Original Article

Improvements of Atherosclerosis and Hepatic Oxidative Stress are Independent of Exercise Intensity in LDLr$^{-/-}$ Mice

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***Background*: Cardiovascular diseases are the main causes of death in the Western world and are manifested by atherosclerosis. Depending on its intensity, regular aerobic exercise may be either beneficial or harmful to the atherosclerosis process.**
***Aim*: The aim of this study was to verify the effects of aerobic exercise training of different intensities on the profile of atherosclerotic lesions and serum lipid, and in the hepatic oxidative balance of low-density lipoprotein receptor-deficient (LDLr$^{-/-}$) mice previously developed with atherosclerosis.**
***Methods*: All animals were submitted to a three-month high-fat and high-cholesterol diet regime. The animals were then randomly divided into no exercise (G1, $n=9$), low-intensity aerobic exercise (G2, $n=10$, 8 weeks of treadmill running, 30 min/day^{-1} at 8-10 m/min^{-1}) and moderate-intensity aerobic exercise (G3, $n=10$, 8 weeks of treadmill running, 30 min/day^{-1} at 10-16 m/min^{-1}) groups. Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG), and oxidative damage (protein carbonyls and lipid hydroperoxides) were measured. The activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the liver tissue was assessed.**
***Results*: G2 (0.015 ± 0.005 cm^2) and G3 (0.014 ± 0.001 cm^2) presented lower aortic fat deposition than G1 (0.039 ± 0.005 cm^2). G2 and G3 exhibited higher HDL-C, TG and CAT activity, but lower lipid peroxidation and carbonyl protein than G1. SOD values were higher in G3 than G2 and G1, and GPx was higher in G2 than in G3 and G1.**
***Conclusions*: Our protocols of low- and moderate-intensity aerobic exercise training (30 min daily for 8 weeks) induced similar benefits in LDLr$^{-/-}$ mice with atherosclerosis.**

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Key words; Aerobic exercise, Physical activity, Lipid profile, Atherosclerotic plaque, Antioxidant

Introduction

Atherosclerosis is characterized by a chronic inflammatory and degenerative process which affects the vessels by the accumulation of lipid inflammatory cells and fibrous elements in the subendothelial space[1)]. Mouse models of atherosclerosis have proved to be useful to investigate the development and progression of atherosclerotic lesion, and such models have been extensively discussed in reviews[2)]. Low-density lipoprotein receptor-deficient (LDLr$^{-/-}$) mice are a model of familial hypercholesterolemia due to one of the mutations affecting the LDLr, and the plasma lipoprotein profile resembles that of humans[3)]. In addition, the severity of hypercholesterolemia and ath-

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erosclerotic lesions in LDLr$^{-/-}$ mice can be accelerated by feeding high-fat and high-cholesterol diets[4]. Moreover, this model permits the manipulation of atherosclerotic stages, avoiding the possible risks of clinical trials and thus providing important investigative insights into the physiopathology of the disease.

The control of oxidant status and metabolism of lipoproteins in the body has become an important tool for combating atherosclerosis. Polyunsaturated fatty acids present in cell membranes may be a target of reactive oxygen species (ROS) triggering chemical reactions, so-called lipid peroxidation[5]. The liver produces enzymes with great antioxidant capacity[6, 7] being a major location for LDL oxidation by cytochrome P450[8]. Thus, the liver oxidative balance is important for understanding atherosclerosis mechanisms and has been studied in experimental designs[9-11]. In addition, the cellular oxidative process may also be involved in the oxidation of proteins, which may be related to the processes of aging and modification of apoprotein constituents of plasma lipoproteins. When oxidized, proteins become targets of degradation by endogenous proteases, increasing the risk of cardiovascular diseases[1].

Aerobic exercise training leads to beneficial changes in atherosclerosis, oxidative stress and lipid profile both in humans and in animal models[12-14], although the mechanisms are not completely understood. Regular aerobic exercises of different intensities are recommended as non-pharmacological strategies for the prevention and treatment of cardiovascular diseases[15-17]; however, there is a lack of studies addressing aerobic exercise intensity in this field of investigation. Such an issue is of relevance as, depending on its intensity, exercise may have either harmful or beneficial effects. For example, exercise may cause an increase in free radical production[18] or improve the antioxidant enzymatic defense system[19].

