Thrombosis Research 175 (2019) 21–31

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Physical exercises decreases thrombus and neointima formation in atherosclerotic mice

Maiara F. Terra[a], Denise G. Pedrosa[a], Cláudio Cesar Zoppi[a], Claudio C. Werneck[b], Cristina P. Vicente[a,*]

[a] Department of Structural and Functional Biology, State University of Campinas (UNICAMP), São Paulo, Brazil
[b] Department of Biochemistry and Tissue Biology, Institute of Biology, State University of Campinas (UNICAMP), São Paulo, Brazil

ARTICLE INFO

Keywords:
Physical exercise
Thrombosis
Atherosclerosis
Metalloproteinases
Endothelial progenitor cells
Endothelial nitric oxide synthase

ABSTRACT

The practice of physical exercise is highly indicated to prevent cardiovascular diseases and is directly related to the improvement of endothelial function and the regulation of arterial blood pressure. The objective of this study was to analyze the effect of physical exercise in vascular remodeling after $FeCl_3$ chemically induced arterial injury on atherosclerotic mice. To analyze the effect of exercises on thrombus formation, LDL receptor-deficient mice were fed for 6 weeks with a high-fat diet and performed or not physical exercises for 2 weeks before the arterial injury. To verify endothelium recovery the animals were exercised or not 2 weeks before the injury, and 3 weeks after it, when the vessels were analyzed. In this work, we observed that physical exercises done only before arterial injury reduced thrombosis time, protected the endothelial layer, promoted the recruitment of CD34 positive progenitor cells, increased the level of eNOS and gelatinases activities and decreased the number of inflammatory cells in the vessel, but do not avoid the growth of neointima. Otherwise exercises done before and continued after injury, increased gelatinase activities, reduced lipid deposition in the aortic arch and prevented neointima formation. Thus, we could conclude that physical exercises are done before and continued after endothelial injury stimulate endothelial recovery by promoting endothelial cell growth, matrix remodeling and decreasing inflammation in the vessel wall.

1. Introduction

Strategies that can improve vascular health are the primary target in cardiovascular studies nowadays. According to WHO 2018, cardiovascular diseases kill 17,9 million people a year, accounting for 31% of the total deaths in the world and physical inactivity is the fourth leading cause of death globally [1]. The World Health Organization (WHO) recommends the practice of regular exercises to prevent and ameliorate cardiovascular diseases. Exercises, when performed regularly, can improve endothelial function and arterial blood pressure, reducing the presence of inflammatory molecules and oxidative stress mainly by increasing endothelial oxide nitric synthase activity (eNOS) and dismutase superoxide [2]. It was observed a vasomotor response to physical exercise that is directly related with mechanical stress due to blood flow, leading to vasodilatation and an increase on endothelial progenitor cells (EPCs) mobilization to regenerate vascular endothelium in consequence of eNOS and oxide nitric dependent signalization [3].

Atherosclerosis is one of the leading causes of cardiovascular dysfunction and is a chronic inflammatory disease that affects medium and large caliber arteries [4]. It begins with an endothelial injury caused by oxidative stress associated with risk factors, like diabetes mellitus, hypertension, smoke, obesity, and metabolic syndromes. A migratory cell process starts to the inflammation site involving primarily a transient adhesion stage, where endothelial P- and E-selectins ligate to carbohydrate on leukocyte surface, attracting these cells for local lesion [5]. These inflammatory events can also lead to smooth muscle cells migration to the intima layer, forming neointima and a fibrous cap on atherosclerotic plaques. These plaques may presence proteolytic enzymes, like metalloproteinases (MMPs), which are responsible for the local extracellular matrix degradation and with the plaque instability [6]. When in an advanced stage, atherosclerotic plaques can rupture, leading to platelets activation, vessel subendothelial layers exhibition and thrombus formation [7].

Thrombosis can also be caused by a direct injury on the vessel wall, promoting a blood overflow from circulation and the beginning events to homeostasis restoring of vascular system [8]. The endothelial cell loss due to a vascular injury leads to an inflammatory process that involves cytokines and growth factors expression, migration and

* Corresponding author at: UNICAMP, Institute of Biology, Charles Darwin Street s/n, Bloc N 3rd floor, 13083-863, P.O. Box: 6109, Brazil.
E-mail address: cvicente@unicamp.br (C.P. Vicente).

https://doi.org/10.1016/j.thromres.2019.01.003
Received 5 October 2018; Received in revised form 10 December 2018; Accepted 8 January 2019
Available online 19 January 2019
0049-3848/

proliferation of smooth muscle cells with a high quantity of MMPs, that can result on neointima formation and vessel stenosis. Therefore, prevention of inflammation and stimulation of EPCs migrations to the vessel wall can promote neovascularization of the damaged tissue and the inhibition smooth muscle cells layer formation that is crucial to a positive endothelium repair [9].

The objective of this work was to evaluate the effects of physical exercise on thrombosis and vascular remodeling on atherosclerotic mice and to correlate these effects with the activity of metalloproteinases, eNOS, and the presence of progenitor cells in the vascular wall.

2. Materials and methods

2.1. Mice

Male LDL receptor-deficient mice (LDLr−/− or B6.129S7Ldlrtm1Her/J) and C57Bl/6J mice (6 weeks old) were obtained from Multidisciplinary Center for Biological Research (CEMIB/UNICAMP), Campinas/SP and kept on light/dark cycles of 12/12 h with free access to food and water. Ethical Committee of State University of Campinas (CEUA), protocol 4144-1, approved all animals care and procedures followed by guides for care and use of animals in the laboratory.

