) kits (R&D Science, USA) per manufacturer instructions.

qRT-PCR

Total RNA of aorta atherosclerotic plaque tissues in the two groups was isolated and reverse transcribed into cDNA following the instructions of RT-PCR kits. The reaction system was as follows: forward/reverse primer 0.4 μL, SYBR Green Real-time PCR Master Mix 10 μL, cDNA 1.6 μL, and ddH2O 8.0 μL. The primer sequences and the reaction condition of each gene are shown in **Table 1**. PCR amplification was conducted. The C_T values of MMP-9, MMP-14, eNOS, iNOS or tNOS gene were calculated using ABI 7300 System software. The relative expression levels of MMP-9, MMP-14, eNOS, iNOS and tNOS mRNA were calculated using $2^{-\Delta\Delta Ct}$ method.

Western blot

The aortic atherosclerotic plaque tissues were ground and protein lysis buffer RIPA was added to extract the total protein. The protein concentration was determined by BCA method. After SDS-PAGE gel electrophoresis, protein samples were transferred to a PVDF membrane. Then the membrane was placed in TBST with 5% non-fat milk powder, and blocked for 1 h at room temperature. The primary antibody (MMP-9: 1:1000, ab76003, Abcam, USA; MMP-14: 1:500, ab51074, Abcam, USA) was incubated

Table 1. Primer sequences and the reaction conditions of each gene

Gene	Primer sequences	Reaction condition
MMP-9	forward primer: 5' CTGGACAGCCAGACACTAAAG 3' reverse primer: 5' CTCGCGGCAAGTCTTCAGAG 3'	95°C for 5 min, denaturation at 95°C for 20 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and a total of 35 cycles;
MMP-14	forward primer: 5' CGAGGTGCCCTATGCCTAC 3' reverse primer: 5' CTCGGCAGTCAAAGTGG 3'	95°C for 5 min, denaturation at 95°C for 25 s, annealing at 57°C for 30 s, extension at 72°C for 1 min and a total of 35 cycles;
iNOS	forward primer: 5' GATCAATAACCTGAAGCCC 3' reverse primer: 5' GCCCTTTTTTGCTCCATAGG 3'	95°C for 5 min, denaturation at 95°C for 15 s, annealing at 61°C for 30 s, extension at 72°C for 1 min and a total of 35 cycles;
eNOS	forward primer: 5' CACCCTCAGGTTCTGTGTGTT 3' reverse primer: 5' GTAGCCTGGAACATCTTCCGT 3'	95°C for 5 min, denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min and a total of 35 cycles;
tNOS	forward primer: 5' AATCCAGGTGGACAGAGACC 3' reverse primer: 5' TCCTTGAGCTGGTAGGTGCT 3'	95°C for 5 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 65 s and a total of 35 cycles.

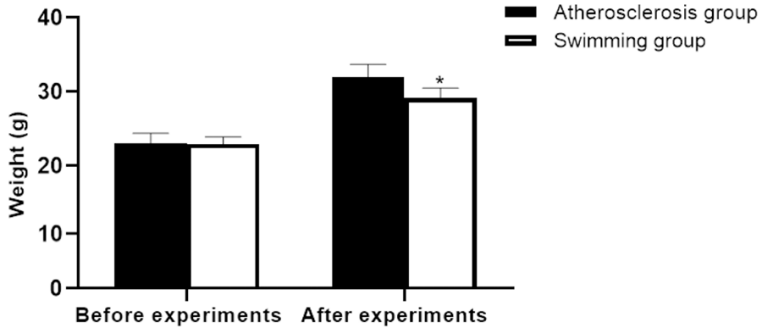

Figure 1. Comparison of weight change in mice between groups. Note: Compared with atherosclerosis group, *P<0.05.