Therefore, the aim of this study was to verify the influence of aerobic exercise training of different intensities on the profile of atherosclerotic lesions and serum lipid, and on the hepatic oxidative balance of LDLr$^{-/-}$ mice previously developed with atherosclerosis.

Methods

Study Design, Animals and Exercise Protocols

Twelve-week-old male C57/Bl/6J LDLr$^{-/-}$ mice (n=29, initial body weight 23.80±1.91 g) from the Federal University of Viçosa animal facilities were fed a hyperlipidic and hypercholesterolemic diet (cholesterol=1.5 g/kg; total fat=210 g/kg) for 3 months.

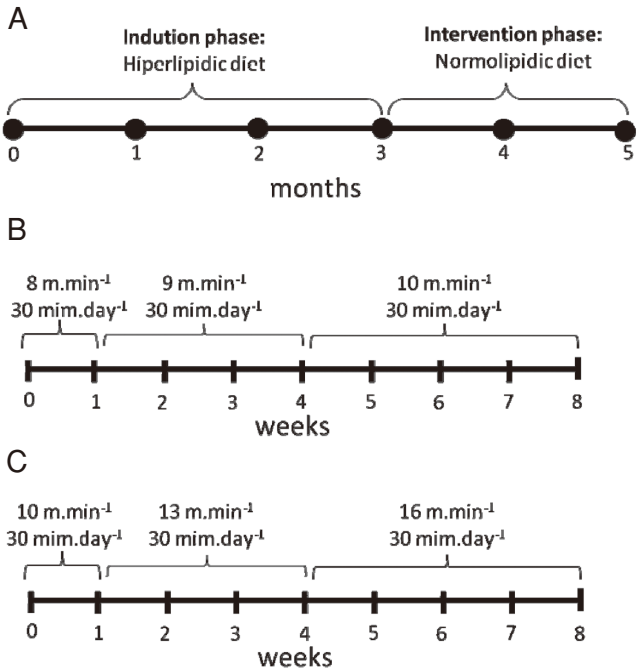

Fig. 1. Study design (A), low-intensity exercise protocol (B) and moderate-intensity exercise protocol (C).

This phase was referred to as the atherosclerosis induction phase according to Ramachadran *et al.*[13] (**Fig. 1A**). The animals then received a normolipidemic commercial diet (cholesterol=0.002 g/kg; total fat=45 g/kg) for 2 months (intervention phase, Figure 1A) and randomly divided them into 3 groups: no exercise (G1; n=9), low-intensity exercise (G2; n=10) and moderate-intensity exercise (G3; n=10).

G2 and G3 animals ran on a motor-driven treadmill (Insight Equipamentos Científicos, Ribeirão Preto, Brazil) for 8 weeks. Exercise training consisted of a progressive running protocol of 30 min/day, five days/week (Monday to Friday) at speeds of 8 to 10 m/min^{-1} (low-intensity exercise, G2, **Fig. 1B**) or 10 to 16 m/min^{-1} (moderate-intensity exercise, G3, **Fig. 1C**). Exercise intensity was determined by adjusting the running speed to the oxygen consumption according to Høydal *et al.*[20]. This study was approved by the institutional ethics committee (protocol nº 46/2008).

Measurement of Citrate Synthase (CS) Enzyme Activity in Skeletal Muscle

At euthanasia, a fragment of the gastrocnemius (red portion) was harvested and stored at −80℃. The muscle fragment was later weighed and homogenized with a glass homogenizer on ice in a solution of Tris-HCl (100 mmol/L^{-1}), at a constant weight/volume rate. The homogenate was then added to a reaction

mixure containing Tris-HCl (100 mmol/L^{-1}), dithiobis (2-nitrobenzoic acid) (1.0 mmol/L^{-1}) and acetyl coenzyme A (3.9 mmol/L^{-1}). After the addition of oxaloacetate (1.0 mmol/L^{-1}), absorbance was read at 412 nm for a two-minute period. The mean absorbance in variation/min^{-1} was recorded for each sample and the activity of CS was calculated using an extinction coefficient of 13600 mol/L^{-1}.cm^{-1} according to Alp *et al.*[21].