2.2. Induction of atherosclerosis

To induce atherosclerosis, 6 weeks-old LDLr−/− mice were fed with a high-fat diet (HFD) (18% of protein, 22% of total fat, 45% of carbohydrate and 0.2% of cholesterol, on a total of 448 Kcal/100 g obtained from PragSoluções, Jaú, São Paulo - Brazil), for 6 or 9 weeks. These animal groups were denominated HFD 12 wks or 15 wks.

2.3. Physical exercise protocol

Exercise protocol was done on a treadmill for 30 min at a speed of 12 m/min, five times/week, during the last 2 or 5 weeks of the high-fat diet feeding, with an adaptation period a week before the beginning of the training, increasing time and speed gradually. The exercise protocol was adapted from Laufs et al. [2], and the protocol to analyze vessel recovery was determined according to Vicente et al. [10], that observed that 3 weeks was the time necessary after the arterial injury to the full vessel recovery or to observe a significant growth of neointima. The animal groups were denominated HFD + Ex 2 wk or HFD + Ex 5 wk. In the injured group, to analyze thrombus formation, the animals were exercised for two weeks, then submitted to arterial injury (HFD − EX + lesion) or not, sedentary (Sed) (HFD Sed + lesion). Also, to analyze neointima formation, the animals were exercised for 2 weeks, injured and analyzed three weeks later without further exercises (HFD − EX + lesion + 21 d) or exercised for more three weeks completing five weeks of exercises (HFD − Ex + lesion + ex 21 d).

2.4. VO_2max test

The objective of the VO_2max test was to determine if the exercises improved mice cardiorespiratory fitness and the effect of HFD in this process. This test was performed on the last day of exercise protocol and was done on a metabolic treadmill associated with a gas analyzer (Oxylet system; Panlab/Harvard Apparatus, Barcelona, Spain), using a 25° angle inclination. It began with a period of 4 min to measure ambient air, followed by 1 min at 5 cm/s, 2 min at 10 cm/s and with speed increasing of 3 cm/s every 2 min, until exhaustion, oxygen capitation was recorded every 1 s with Metabolism software (Panlab/Harvard Apparatus) coupled with gas measurement. We compared the VO_2max values among the sedentary and exercised groups after 2 or 5 weeks of exercise, and besides maximal speed, we also analyzed total distance and the exhaustion time.

2.5. Body weight and lipid profile analysis

To determine if HFD and exercises can alter the levels of cholesterol, triglycerides and body weight, C57Bl/6J and LDLr−/− mice were weighted on the first day of each week of training, and at the end of the protocol, the animals were euthanized and the levels of cholesterol and triglycerides analyzed. Blood was collected from the cava vein with sodium citrate 3.8% as anticoagulant and plasma were obtained by centrifugation at 3000 rpm for 5 min. Total cholesterol and triglycerides were analyzed using an enzymatic method on spectrophotometer with kits from LaborLab Liquid Stable, according to the manufacturer's instructions.

2.6. Carotid artery injury and thrombosis time determination

To determine if exercises can influence in the time required for thrombus formation after carotid artery arterial lesion, we used 12 weeks-old LDLr−/− male mice fed with HFD for six weeks exercised or not, for 2 weeks and weighing 25 ± 2 g. The animals were anesthetized with 100 mg/kg of ketamine and 16 mg/kg of xylazine *via* intramuscular injection. The arterial injury was induced chemically with 15% Ferric Chloride ($FeCl_3$) using a 1 mm^2 filter paper soaked overnight in the $FeCl_3$ solution and dried for 1 h at 37 °C before surgery. The right common carotid artery was isolated, and a filter paper applied for 2 min. To determine carotid artery thrombosis occlusion-time, we used an ultrasound probe (Transonic Flowprobe MAO 5 PSB, Ithaca - USA). The blood flow was recorded by DATAQ program (Transonic System TS 420, Ithaca- USA) and the occlusion time was considered the interval between initial the flow time and the time at which the flow cessation after injury. These animal groups were denominated HFD Sed + lesion and HFD ex 2 wk + lesion.

2.7. Determination thrombus areas using histochemistry

To confirm thrombus formation and determining thrombus area in the vessel lumen after arterial lesion, HFD fed mice exercised or not 2 weeks before arterial injury were euthanized with 500 mg/kg of ketamine 1 h after injury. The right common carotid artery was harvested and embedded in Tissue-Tek OCT (Tissue Tek Optimal Cutting temperature compound, Torrance – USA) and 8 μm cross-sections were cut along the whole vessel. We used Hematoxylin-Eosin staining (HE), and slides were mounted with Cytoseal 60 (Richard Allan Scientific, Kalamazoo- USA), examined on OlympusBX60 microscope (camera QColor 3). The amount of lumen occupied by thrombus was measured with ImageJ® 1.42q program. The slides with the highest amount of thrombus were used for the area calculation. This area was calculated by subtracting the lumen area with thrombus from the total area of the vessel lumen delimited by the internal elastic lamina. We used six animals per analyzed group.