Table 2. Comparison of serum lipid levels between groups (mmol/L)

Group	TC	TG	LDL	HDL
Atherosclerosis group	9.08±0.77	2.87±0.21	5.02±0.63	2.05±0.34
Swimming group	7.94±0.68	1.37±0.18	3.48±0.52	2.74±0.41
T value	3.509	17.150	5.962	4.097
P value	0.003	<0.001	<0.001	<0.001

Note: TC: Total cholesterol; TG: Triglyceride; LDL: Low density lipoprotein; HDL: High density lipoprotein.

overnight at 4°C. After washing, PVDF membrane was incubated with the secondary antibody (1:1000, ab6721, Abcam, USA). Finally, ECL reagent was added to develop the color and the integrated optical density of the target brand was detected using Bio-Rad image software. GAPDH served as an internal reference.

Statistical analysis

All data in this study were analyzed by SPSS 22.0 software. The measured data are presented as mean ± standard deviation (SD). Comparison between two groups was conducted by independent sample t test. Counted data are presented as a percentage, and comparison between two groups was performed by chi-square test. Statistical significance was set at P<0.05.

Results

Comparison of weight change between two groups

As shown in **Figure 1**, there were no significant differences in the weight before experiment between the two groups. After the experiment, mean weight in the swimming group was significantly lower than that of the atherosclerosis group (P<0.05).

Comparison of serum lipid levels between two groups

As show in **Table 2**, the serum levels of TC, TG and LDL in the swimming group were lower than those in atherosclerosis group, while the serum HDL level was higher (all P<0.01).

Comparison of H&E staining results among groups

The results of H&E staining are shown in **Figure 2**. In the atherosclerosis group, the aortic walls of mice were obviously thickened, and a disordered arrangement and proliferation of smooth muscle cells was found. In addition, the collagen fibers were significantly increased, large plaques were formed in the protruding aortic intima, and abundant foam cells accumulated in the atheromatous plaque. However, in the

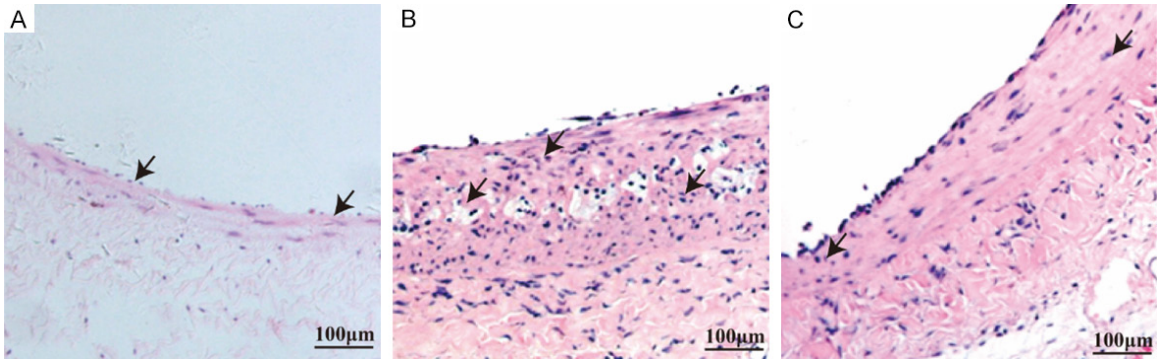

Figure 2. H&E staining for aortic atherosclerosis in mice (×100). A: Normal aorta; arrows indicate the normal aorta. B: Atherosclerosis group; arrows indicate the development of aortal atherosclerosis. C: Swimming group; arrows indicate the degree of aortic lesions which is lighter than that in the atherosclerosis group.