Assessment of Lipid Deposition (Aorta Lesion Area)

Lipid deposition was measured in the aortic arch and in the thoracic and abdominal aortas using en face analysis with Sudan IV dyes[22]. At euthanasia, the aortas were dissected and all the adventitia from the aortic valve to the iliac bifurcation was carefully removed. The aorta was then opened longitudinally and fixed for 12 hours in a formalin-sucrose solution (4% paraformaldehyde, 5% sucrose, 20 μmol/L BHT, and 2 μmol/L EDTA, pH 7.4) at 4℃. The aorta was then placed in 70% ethanol solution for five minutes. Subsequently, it was stained for 10 minutes under agitation in a solution containing 0.5% Sudan IV, 35% ethanol and 50% acetone, and then bleached in 80% ethanol solution for five minutes. The stained aortas were photographed using an 8.1 megapixel digital camera, with the macro function turned on and with distance, zoom and luminosity controlled. Analyses were performed using Image-Pro Plus software. Pixels were converted into square centimeters using a standard microscopic scale in the same condition that the aortas were submitted, according to the software. The sum of the areas of atherosclerotic lesions (where there was lipid accumulation) was calculated by the software and the results were expressed in square centimeters. To ensure that there was no differences in the total size of the aorta among animals, this area was also measured, and no statistical differences ($p=0.96$) were observed among groups.

Determination of Lipid Profile

Serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were determined using commercial kits (Bioclin, Brazil) for an enzymatic colorimetric method and an automatic Cobas analyzer.

Biochemical Analysis of the Liver Tissue

The liver was dissected at euthanasia and stored at −80℃. Superoxide dismutase (SOD) activity was determined according to Bannister and Calabrese[23]. Briefly, a fragment of liver tissue was homogenized in glycine buffer (50 mmol.L^{-1}, pH 10.1) and enzymatic activity was estimated by the inhibition of adrenaline auto-oxidation using spectrophotometric measurements (480 nm).

To determine catalase (CAT) activity, a portion of liver tissue was homogenized in 50 mmol/L^{-1} phosphate buffer and the resulting suspension was centrifuged at 3000 g and 4℃ for 10 minutes. The supernatant was used to measure enzymatic activity. CAT activity was determined by the decay rate of hydrogen peroxide (10 mmol/L^{-1}) read on a spectrophotometer at 240 nm, according to Aebi[24].

For glutathione peroxidase (GPx) activity, a fragment of the liver was homogenized in 50 mmol/L^{-1} phosphate buffer and the resulting suspension was centrifuged at 3000 g at 4℃ for 10 minutes. The supernatant was analyzed by a spectrophotometer using reduced glutathione, glutathione reductase and sodium azide. GPx activity was determined from the decay rate of NADPH (molar extinction coefficient = 6220) using a spectrophotometer (340 nm), as described by Flohé and Günzler[25].

Lipid hydroperoxide was detected as described previously by Jiang *et al.*[26]. When Xylenol Orange binds to ferric ions, it produces a blue-purple chromophore with extinction coefficient of 1.5×10^{-4} M^{-1} cm^{-1} at 560 nm. The hydroperoxide concentration can be estimated since the extinction coefficient of hydroperoxide is 4.3×10^{-4} M^{-1} cm^{-1} at 560 nm.

Oxidative damage to proteins was assessed by determining carbonyl groups based on the reaction with dinitrophenylhydrazine, as described by Levine *et al.*[27]. Briefly, liver proteins were precipitated by adding 20% trichloroacetic acid to dinitrophenylhydrazine. The samples were re-dissolved in guanidine hydrochloride and the carbonyl content was spectrophotometrically determined at 370 nm using a molar absorption coefficient of 22.000 mol/L^{-1}.cm^{-1}.

The amount of proteins of CAT, SOD, GPx, hydroperoxides, protein carbonyls and CS were measured by the method of Lowry *et al.*[28].

Statistical Analysis

The Kolmogorov-Smirnov test for normality was initially performed for all data. Groups were compared using one-way ANOVA followed by the post-hoc Duncan test as appropriate. A p value less than 0.05 was considered significant. All data are presented as the mean ± standard error (SE). Data analyses were performed using the statistical software program SPSS, version 12.0 for Windows.