2.8. Quantification of neointima formation using histochemistry

Neointima formation was analyzed in HFD fed mice exercised or not 2 weeks before the injury and exercised or not for more 3 weeks after injury. Previous observations from our lab [10] determined that 21 days were necessary to promote the complete occlusion of the vessel lumen with neointima after arterial injury. The right common carotid artery was harvested and embedded in Tissue-Tek OCT (Tissue Tek Optimal Cutting temperature compound, Torrance – USA) and 8 μm cross-sections were cut along the vessel. We used Hematoxylin-Eosin staining (HE), and slides were mounted with Cytoseal 60 (Richard Allan Scientific, Kalamazoo- USA), examined on OlympusBX60 microscope (camera QColor 3). The amount of lumen occupied with neointima was measured with ImageJ® 1.42q program. The slides with the highest amount of neointima were used for the calculation. This area was calculated by subtracting the lumen area with neointima from the total

area of the vessel lumen delimited by the internal elastic lamina. We used six animals per analyzed group.

2.9. Quantification of the presence of endothelial cells, progenitor cells and eNOS, ICAM and LY6G in the vessel wall by immunofluorescence

The Immunofluorescence assay for endothelial cells (CD31) and progenitor cells (CD34) was done on frozen sections of HFD fed exercised or not animals 1 h after lesion. For endothelial cells, we used rat anti-mouse CD31 (eBioscience, San Diego, CA, dilution 1:80), to analyze the lesion extension and if the exercises promoted protection against the endothelial layer lost after injury. For progenitor cells, we utilized rat anti-mouse CD34 (eBioscience, San Diego, CA, dilution 1:100), a hematopoietic marker that can indicate the migration of progenitor cells to the lesion site. For eNOS, that is a positive marker for endothelial functionality, and we used rabbit anti-mouse NOS3 (Santa Cruz Biotechnology, dilution 1:100) in 2 different moments: 1 h after the lesion to verify endothelial functionality after lesion and 3 weeks after injury in animals exercised or not before and after lesion. ICAM labeling was done using rat anti-mouse ICAM antibody (R&D systems, Minneapolis, USA, dilution 1:80) to illustrate inflammation due to atherosclerosis since it is an adhesion molecule and can be considered a marker of this process. LY6G is a marker for neutrophils, and we used a rat anti-mouse LY6G antibody (Sigma-Aldrich, St. Louis, USA, diluted 1:100) to analyze the presence of inflammatory cells. ICAM and LY6G presence was observed in sedentary and 2 weeks exercised mice 1 h after arterial lesion. The slides were fixed with cold acetone (20 min at 4 °C), then perform blocking with PBS/BSA (0.05%). Then, the sections were washed three times with PBS (5 min each), and the labeling was detected using secondary antibodies conjugated with FITC (0.2 mg/ml eBioscence, San Diego, CA) diluted 1:250 and incubated for 40 min at RT. The nuclei were labeled with DAPI (0.5 mg/mL in methanol) for 5 min at 37 °C. The slides were mounted and analyzed by fluorescence microscope (Olympus BX60, camera QColor 3). The images were captured by QCapture 4.0 software and the cells double marked with DAPI and FITC were counted using ImageJ 1.42q software. We performed this score by choosing randomly 3 cuts of each slide for each analyzed marker.

2.10. Gelatinases activities using in situ zymography

Frozen sections of carotid artery from animals HFD fed animals exercised or not 2 weeks before injury or exercised 2 weeks before injury and 3 weeks after, were incubated 5 min with a gelatinase buffer 1× (Tris 0.5 M, NaCl 1.5 M, $CaCl_2$ 50 mM on pH 7.6) and after on a solution of DQ-Gelatin (a green fluorogenic substrate to gelatinases) 1 mg/mL (Invitrogen, Eugene, Oregon, USA). The gelatinase activity was measured using incubation of 3 h on a dark, moist chamber, at 37 °C. Slides were then washed three times with PBS 1×, mounted and examined on the fluorescence microscope Observer Z.1 (Zeiss, Oberkochen, Germany) using Axio Vision 4.8 Software. Proteolytic activity was detected by the fluorescence increased due to gelatinolytic activity on the chromogenic substrate. The fluorescence intensity on different slides was quantified on ImageJ® 1.42q program (integrated density), with an $n = 5$ per analyzed group.

2.11. Statistical analysis

Groups were analyzed using $n \geq 4$ animals and $n = 3$ when indicated on methodology. Statistical significance was determined through the Mann Whitney U test. Values of p smaller or equal 0.05 were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

3. Results

3.1. VO_2max test indicated an improvement of mice physical capacity

We observed an increase in the distance, aerobic maximum speed and exhaustion time on the exercised groups of both 2 weeks and 5 weeks, when compared to controls or non-exercised animals.