Table 3. Comparison of plaque area, fibrous cap thickness, plaque rupture rate, and vulnerable index between groups

Group	plaque area (μm^2)	fibrous cap thickness (μm)	plaque rupture rate	vulnerable index
Atherosclerosis group	33256±349	4.34±0.59	50% (25/50)	2.78±0.82
Swimming group	27876±305	6.58±0.74	20% (10/50)	1.39±0.58
T/χ^2 value	36.710	7.485	9.890	4.376
P value	<0.001	<0.001	0.002	<0.001

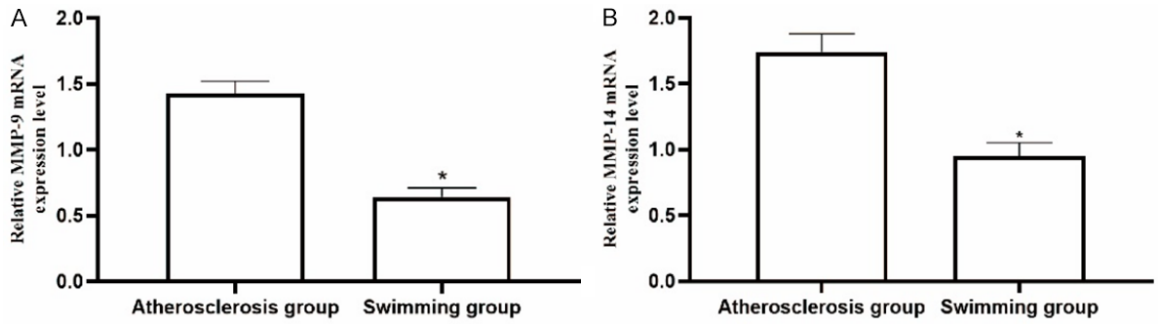

Figure 3. *qRT-PCR* analysis for MMP-9 and MMP-14 mRNA expression levels between two groups. A: MMP-9 mRNA expression; B: MMP-14 mRNA expression. Note: Compared with atherosclerosis group, *P<0.05; MMP-9: Matrix metalloproteinase-9; MMP-14: Matrix metalloproteinase-14.

swimming group, the aortic walls of the mice were slightly thickened. The structure of the aorta was relatively complete. The arrangement of smooth muscle cells and collagen fibers was relatively regular. The collagen fibers were sparse. A small amount of foam cells accumulated in the aortic intima.

Comparison of plaque area, fibrous cap thickness, plaque rupture rate, and vulnerable index between groups

As shown in **Table 3**, the plaque area, fibrous cap thickness, plaque rupture rate and vulnerable index in swimming group were 27876±305 μm^2, 6.58±0.74 μm, 20% and 1.39±0.58, respectively. The plaque area, plaque rupture rate and vulnerable index in the swimming group were significantly lower than those of the atherosclerosis group (all P<0.01), while the fibrous cap thickness in the swimming group wasobviously higher than that of the atherosclerosis group (P<0.001).

Comparison of mRNA expression levels of MMP-9 and MMP-14 between two groups

As shown in **Figure 3**, the mRNA expression levels of MMP-9 and MMP-14 in the swimming group were 0.64±0.07 and 0.92±0.10, respec-

Table 4. Comparison of eNOS, tNOS, and iNOS mRNA levels between two groups

Groups	eNOS	tNOS	iNOS
Atherosclerosis group	0.35±0.07	0.58±0.10	0.38±0.08
Swimming group	0.76±0.10	0.95±0.13	0.82±0.12
T value	10.620	7.134	9.648
P value	<0.001	<0.001	<0.001

Note: eNOS: endothelial nitric oxide synthase; tNOS: Total endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase.