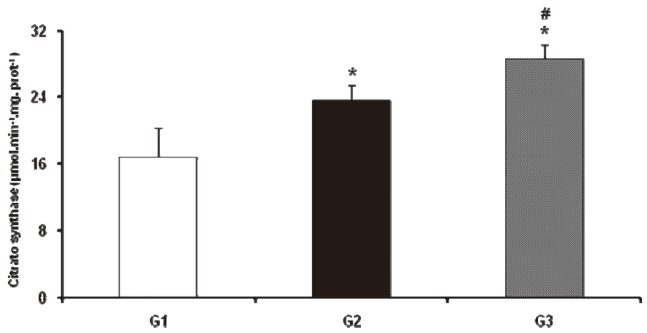

Fig. 2. Citrate synthase activity in LDLr$^{-/-}$ mice.

G1, non-exercised (n=9); G2, low-intensity aerobic exercise (n=10); G3, moderate-intensity aerobic exercise (n=10). *, denotes statistical difference from G1 and G2 ($p<0.05$). #, denotes statistical difference from G2 ($p<0.05$). Data are the mean ± SE.

Results

Body Weight

Animals from G1 exhibited a significant body weight gain throughout the experimental period (24.59 ± 0.39 g to 27.65 ± 0.64 g, $p<0.05$); however, it was not observed in animals from G2 (24.58 ± 1.15 g to 26.41 ± 0.94 g, $p>0.05$) and G3 (24.57 ± 1.14 g to 25.34 ± 0.69 g, $p>0.05$) groups.

Citrate Synthase Activity

CS activity was higher in the gastrocnemius' red portion of animals from G2 (23.61 ± 1.85 μmol/min^{-1}/mg^{-1} of protein) than from G1 (16.87 ± 3.47 μmol/min^{-1}/mg^{-1} of protein) (**Fig. 2**). Likewise, CS activity was higher in G3 (28.60 ± 1.69 μmol/min^{-1}/mg^{-1} of protein) than in G2 and G1.

Atherosclerotic Lesion Area

Animals from G2 (0.015 ± 0.005 cm^2) and G3 (0.014 ± 0.001 cm^2) showed lower total lesion area ($p<0.05$) than those from G1 (0.039 ± 0.005 cm^2) (**Fig. 3A**). In addition, animals from G2 and G3 had a lower ($p<0.05$) percentage of lesion area (total lesion area/total aorta area) than those from G1 (**Fig. 3B**). **Fig. 3C** shows images of atherosclerotic lesions in the aorta of representative animals from the experimental groups.

