Effect of Swimming on Lipid Metabolism, LDL Oxidation Resistance, and Atherogenesis in Apolipoprotein E Knock-Out Mice

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¹Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, MG, Brazil. ²Institute of Genetics and Biochemistry, Federal University of Uberlândia, MG, Brazil. ³University Center of Belo Horizonte UnibH, MG, Brazil. ⁴Laboratory of Exercise Physiology, School of Physical Education, Physiotherapy and Occupational Therapy, Federal University of Minas Gerais, MG, Brazil.

ABSTRACT
Fonseca TR, Botelho FV, De Lima DC, Ferraz FO, Dos Santos LAVDR, Mendes TT, Lima NRV, Alvarez-Leite JI. Effect of Swimming on Lipid Metabolism, LDL Oxidation Resistance, and Atherogenesis in Apolipoprotein E Knock-Out Mice. JEPonline 2011;14(4):49-58. This study evaluated the effects of 6 weeks of swimming on lipid metabolism, oxidation resistance, and atherogenesis in 6-week-old female apolipoprotein E-deficient mice (apoE⁻/⁻). The mice were divided into a control group (n=7) and a treatment group (n=7). Mice in the treatment group were required to swim 10 min·d⁻¹, 5 d·wk⁻¹ for 6 weeks (which was increased by 10 min·d⁻¹ until the mice swam continuously for 1 hr). Plasma total cholesterol was evaluated before and after the experiment. Liver and fecal lipids, lipid oxidation resistance, and atherosclerotic area of thoracic and abdominal aorta were evaluated after euthanasia. Body weight, lipid metabolism, and lipid oxidation resistance were similar in both groups. Relative index was statistically decreased in the experimental group, thus suggesting that the apoE⁻/⁻ mice that underwent the swimming exercise exhibited decreased atherosclerotic lesions in the aorta. However, the decrease was not due to changes in lipid metabolism or lipid oxidation resistance profile.

Key Words: Physical Exercise, Atherosclerosis, Lipid Metabolism
INTRODUCTION

Physical exercise is currently recommended as a nonpharmacologic strategy for prevention and/or treatment of cardiovascular diseases (20,22). Research from numerous sources indicate that regular exercise is helpful in reducing and/or reversing the progression of atherogenesis, lowering blood pressure, increasing HDL cholesterol, diminishing the LDL cholesterol concentration in plasma, and decreasing body weight (5,9,14,20-22). Yet, despite the association between exercise and reduced risks of cardiovascular morbidity and mortality, the mechanisms by which physical activity promotes a healthier lifestyle are still unknown.

Lipoproteins have been associated to coronary diseases. Low-density lipoprotein cholesterol and HDL have contrary actions in atherogenesis. High-density lipoprotein cholesterol presents protective properties while LDL favors the formation of atherosclerotic lesions (4,7). Oxidative stress is another cause of atherosclerosis. Oxidized lipids such as oxidized LDL (LDLOx) have pro-atherogenic effects. LDLOx is internalized and accumulates in macrophages, turning them into foam cells that remain in subendothelial space. Those events culminate in atheroma development (2,19).

Regular physical exercise increases the oxidative stress. At the same time, antioxidative defenses are induced, as some in vivo studies have demonstrated (9,10,14). Therefore, regular physical exercise can induce an antioxidative response in arteries, being beneficial for preventing atherosclerotic disease. In fact, since apolipoprotein-E deficient mice (apoE^{-/-}) are susceptible to atherosclerosis and spontaneously develop atherosclerotic lesions on a standard chow diet (11), this study evaluated the effect of 6 weeks of swimming on lipid metabolism, oxidation resistance, and atherosclerosis in 6-week-old female apolipoprotein E-deficient mice (apoE^{-/-}).