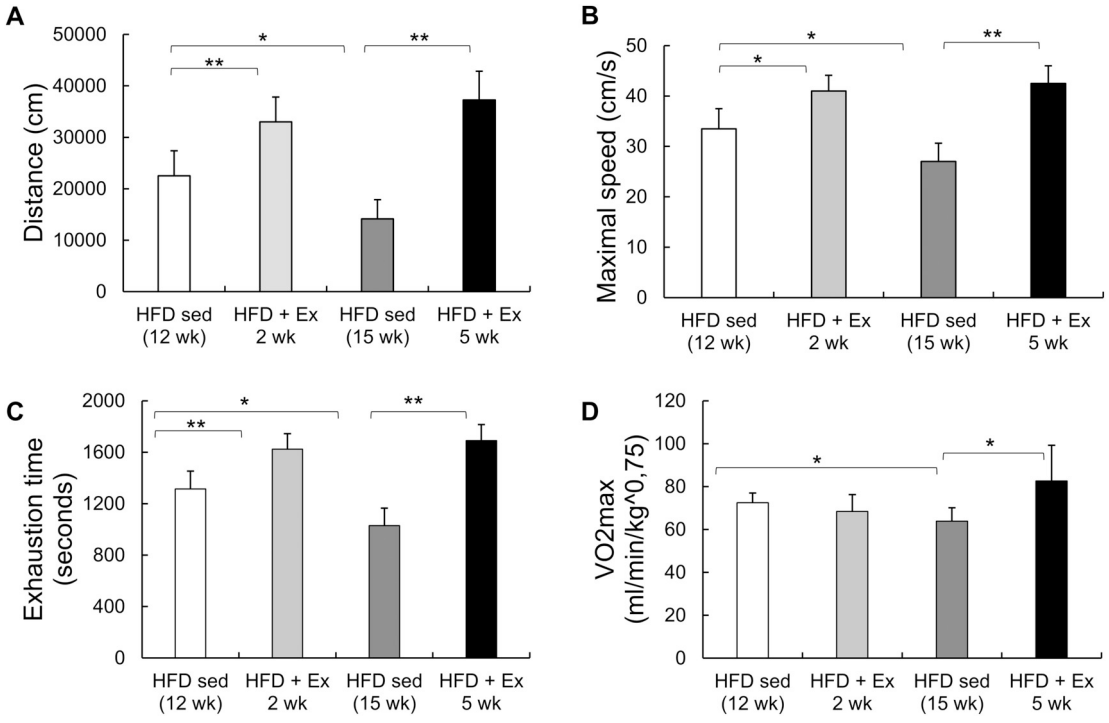

Fig. 1. Effect of the exercises on VO_2max, distance, maximal speed and exhaustion time. (A) Distance (B) Maximal speed (C) Exhaustion time (D) VO_2max. HFD sed 12 wks (animals LDLr−/− 12 weeks-old fed 6 weeks with HFD); HFD + ex 2 weeks (animals LDLr−/− 12 weeks-old fed 6 weeks with HFD exercised the last 2 weeks); HFD sed 15 wks (animals LDLr−/− 15 weeks-old fed 9 weeks with HFD); HFD ex 15 wks (animals LDLr−/− 15 weeks-old fed 9 weeks with HFD and exercised the last 5 weeks). $n = 6$ per group, $^{*}p < 0.05$ and $^{**}p < 0.01$.

Exercised mice for two weeks ran 32,995.7 ± 4837 cm *vs.* 22,500.8 ± 4869 cm of non-exercised mice and 5 weeks exercised mice ran 37,256.7 ± 5586 cm *vs.* 14,160 ± 3717 cm, respectively ($p = 0.01$; Fig. 1A). Maximal speed was also increased where two weeks exercised mice reached 41.5 ± 3.0 cm/s *vs.* 33.5 ± 4 cm/s for controls mice. This improved condition was sustained after 5 weeks exercise (42.5 ± 3.5 *vs.* 27 ± 3.6 cm/s) ($p = 0.02$; Fig. 1B). The same was verified for the exhaustion time that was 1314.83 ± 138 s for control *vs.* 1624.17 ± 120.8 s, for 2 weeks of exercises and 1030 ± 135 s for control *vs.* 1689.67 ± 125.8 for 5 weeks of exercises ($p = 0.01$; Fig. 1C). It was not observed a significant difference in these parameters between the exercised groups for 2 or 5 weeks. The VO_2max presented no significance in the animals exercised two weeks when compared to the non-exercised group of the same age (12 weeks - 68.45 ± 7.7 *vs.* 72.45 ± 4.5 ml/min/kg, $p = 0.39$) but exercised five weeks group presented a 26,96% increase when compared to its respective control (15 weeks - 82.63 ± 16.6 *vs.* 63.86 ± 6.2 ml/min/kg, $p = 0.03$) (Fig. 1D).

3.2. Body weight and cholesterol levels were decreased after 5 weeks of exercises

In our study, physical activity for five weeks decreased body weight significantly in 31.9% (27.64 ± 3.1 *vs.* 19.375 ± 1.8 g, $p = 0.006$), while two weeks of exercise did not affect it (22.94 ± 3.1 *vs.* 23.92 ± 1.4 g, $p = 0.24$). Besides, there was an increase in body weight of 18.8% from 12 to 15 weeks animals due to HFD ($p = 0.03$) (Fig. 2A).

Analyzing lipid profile, we could see an increase on the total cholesterol and triglycerides levels of HFD fed group when compared to normal diet fed animals (standard diet), of 159.7% and 111.2%, respectively (256.6 ± 61 *vs.* 638.722 ± 80 mg/dl, $p = 0.002$ and 74.25 ± 9 *vs.* 175.93 ± 40 mg/dl, $p = 0.002$). Only the five weeks exercised group presented a reduction on the cholesterol levels of 19.8% (638.722 ± 80 *vs.* 533.358 ± 65 mg/dl, $p = 0.03$) (Fig. 2B). The levels of triglycerides (175.93 ± 40 *vs.* 152.87 ± 33.4 mg/dl, $p = 0.12$) were not altered by the exercises (Fig. 2B and C).