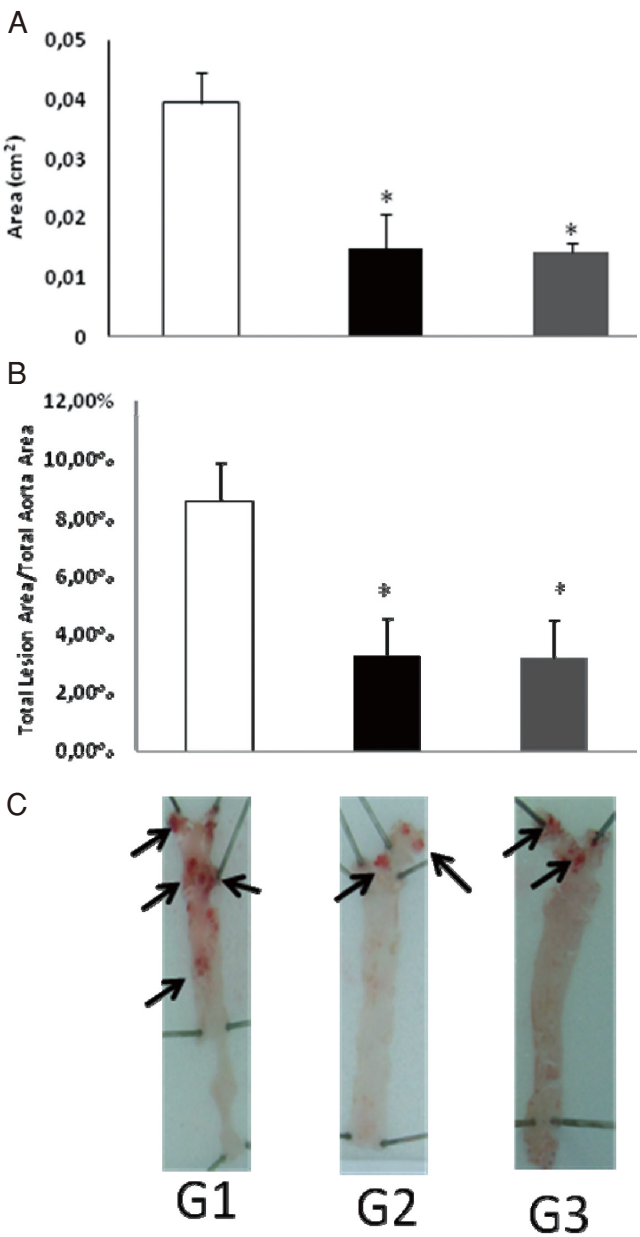

Fig. 3. Total lesion area (A), percentage of lesion area (B) and representative illustrations of lesion area (C) in the aorta of LDLr$^{-/-}$ mice.

G1, non-exercised (n=9); G2, low-intensity aerobic exercise (n=10); G3, moderate-intensity aerobic exercise (n=10). *, denotes statistical difference from G1 ($p<0.05$). Data are the mean ± SE.

Serum Lipid Profile and Hepatic Oxidative and Antioxidative Balance

Serum TC did not differ among groups (**Table 1**); however, HDL-C and TG showed higher values in G2 and G3 than G1. Animals from G3 exhibited significantly increased values of SOD than those from G2 and G1. CAT activity increased in animals from G2 and G3 as compared to G1 animals. Animals from G2 showed a significant increase in GPx activity compared with those from G3 and G1. Hepatic lipid peroxidation in animals from groups G2 and G3 was lower than in those from G1. Likewise, the oxidation of proteins in the liver tissue of animals from G2 and G3 was lower than in those from G1.

Table 1. Serum lipid levels and hepatic oxidative and antioxidative balance in LDLr$^{-/-}$ mice

	G1	G2	G3
HDL-C (mg.dL^{-1})	60.5 ± 3.83^{a}	75.33 ± 4.76^{b}	69.33 ± 4.61^{b}
TC (mg.dL^{-1})	308.87 ± 22.75^{a}	318.44 ± 15.83^{a}	331.5 ± 25.77^{a}
TG (mg.dL^{-1})	141.25 ± 18.21^{a}	212.77 ± 19.74^{b}	183.3 ± 24.79^{b}
SOD (U.mg.protein^{-1})	0.62 ± 0.03^{a}	0.82 ± 0.04^{a}	1.33 ± 0.14^{b}
CAT (U. mg.protein^{-1})	0.65 ± 0.02^{a}	0.93 ± 0.05^{b}	0.90 ± 0.05^{b}
GPx (uM.min^{-1}. mg.protein^{-1})	0.09 ± 0.01^{a}	0.20 ± 0.03^{b}	0.12 ± 0.008^{a}
LP (nmol mg.protein^{-1})	0.81 ± 0.10^{a}	0.55 ± 0.02^{b}	0.56 ± 0.01^{b}
PC (nmol mg.protein^{-1})	1.59 ± 0.51^{a}	0.70 ± 0.19^{b}	0.91 ± 0.17^{b}

Abreviations: TC, serum total cholesterol; TG, triglycerides; SOD, superoxide dismutase; CAT, catalase; GPx, glutatione peroxidase; LP, lipid peroxidation; PC, protein carbonilation; G1, non-exercised (n=9); G2, low-intensity aerobic exercise (n=10); G3, moderate-intensity aerobic exercise (n=10). $^{a\,b}$values with different letters in the same row are statistically different ($p<0.05$). Data are the mean ± SE.