METHODS

Experimental design

Six-week-old female apoE knock-out mice were maintained in collective cages in an appropriate room with controlled temperature and a 12-h light cycle. Mice were distributed based on their initial weight and plasma cholesterol into two groups: control (CT; n=7); swimming (SW; n=7). The trained mice underwent a 6-week swimming program. Animals were fed chow diet and water ad libitum for the duration of the experiment. The protocol was approved by the Animal Care Committee of Federal University of Minas Gerais, Brazil (CETEA # 055/2003).

Animal body weight was measured on an animal scale at the beginning, and at 2, 4, and 6 weeks after. Blood samples were collected on the caudal vein using heparinized capillary tubes for total cholesterol determination at the beginning and 6 weeks after. At the end of the 6th week, after 8 to 10 hrs of fast and under anesthesia (130 mg/kg of Ketamine and 0.3 mg/kg of Xylazine), all animals were euthanized, the blood was drawn from the axillary region and plasma was separated by centrifugation at 2500 rpm for 15 min. The plasma collected at the 6th week was used to determine total cholesterol as well as to evaluate the lipid oxidation resistance using the conjugated diene assay. Liver and feces were collected at the end of experiment to determine total lipids and cholesterol in the liver and OH-a-sterols in feces. The aorta was collected to assess atherosclerotic lesions.

Procedures

Exercise protocol

The swimming protocol was based on the one described by Nakao et al. (12) and Oh-ishi et al. (13). The animals in the treatment (swim) group underwent a 6-week swim program 5 times per week in
water at 34°C. The program consisted of a period of acclimatization on the first week, beginning at 10 min of swimming, which was further extended by 10 min daily until the animals swam continuously for 1 hr. On the subsequent weeks, the animals swam for 1 hr 5 times per week. After the exercise routine, the animals were dried with towels and remained in their cages under light until they were totally dry.

**Plasma total cholesterol**
Blood samples were collected at the beginning and at 6 weeks using heparinized capillary tubes and centrifuged at 2500 rpm for 15 min. The cholesterol concentration was determined by the Cholesterol Oxidase enzymatic assay according to Allain et al. (1) using a commercial cholesterol determination kit (KATAL, Belo Horizonte, MG, Brazil). Samples were diluted at a 1:100 ratio and the assay was performed using 96 wells microplates and further read on a ELISA plate reader at 500 nm. The cholesterol concentration was derived from the equation obtained from the cholesterol standard curve.

**Liver and fecal lipids, liver cholesterol, and fecal OH-a-sterols**
Liver and fecal lipids were extracted using the technique described by Folch et al. (6): one hundred milligrams of liver or feces were minced in the presence of 1.9 ml of a chloroform/methanol 2:1 solution in a LABO STIRRER LR 41 B homogenizer for 7 min. Next, 400 µL of methanol were added to the tubes and they were centrifuged at 3000 rpm for 10 min. The pellet was discarded and the supernatant was homogenized with 800 µL of chloroform and 640 µL of NaCl 0.73%. Next, the samples were centrifuged again at 3000 rpm for 10 min and the upper phase was discarded. The inner wall of the tubes was washed 3 times with 300 µL of FOLCH solution (chloroform 3%, methanol 48%, water 47%, and 2% of NaCl 0.29%). The lipids extracted were dried at 37°C for 24 hrs, frozen at -20°C and resuspended in 1.5 ml of isopropanol for determining total cholesterol. The same method for determining total cholesterol in plasma was applied to determine cholesterol concentration in the liver and OH-a-sterols concentration in feces, using different dilution ratios.

**Lipid oxidation resistance**
The kinetics of copper-induced lipid oxidation of plasma preparations (diluted 100-fold in PBS) was monitored at 245 nm at 37°C for 4 hrs and used as a measure of resistance to lipid peroxidation. The final concentration of copper was 30 µmol/L (18). The lag phase (t lag) of conjugated diene formation (product of lipid oxidation) was determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption (8,18).