3.3. Exercises decreased the arterial thrombosis time

We analyzed the effect of 2 weeks of exercises done before the arterial injury. We observed that the arterial thrombosis time was 5.2 min in HFD sedentary animals and 10.7 min in on HFD exercised animals, a significant increase of 107.9% on the thrombosis time with $p = 0.002$ (Fig. 3).

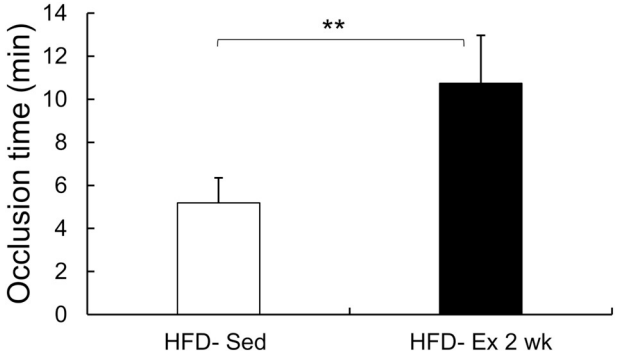

Fig. 3. Effect of the exercises on the occlusion time of carotid artery from LDLr−/− mice. Occlusion time was determined after $FeCl_3$ carotid artery lesion using an ultrasound probe. HFD sed lesion (animals LDLr−/− 12 weeks-old HFD fed 6 weeks and lesioned); HFD ex 2 wks + lesion (animals LDLr−/− 12 weeks-old fed 6 weeks and exercised the last 2 weeks). $n = 7$ animals per group, $^{**}p < 0.01$.

3.4. Thrombus area was decreased by exercises done 2 weeks before the arterial injury

We analyzed thrombus area in the vessel lumen 1 h after injury on sedentary and two weeks exercised animals using histochemical analysis of the lesioned vessel (Fig. 4A), and we observed a significant reduction on thrombus area on the exercised group of 28.1% (97 ± 1,9 *vs.* 67.7 ± 17.8%, $p = 0.002$) (Fig. 4B).

3.5. Neointima formation was inhibited only by exercises executed before and after arterial lesion

We observed a significant reduction of 9.2% on neointima area on animals exercised three weeks after injury compared to sedentary group (90.5 ± 8.9 *vs.* 79.9 ± 6.7%, $p = 0.04$) (Fig. 5A.1 and A.2). On exercised only two weeks before the injury, we did not observe the difference when compared to the not exercised group (90.5 ± 8.9 *vs.* 86.3 ± 11.4%, $p = 0.4$) (Fig. 5A.1 and A.3). Finally, on exercised two weeks before the injury and three weeks after it (Fig. 5A.4), we found a decrease of 24.6% when compared to the non-exercised lesioned group (90.5 ± 8.9 *vs.* 66.4 ± 14.3%, $p = 0.01$). The neointima areas were calculated using the Image J program and analyzed with $n = 5$ animals (Fig. 5B).

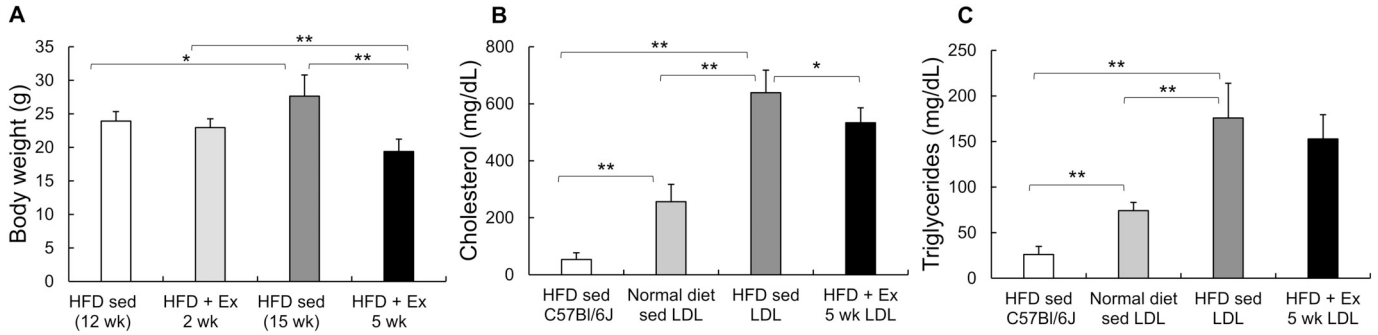

Fig. 2. Effect of exercises on Body weight, triglycerides, and cholesterol levels. (A) Body weight of LDLr−/− mice (B) Cholesterol levels (B) Triglycerides levels. HFD sed 12 wks (animals LDLr−/− 12 weeks-old fed 6 weeks with HFD); HFD + ex 2 weeks (animals LDLr−/− 12 weeks-old fed 6 weeks with HFD exercised the last 2 weeks); HFD sed 15 wks (animals LDLr−/− 15 weeks-old fed 9 weeks with HFD); HFD ex 5 wks (animals LDLr−/− 15 weeks-old fed 9 weeks with HFD and exercised the last 5 weeks); HFD sed C57BL/6J (wild-type animals 15 weeks-old HFD fed 9 weeks); Normal diet LDL (animals LDLr−/− 15 weeks-old fed regular chow). $n = 6$ per group, $^{*}p < 0.05$ and $^{**}p < 0.01$.