Discussion

The main findings of this study were that LDLr$^{-/-}$ mice previously developed with atherosclerosis that underwent aerobic exercise training of different intensities had beneficial effects regarding aorta lipid deposition, liver oxidative balance and serum lipid profile, and that these benefits occurred independently of the exercise intensity.

Initially, our results showed that both training protocols used here were effective in interfering with muscle aerobic metabolism. CS activity in the gastrocnemius' red portion increased in response to both exercise protocols, although it was more pronounced in moderate-intensity exercise training. Citrate synthase is a regulatory enzyme in the Krebs cycle and the increase in its activity suggests an improvement in muscle oxidative capacity induced by aerobic training, which has been demonstrated in rats[29] and mice[30] elsewhere with different exercise models.

The effects of exercise training on the reduction of atherosclerosis have been evaluated previously[12-14, 31]. Most of these studies suggest that moderate-intensity training is effective in controlling and/or reducing the size of atherosclerotic plaque. Such an effect of moderate-intensity training was also observed in the present study, but our results demonstrate additionally that such a positive benefit of regular exercise may be achieved by low-intensity aerobic exercise. This is the first study to demonstrate that low-intensity aerobic exercise has beneficial effects in reducing the development of atherosclerotic lesions in this animal model that are similar to the effects of moderate-intensity exercise. Studies have demonstrated regression or a lower degree of atherosclerosis in experimental animals prone to the development of atherosclerosis, especially in LDLr$^{-/-}$ and ApoE$^{-/-}$ mice subjected to different aerobic exercise models, such as voluntary running[31], treadmill running[13] and swimming[14].

We demonstrated that the fraction of HDL-C increased in both exercise groups, which may have contributed to the reduction of atherosclerotic plaque since this increase in serum HDL-C is inversely related to the appearance of atherosclerotic plaque[32]. According to Kodama *et al.*[33], the variable that best explains the increase in HDL-C is the exercise duration and this may explain the lack of difference between the HDL-C levels in the exercise groups observed here since both low- and moderate-intensity training had the same duration.

Our results did not show exercise training effects on TC and TG levels. Ramachadran *et al.*[13] found similar results in a study using the same animal model. In agreement, Fukao *et al.*[31] demonstrated that voluntary exercise decreases atherosclerosis with no change in TC and TG serum levels. It is noteworthy that in ApoE$^{-/-}$ mice the decrease in the atherosclerotic lesion induced by Benzo(a)pyrene may occur with no changes in TC and TG serum levels[34].

Serum levels of TG normally decrease in response to aerobic exercise when a normal chow diet is used. Although unexpected, the increased TG levels in exercised animals in our study may be explained by the fact that exercise increases the mobilization of TG from the liver as an energy source, which might have increased the serum levels of very low-density lipoprotein (VLDL)[35, 36]. Such accumulation in the serum is due to its lower clearance, since the half-life of this lipoprotein may be increased by up to 30-fold in LDLr$^{-/-}$ mice, as compared to wild animals[37]. For instance, Peng *et al.*[38] examined the effect of long-term activation of hepatic nuclear receptor (LXR) on plasma TG homeostasis in wild-type C57BL/6 and LDLr$^{-/-}$ mice by treating these animals with the LXR

agonist T0901317 for 4 weeks. They observed that LXR agonist treatment decreased plasma total triglycerides in wild-type mice by 35% due to a significant reduction in plasma VLDL triglycerides. In contrast, in $LDLr^{-/-}$ mice, T0901317 treatment increased plasma VLDL and triglycerides. These changes in circulating lipoprotein profiles in response to T0901317 treatment in these two animal models reflect the balance between synthesis and secretion on the one hand and lipolysis and clearance on the other. In both models there was both an increase in VLDL production and secretion and an increase in LPL production and activity in T0901317-treated animals. In wild-type mice, lipolysis and clearance predominates, while in the absence of LDLr, which plays a major role in the clearance of apoB-containing lipoproteins, increased output predominates[38]. The reduction of atherosclerotic lesions observed in the present study may be explained by factors other than the decrease in TC and TG, such as inflammation, endothelial dysfunction[31], oxidative stress[37] and genetic factors.