**Thoracic and abdominal aortic lesions**
After removal of the surrounding adventitial fat tissue, thoracic and abdominal aortas from the arch to renal bifurcation were opened longitudinally, fixed in formal-sucrose, and stained with Sudan IV. Then aortas were extended upon glass slides and scanned at high resolution. Images obtained were submitted to morphometric analyses using an image analyzer (Image pro plus, version 4.5.1). The extent of atherosclerosis was determined by morphometry of the areas stained by Sudan IV (15). A relative index (area of the lipid deposition over the total area of the aorta in each image) was assigned (3). The investigator performing the analysis was blinded to the animal condition.

**Statistical Analyses**
The Lilliefors test was used to verify the normality of the samples, and the Bartlett test was used to verify the homogeneity of variances. The statistical analysis methods used were Student's t test to compare treatments and ANOVA two-way with repeated measurements for compare responses along the treatments. The data shown displays the mean and standard error (SE) of each group. The difference was considered statistically significant when P=0.05.
RESULTS

Mice in both the control group and the treatment (swim) group showed an increase in body weight (Figure 1) and a reduction in plasma total cholesterol (Figure 2) after 6 weeks of regular exercise. There was no difference between the groups.

Figure 1. Body weight (g) of apoE−/− mice during the 6 weeks of experiment. CT = control group, SW = swimming group. Vertical lines represent the standard error (SE) from the mean. #Statistically different from “0” in the same group (P=0.05).

Figure 2. Plasma total cholesterol (mg/dL) of apoE−/− mice before and after 6 weeks of experiment. CT = control group, SW= swimming group. Vertical lines represent the standard error (SE) from the mean. #Statistically different from “0” in the same group (P=0.05).

Liver lipid and cholesterol concentration and fecal excretion of lipids and OH-a-sterols were analyzed to evaluate the influence of physical exercise on lipid metabolism. No significant difference could be detected between the groups when total lipids and cholesterol in liver and OH-a-sterols excretion were analyzed (Table 1).
Table 1. Liver and fecal lipids, liver cholesterol, and fecal OH-a-sterols in apoE⁻/⁻ mice.

<table>
<thead>
<tr>
<th></th>
<th>CT (n=7)</th>
<th>SW (n=7)</th>
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</thead>
<tbody>
<tr>
<td><strong>Liver lipids</strong> (mg/g liver)</td>
<td>10.2 ± 0.73</td>
<td>11.2 ± 1.12</td>
</tr>
<tr>
<td><strong>Liver cholesterol</strong> (mg/g liver)</td>
<td>7.04 ± 0.41</td>
<td>6.49 ± 1.15</td>
</tr>
<tr>
<td><strong>Fecal lipids</strong> (mg/g feces)</td>
<td>4.21 ± 0.54</td>
<td>4.07 ± 0.22</td>
</tr>
<tr>
<td><strong>Fecal OH-a-sterol</strong> (mg/g feces)</td>
<td>3.99 ± 0.49</td>
<td>3.73 ± 0.21</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard error.

Although no quantitative difference was observed in plasma, liver and fecal lipid content, it was hypothesized that regular exercise could improve the antioxidant status of plasma in the mice. Upon evaluating the kinetic of plasma lipid oxidation, lag time phase for both groups was similar (Figure 3).

![Figure 3. Plasma lipid oxidation resistance of apoE⁻/⁻ mice. CT = control group, SW= swimming group. Vertical lines represent the standard error (SE) from the mean.](image)

Even though no changes in lipid metabolism and lipid oxidation resistance were related to physical exercise, when the thoracic/abdominal aorta was analyzed, a reduction in lesion area was observed in mice which performed the exercise routine (Figure 4).
Figure 4. (A) Atherosclerotic lesion in the thoracic and abdominal aorta expressed as percentage of the Sudan IV positive area of internal surface. Vertical lines represent the standard error (SE) from the mean. *Statistically different from CT group (P=0.05). (B) Representative aortas from apoE−/− mice from Control group and (C) Swimming group. B and C scale = mm.
DISCUSSION

Cardiovascular diseases are an important cause of death worldwide (19) and physical exercise is one of the most effective strategies for reducing the risk of dying of heart disease (20,22). In fact, exercise is a main component of cardiac rehabilitation along with diet, smoking cessation, and blood pressure and cholesterol management. Thus, the purpose of this study was to investigate the effect of regular exercise in lipid metabolism and atherosclerosis in apoE\(^{-/-}\) mice, given that they are susceptible to atherogenesis.