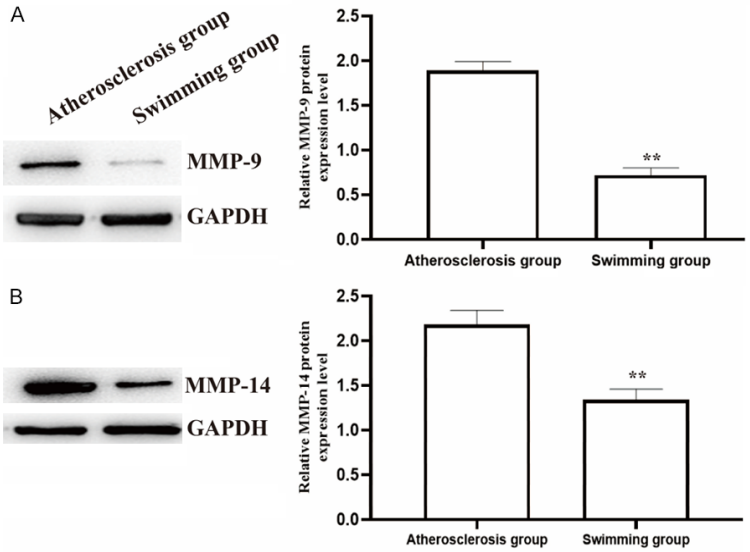

Figure 4. Western blot analysis for MMP-9 and MMP-14 protein expression levels. A: MMP-9 expression; B: MMP-14 expression. Note: Compared towith atherosclerosis group, **P<0.01; MMP-9: Matrix metalloproteinase-9; MMP-14: Matrix metalloproteinase-14.

Table 5. Comparison of MDA and SOD levels in plasma between groups

Group	MDA (nmol/L)	SOD (U/ml)
Atherosclerosis group	4.32±0.58	42.05±4.92
Swimming group	3.34±0.62	35.76±4.21
T value	3.650	3.072
P value	0.002	0.007

Note: MDA: Malondialdehyde; SOD: Superoxide dismutase.

tively, which were significantly lower than 1.43±0.09 and 1.74±0.14 in the atherosclerosis group (all P<0.05).

Comparison of expression levels of eNOS, tNOS, and iNOS mRNA between groups

As shown in **Table 4**, the expression levels of eNOS, tNOS, and iNOS mRNA in the atherosclerosis group were 0.35±0.07, 0.58±0.10 and 0.38±0.08, respectively, which were lower than the levels of 0.76±0.10, 0.95±0.13 and 0.83±0.12 in the swimming group (all P< 0.001).

Comparison of protein expression levels of MMP-9 and MMP-14 between groups

As shown in **Figure 4**, western blot results showed that protein expression levels of MMP-9 and MMP-14 in the swimming group were significantly lower compared to those of the atherosclerosis group (all P< 0.01).

Comparison of MDA and SOD levels between groups

Biochemical enzyme assays revealed that levels of MDA and SOD in the atherosclerosis group were 4.32±0.58 nmol/L and 42.05±4.92 U/ml, respectively, while the MDA and SOD levels in the swimming group were 3.34±0.62 nmol/L and 38.76±4.21 U/ml. The MDA level in the swimming group was signifciantly lower (P=0.002) while the SOD level was significantly higher (P=0.007) than those of the atherosclerosis group, as shown in **Table 5**.

Comparison of serum IL-6, Lp-PLA2, and TNF-α expression levels between groups

As shown in **Table 6**, ELISA results showed that serum levels of IL-6, Lp-PLA2, and TNF-α in swimming group were 59.38±10.67 pg/mL, 23.46±5.33 ng/mL, and 7.46±1.96 pg/ml, which were lower than those (83.41±12.18 pg/mL, 35.48±7.82 ng/mL and 11.39±2.85 pg/ml) of the atherosclerosis group (all P<0.01).