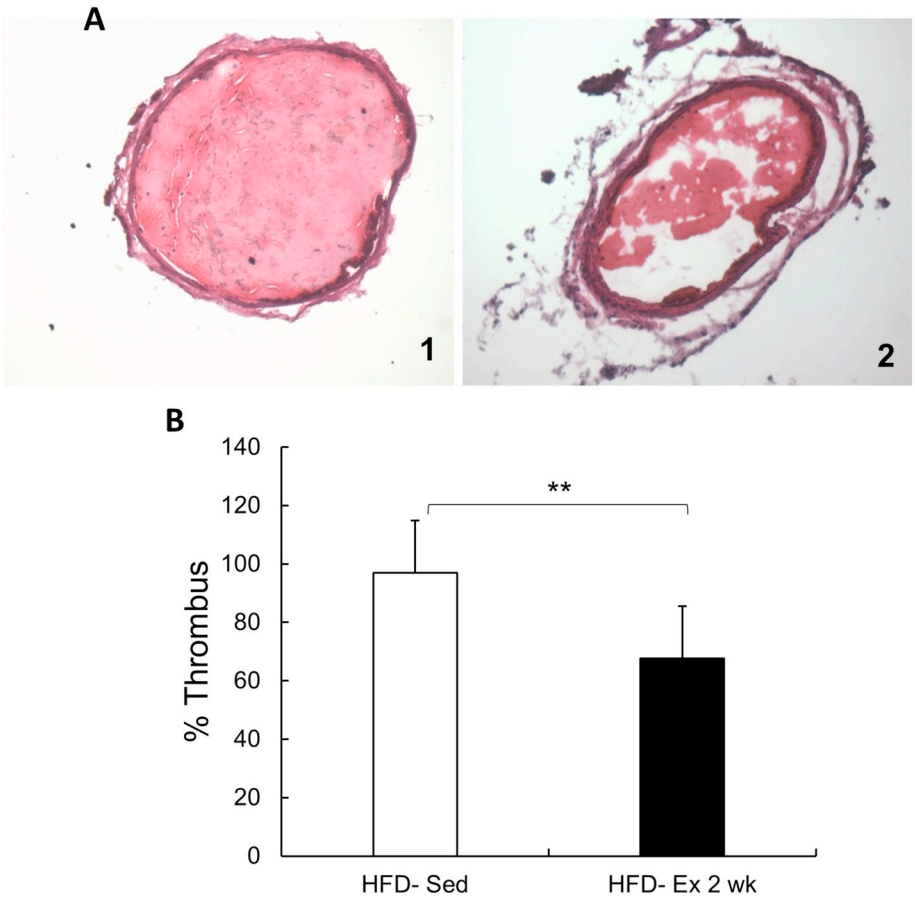

Fig. 4. Determination of thrombus area 1 h after lesion. Thrombus area was determined histologically using HE staining of the injured vessels. (A) Histological analysis of injured carotid artery. 1 - Sedentary + 1 h after lesion; 2 - Exercise 2 weeks + 1 h after lesion. (B) Percentage of thrombus on carotid artery 1 h after arterial lesion. $n = 6$ per group, $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.6. Exercises executed 2 weeks before arterial lesion protect the integrity of the endothelial layer

We labeled the cells with the anti-CD31 antibody, a specific marker for the endothelial layer and its integrity by immunohistochemistry and counted the number of positive cells for this marker (Fig. 6A). On the sedentary groups with the arterial lesion, we observed a significant reduction of 67.74% on CD31 labeled cells compared with non-lesioned animals (28.9 ± 3.2 *vs.* 10.6 ± 2.3 cells, $p = 0.008$), indicating the effectiveness of our lesion method. On the exercised lesioned group, the endothelial loss was 70% less than in the control group (10.6 ± 2.3 *vs.* 18 ± 3.1 cells, $p = 0.008$) (Fig. 6B).

3.7. Exercises increased the number CD34 positive cell and eNOS presence in the endothelial layer after arterial lesion

We labeled the cells with anti CD34 antibody detected with a FITC conjugated secondary antibody and counted the cells using Image G program. We observed that the number of CD34 positive cells increased 111.4% in animals exercised 2 weeks, lesioned and sacrificed 1 h after that, when compared to HFD sedentary animals (13.3 ± 3.6 *vs.* 24.1 ± 3.9 cells, $p = 0.008$) (Fig. 7A and B).

We also analyzed the presence of eNOS by immunohistochemistry in animals without lesion (Fig. 8A.1), 1 h (Fig. 8A.2–A.3), and 21 days (Fig. 8A.4–A.7) after injury, exercised or not. On sedentary animals sacrificed 1 h after lesion, we observed a significant reduction of 65% of labeled cells (24 ± 1.9 *vs.* 7.7 ± 1.6 cells, $p = 0.007$), and in exercised animals, this decrease was of 43.8% when compared to the group without lesion (24 ± 1.9 *vs.* 14.5 ± 2.1 cells, $p = 0.008$). Also, physical exercise promoted the recovery of eNOS, increasing it in 64.1% on the exercised group when compared to the sedentary animals (7.7 ± 1.6 *vs.* 14.5 ± 2.1 cells, $p = 0.007$) (Fig. 8B).

After 21 days, there was a reduction of 67.12%, 42.46% and 57.54% on lesioned sedentary group, exercised only after lesion and exercised before the injury, respectively, when compared to animals without lesion (24 ± 1.9 *vs.* 8.3 ± 1.9 cells, $p = 0.008$; 24 ± 1.9 *vs.* 15 ± 1.6 cells, $p = 0.005$; 24 ± 1.9 *vs.* 9.9 ± 2.2 cells, $p = 0.007$). We also observed that on the group with exercise before and after injury, the number of positive cells for eNOS did not differ significantly from the group without lesion (24 ± 1.9 *vs.* 30 ± 5 cells, $p = 0.09$) (Fig. 8C).