Although changes in lipoproteins contents are directly related to atherosclerosis progression, other variables must be considered. Stocker and Keaney[39] suggest that risk factors for atherosclerosis are associated with increased production of ROS. These ROS lead to oxidative modification of LDL to oxLDL, which stimulates the migration of circulating monocytes into the subendothelial space, causing endothelial injury and the appearance of atherosclerotic plaques[1]. In contrast, the activity of antioxidants can help to slow atherosclerosis progression[5], and exercise training is considered an important tool in increasing the expression and activity of antioxidant enzymes[29].

Our data show that moderate-intensity aerobic training increased SOD activity. Such result may be explained by the fact that exercise training at higher intensities increased oxygen (O_2) consumption with a concomitant increase in superoxide production[39]. Using a similar protocol, Silva *et al.*[30] showed an increase SOD enzyme activity in the liver of CF1 mice. SOD plays a fundamental role in the control of atherosclerosis. For example, SOD overexpression along with CAT decreased Benzo[a]pyrene-induced atherosclerosis in $ApoE^{-/-}$ mice[34]. It was suggested that increased SOD may reduce atherosclerosis through the co-activation of vascular relaxation mediated by nitric oxide (NO) as the radical O_2 dismutation increases the bioavailability of NO in endothelial cells; in the presence of the O_2 radical, NO shifts to the formation of peroxide nitrite[34].

The aerobic exercise protocol employed in the present study increased CAT activity. Such exercise benefits have been demonstrated by others[40, 41]. The co-expression of CAT is important to decrease the proliferation of smooth muscle cells induced by oxLDL[42]. In addition, hydrogen peroxide increases the proliferation of these cells, resulting in an increased atherosclerotic process, but in the presence of CAT such proliferation does not occur[42].

In the present study the low-intensity exercise training protocol induced a significant increase in GPx activity. Lewis *et al.*[43] demonstrated the importance of GPx in the prevention and control of atherosclerosis. They showed that mice not expressing GPx increased the process of atherosclerosis compared to controls, and the lack of GPx was associated with increasing pro-inflammatory molecules such as VCAM and pro-inflammatory macrophages. Thus, the increased GPx activity in response to our exercise protocol may have contributed to the reduction of atherosclerosis in $LDLr^{-/-}$ mice.

We observed a reduction of lipid hydroperoxides and protein carbonyl content in exercise groups, which may have helped in the control of atherosclerosis in these animals. The increase in antioxidant enzyme activities shown in this study may have contributed to the reduction of hydroperoxides and protein peroxidation observed in exercised mice. The reduction of protein carbonylation is a significant indicator of oxidative damage reduction. It is known that atherosclerosis may facilitate the action of free radicals and lead to oxidative damage in lipids and membrane proteins. Such lipid peroxidation can covalently modify residues of lysine amino groups, which can lead to the formation of oxLDL, and the hydroperoxide excess can change ApoB protein structure, a finding that contributes to the recognition of oxLDL by macrophages resulting in the formation of foam cells[1]. Moreover, liver ROS can modify amino acids through a chain of reactions by the aggregation of proteins susceptible to proteolytic degradation[44].

Another possible explanation for the reduction of oxidative damage in response to aerobic exercise training may be associated with the activation of hepatic nuclear receptor (LXR). This receptor is responsible for the removal of oxidized cholesterol derivatives. Lower oxidative stress[45] and exercise[46] can activate this receptor and its activation has been related to decreased atherosclerosis by reducing the proliferation of smooth muscle cells, suppressing the gene expression of inflammatory factors such as tumor necrosis factor α, interleukin 1b, monocyte chemotactic protein-1, inter-cellular adhesion molecule and matrix metallopeptidase 9, all involved in the atherosclerosis process, and inhibiting tissue factors[45]. Nevertheless,

the assessments of such possibilities warrant future investigations.

In conclusion, our protocols of low- and moderate-intensity aerobic exercise training (30 min daily for 8 weeks) promote similar benefits to LDLr$^{-/-}$ mice previously developed with atherosclerosis. These benefits were noted in the atherosclerotic lesions and serum lipid profiles, and in the hepatic oxidative balance, thus suggesting the inclusion of low-intensity aerobic exercise training protocols in clinical trials to ascertain their effects on humans affected with atherosclerosis.

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Conflict of Interest

No.

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