Discussion

Atherosclerosis (AS) is characterized by disorders of lipid metabolism and a pathologic state of many vessels. It can cause loss of productive

Table 6. Comparison of serum IL-6 and Lp-PLA2 levels between groups

Groups	IL-6 (pg/mL)	Lp-PLA2 (ng/mL)	TNF-α (pg/ml)
Atherosclerosis group	83.41±12.18	35.48±7.82	11.39±2.85
Swimming group	59.38±10.67	23.46±5.33	7.46±1.96
T value	4.693	4.016	3.593
P value	<0.001	<0.001	0.002

Note: IL-6: Interleukine-6; Lp-PLA2: Lipoprotein-associated phospholipase A2; TNF-α: tumor necrosis factor-α.

life and mortality. Atherosclerotic plaques are composed of lipid cores and fibrous caps. It was reported that the stability of atherosclerotic plaques depended mainly on the fibrous cap thickness and vulnerable plaques which are considered as high-risk plaques for thrombosis [14]. Some studies reported that the presence of multiple active vulnerable plaques suggested a poor prognosis [15]. In the current study, ApoE-deficient mice supplemented with a high-fat diet were used to construct an atherosclerosis model. An atherosclerotic plaque model was successfully established, which became the groundwork of this study. Our results indicated that the pathologic change of atherosclerotic plaques in the atherosclerosis group was significant. The vascular intima was significantly thickened and injured. A disordered arrangement and proliferation of smooth muscle cells was observed. The collagen fibers were severely broken. On the contrary, the plaque area in the swimming group was small and distributed superficially and sparsely. The collagen fibers were densely arranged and the vascular intima was slightly injured. Moreover, this study also showed that compared to the atherosclerosis group, the plaque area, plaque rupture rates, and vulnerable index in swimming group were significantly decreased while the fibrous cap thickness was significantly increased, indicating that the development of atherosclerotic plaques in ApoE-deficient mice was significantly inhibited in the swimming group. Swimming exercise reduced the plaque area, decreased the vulnerability of atherosclerosis plaques, stabilized the plaque, and protected the structure of the vascular intima.

The dyslipidemia in atherosclerosis is characterized by decreased HDL and increased TG, TC and LDL [16]. It was reported that the high levels of TC and LDL could lead to the development of atherosclerosis [17]. A high serum LDL level is widely considered a risk factor for promoting endothelial dysfunction, which is an important factor in development of atherosclerosis. Another study showed that a low serum HDL level was associated with an increased risk of atherosclerosis diseases [18]. In this study, we found that the weight of atherosclerotic rats conducting a swimming exercise was significantly decreased. Compared to the atherosclerosis group, the serum TC, TG, and LDL levels in the swimming group weredecreased and the serum HDL level was markedly increased. Taken together, this demonstrates that swimming had a preventive effect on the formation of atherosclerotic plaques by reducing the levels of TC, TG, and LDL and increasing the HDL level, which is similar to the study of Zhao et al. [19].

It is well known that atherosclerosis is characterized by lipid plaques, which are responsible for both physiologic and mechanical reductions in endothelial function. It was reported that endothelial dysfunction was an important indicator of the development of atherosclerosis. Some studies reported that endoplasmic reticulum stress involved in the initiation and progression of atherosclerosis and modulated endothelial dysfunction by regulating the endothelial nitric oxide synthase (eNOS) signaling pathway [20]. Another study showed that atherosclerosis is correlated with reductions in NO bioavailability. NO, as a potent oxidant, is catalyzed by inducible nitric oxide synthase (iNOS) and can regulate inflammation [21]. Hong et al. reported that exercise training improved endothelial function in coronary arterioles of ApoE-deficient mice through NO-dependent signaling pathways [10]. These NO-mediated beneficial effects of exercise on function of vascular in atherosclerosis confirm previous studies [5]. The results of this study showed that swimming exercise could rescue the endothelial dysfunction in aorta atherosclerosis of ApoE-deficient mice through regulating the expression levels of eNOS, iNOS, and tNOS, indicating that swimming exercises may inhibit the development of atherosclerosis by improving endothelial function.