3.8. Exercises realized 2 weeks before arterial injury decreased inflammation in the vessel wall

We analyzed the presence of ICAM in the endothelial layer using ICAM antibody in sedentary, and animals exercised 2 weeks before the arterial injury, sacrificed 1 h after injury (Fig. 9A.1–2). The number of leucocytes was determined using LY6G labeling (Fig. 9A.3–4). We observed a significant decrease of 53% in the number of ICAM positive cells (31.9 ± 7.9 *vs.* 14.7 ± 1.4 cells, $p = 0.028$) and of 37.1% leucocytes associated to the vessel (21.9 ± 3.9 *vs.* 14.8 ± 3.1 cells, $p = 0.03$) when compared to controls ($p < 0.05$) (Fig. 9B and C, respectively).

3.9. Exercises increased gelatinases activities 1 h and 21 days after arterial injury

Metalloproteinases (MMPs), also known as gelatinases, act by degrading extracellular matrix, helping to promote thrombus degradation and vascular remodeling. Analyzing metalloproteinases (MMPs) by *in*

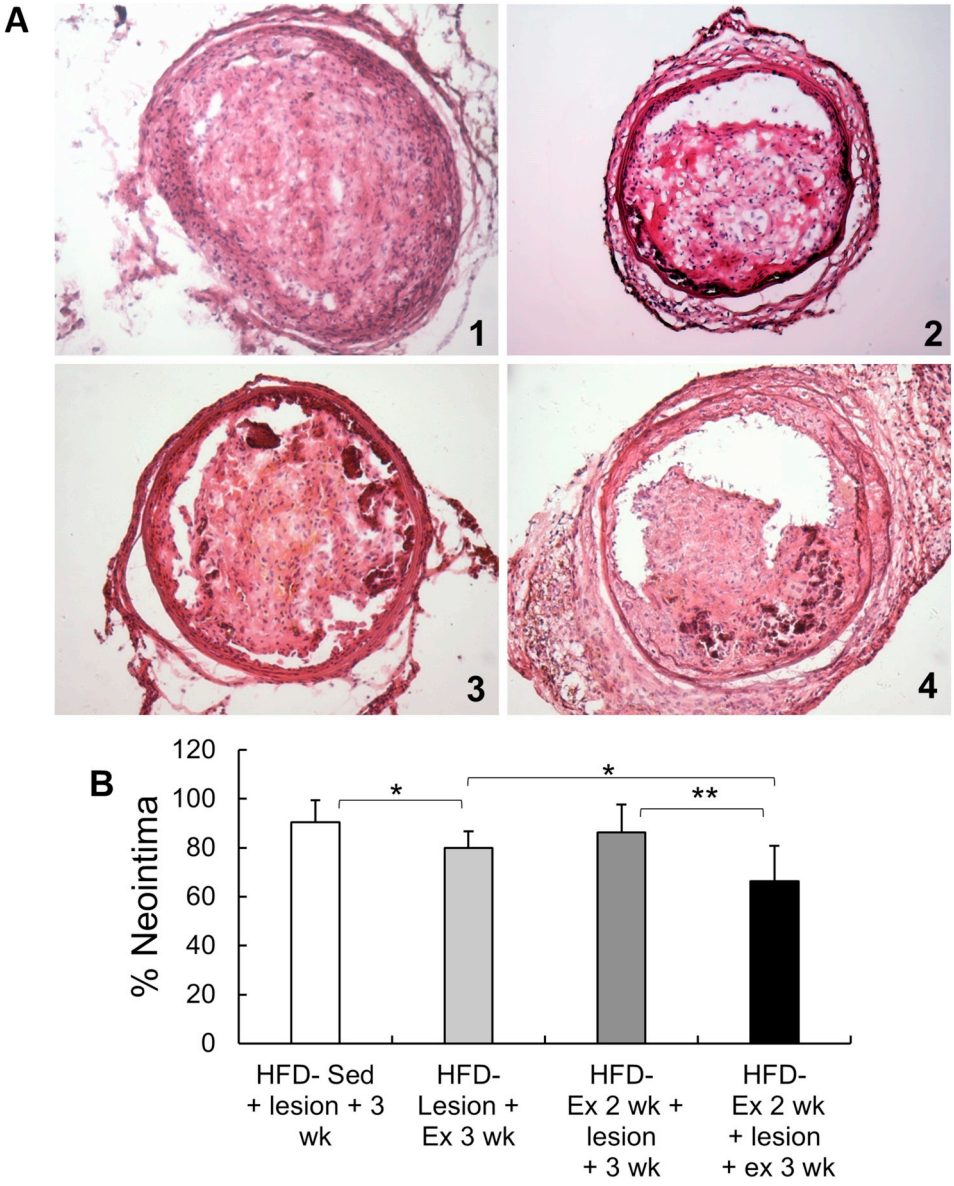

Fig. 5. Determination of neointima area 21 days after lesion. Neointima area was determined histologically using HE staining of the injured vessels. (A) Histological analysis of injured carotid artery. 1 - Sedentary + 21 days after lesion; 2 - Sedentary + lesion + 21 days of exercises after lesion; 3 - Exercise 2 weeks + lesion + 21 days after lesion and 4 - Exercise 2 weeks + lesion + exercise 21 days. (B) Percentage of neointima 21 days after arterial lesion. $n = 6$ per group, $^{*}p < 0.05$ and $^{**}p < 0.01$.

situ zymography, we observed a significant increase of 123.5% (38,395.4 ± 15,741 *vs.* 108,828 ± 24,382 normalized density, $p = 0.008$) on gelatinases activity on animals exercised two weeks before the injury and sacrificed 1 h after that when compared to lesioned sedentary group (Fig. 10A.2–3 and B).