First, we investigated whether 6 weeks of swimming, 1 hr·d\(^{-1}\), 5 d·wk\(^{-1}\), could alter body weight. Throughout the course of the experiment, the changes in body weight were similar between the treatment (swim) group and the control group. Pynn and colleagues (17) have also demonstrated a similar profile when they evaluated body weight in trained and untrained apoE\(^{-/-}\) mice (treadmill, 1 hr a day, 15 m·min\(^{-1}\), 5 d·wk\(^{-1}\) for 6 weeks). Accordingly, Okabe et al. (14) have shown the same result of increase in body weight in trained and untrained apoE\(^{-/-}\) mice (swimming, 45 min·d\(^{-1}\), 3 d·wk\(^{-1}\) for 8 and 16 weeks). On the other hand, Pellegrin and colleagues (16) demonstrated that apoE\(^{-/-}\) mice submitted to exercise (swimming, 50 min·d\(^{-1}\), 5 d·wk\(^{-1}\) for 9 weeks) have shown a reduction in body weight when compared to the control group. Nevertheless, this result may be a consequence of different protocols of exercise and the specific diet used in the study.

A crucial parameter involved in atherogenesis is cholesterol metabolism, especially plasma cholesterol levels (4,7). The findings in the present study indicate that regular exercise in apoE\(^{-/-}\) mice did not change lipid metabolism. Interestingly, the concentration of total cholesterol in plasma, liver and OH-a-sterols in feces and lipid in liver and feces are comparable between the groups. Analogous results were found by Pynn et al. (17), in which total cholesterol, triglycerides, and HDL cholesterol were not different in trained and untrained. Other authors published results that are in accordance with ours, such as Pellegrin et al. (16) who found that regular physical exercise did not cause an alteration in the plasma lipid profile (total cholesterol, HDLc, and triacylglycerols) of apoE\(^{-/-}\) mice under high-cholesterol diet.

Another important mechanism to reduce atherogenesis is blocking or preventing oxidative stress, since oxidation of LDL molecules contributes to the formation of the atherosclerosis lesions (2,19). However we have not found any increase in the anti-oxidative potential in plasma microenvironment in the swimming group. Finally, given the importance of the relative index, regular exercise appears to have reduced the aortic atherosclerosis lesion in apoE\(^{-/-}\) mice. This finding is in accordance with other studies that analyzed the effect of exercise in atherogenesis in apoE\(^{-/-}\) mice (14,17).

Taken together, the findings suggest that the reduction in the atherosclerosis lesion promoted by physical exercise in apoE\(^{-/-}\) was not related to changes in plasma, hepatic and fecal lipids or plasmatic tlag time. We hypothesized that the mechanisms responsible for atherosclerosis prevention through physical exercise involves other parameters, such as reduce endothelial dysfunction.

The reactive oxygen species (ROS) contribute to endothelial dysfunction and an important source of vascular ROS is the NADPH oxidase. However, nitric oxide keeps the vessels surface notthrombogenic and non-adherent to platelets and leukocytes by inhibiting the arterial wall cells adhesion and migration of muscle cells (21, 23). Nevertheless, exercise can decrease vascular NADPH oxidase activity, improving nitric oxide activity and reducing the atherosclerotic lesions (9, 14).
CONCLUSIONS

Physical exercise promotes the reduction of atherosclerotic lesion in aorta of apoE\(^{-/-}\) mice, but this reduction is not due to lipid metabolism and LDL resistance to oxidation.

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