Oxidative stress also participates in the development of atherosclerosis [22]. In the early phase of atherosclerosis, the inflammatory

cells release a large amount of ROS, leading to vascular damage, and ultimately enhancing the progression of atherosclerosis [23]. SOD, a well-known antioxidant enzyme, can convert the highly-reactive radicals into less reactive ones or form conjugates with some harmful compounds [24]. MDA is considered as a key biomarker of oxidative stress and a key mediator of atherosclerotic inflammation [25]. Our results showed that compared to the atherosclerosis group, swimming exercise could significantly reduce the expression level of MDA but promote the level of SOD. Zatiye Ayça et al. reported that exercise may be beneficial to cardiovascular hemodynamics through antioxidant effects [26]. Li et al. revealed that exercises could effectively improve the antioxidant ability of red blood cells in breast cancer patients [27]. Taken together, swimming exercise can decrease oxidative damage and improve atherosclerotic lesions.

Atherosclerosis is an inflammatory disease since inflammatory cells play an important role in atherosclerosis development. Lipoprotein-associated phospholipase A2 (Lp-PLA2) secreted by lymphocyte and macrophage, played a critical role in the progression of atherosclerosis. Lp-PLA2 expression not only worsened the disorders of lipid metabolism and glucose, but also promoted the inflammatory effect in atherosclerosis [28]. It was reported that the decrease in Lp-PLA2 levels was associated with reduced severity of atherosclerosis in the aorta of rats, through inhibiting macrophage infiltration [29]. Moreover, IL-6 and TNF-α expression was deeply involved in the chronic inflammatory response correlated with atherosclerosis. Some studies reported that IL-6 secreted by inflammatory cells could induce the activation of endothelial cells and the dedifferentiation of smooth muscle cells, which eventually results in the reduction and rupture of collage fibrous caps [30]. Another study showed that the proinflammatory mediator TNF-α had atherogenic properties by mainly inducing the early development of atherosclerotic plaques [31]. This study showed swimming exercises could alleviate atherosclerosis progression through inhibiting the expression levels of IL-6, TNF-α, and Lp-PLA2, which is similar to other studies [32, 33].

Matrix reconstruction persists through the development of atherosclerotic plaques. Matrix metalloproteinases belong to the family of decomposable extracellular matrix enzymes, which are involved in the reconstruction and rearrangement of vascular matrix. It was reported that matrix metalloproteinases could lead to the instability or rupture of fibrous caps in atherosclerotic plaques [34]. MMP-9 is excreted by smooth muscle cells and inflammatory cells, and MMP-9 expression can increase the progression of atherosclerosis, thus is considered as a major factor responsible for the instability of atherosclerotic plaques [34]. Moreover, MMP-14, as a membrane protein, degrades the extracellular matrix protein. Some studies reported that multiple atherosclerotic factors can enhance MMP-14 expression [35]. Many studies suggested that MMP-9 and MMP-14 played important roles in affecting the stability of atherosclerotic plaques [36]. Similarly, in this study, the results revealed that swimming could significantly decrease the expression of MMP-9 and MMP-14 in atherosclerotic plaques, indicating that swimming may decelerate the development of atherosclerotic plaque by increasing the stability of plaque.

This study has some limitations. First, the sample size was inadequate. Second, mice, instead of patients, were evaluated. Third, the mechanisms of signal transduction system in the benefits brought by swimming exercises were not measured. Therefore, conducting a multicenter, randomized controlled and large-sample clinical study on swimming exercises to explore the roles of swimming in development of atherosclerosis is warranted to precisely illustrate its preventive and therapeutic effect on atherosclerosis.

In conclusion, swimming exercise can potently inhibit the development of atherosclerotic plaque in ApoE-deficient mice induced by high-fat diet by suppressing lipid levels, inhibiting the expression levels of MMP-9 and MMP-14, decreasing the inflammatory factors, improving endothelial function, and reducing the oxidative stress.

Disclosure of conflict of interest

None.

Address correspondence to: Jian Chi, China Basketball College, Beijing Sport University, No. 48 Xinxi Road, Beijing 100084, China. Tel: +86-010-6298-9028; Fax: +86-010-62989028; E-mail: chijian_bjsport@21cn.com

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