In 21 days after lesion groups, when the neointima layer was already formed (Fig. 10A.4–7), we observed a significant increase on gelatinases activity on exercised group post-lesion of 94.9% (78,110 ± 27,605 *vs.* 125,735 ± 14,777 normalized density, $p = 0.01$) and with exercise before and after lesion of 101.5% when compared with sedentary animals (78,110 ± 27,605 *vs.* 139,836 ± 18,868 normalized density, $p = 0.007$) (Fig. 10C). Also, there was a significant increase of this activity on these same groups concerning exercised group only before arterial lesion, being this increase of 56.8% on exercised group after injury (82,270 ± 19,528 *vs.* 125,735 ± 14,777 normalized density, $p = 0.02$) and of 62.2% on exercised before and after (82,270 ± 19,528 *vs.* 139,836 ± 18,868 normalized density, $p = 0.008$) (Fig. 10C). The Supplemental Fig. 2, we observed an increase in MMP2 expression and a decrease in MMP9 in exercised animals.

4. Discussion

In this work, we observed that 2 weeks of physical exercises done before arterial injury were capable of decreasing thrombosis time and promoting endothelial recovery by attracting endothelial progenitor cells to the site of injury, decreasing the presence of inflammatory markers and promoting vascular remodeling by increasing MMPs activities, inhibiting neointima formation that can promote vascular reocclusion. Also, we observed that continuous exercises performed after injury improved the growth of neointima promoting a better outcome to vascular recovery then exercises done only before the injury.

We realized the VO_2max test to determine the ability of our exercise protocol to improve the aerobic capacity of our animals and also obtained the maximal aerobic speed, total distance and exhaustion time of the animals. VO_2max results after 2 weeks of the physical exercise

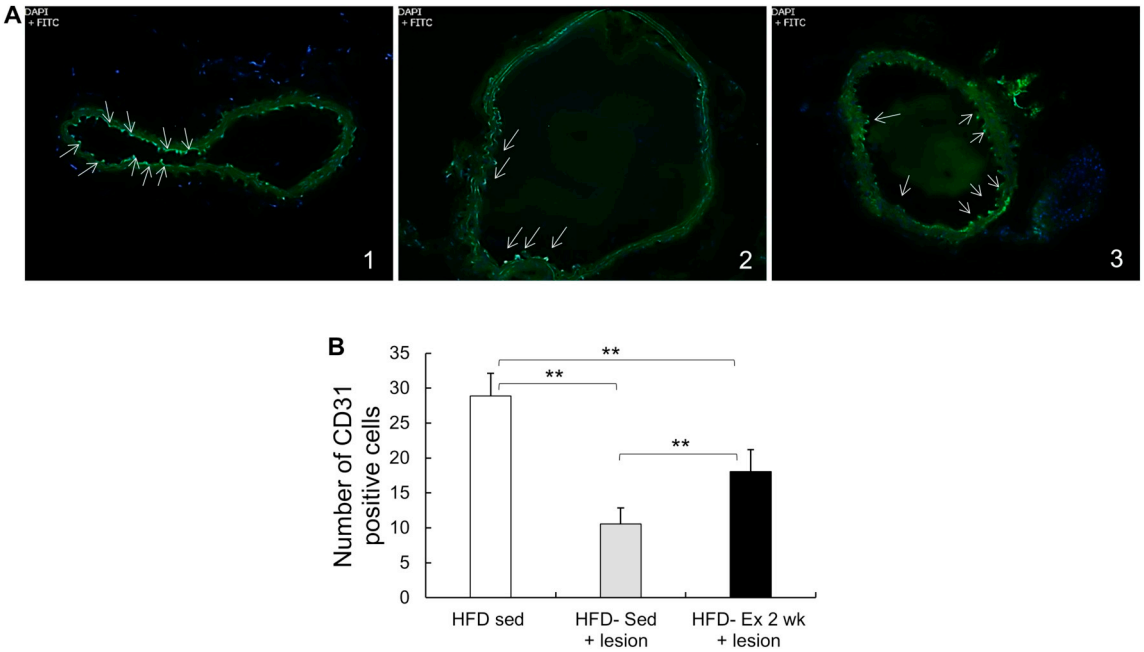

Fig. 6. Analysis of the endothelial layer after lesion. The endothelial layer was analyzed using immunohistochemistry with a CD31 antibody labeled with FITC. The positive cells were counted using Image J program. (A) Immunohistochemistry of Carotid artery of LDLr−/− mice labeled by CD31. 1 - Sedentary group without lesion; 2 - Sedentary + 1 h after lesion; 3 - Exercises 2 weeks + 1 h after lesion, white arrows indicate some labeled cells. (B) Quantification of the number of CD31 positive cells. $n = 5$, $^{*}p < 0.05$ and $^{**}p < 0.01$.

protocol did not vary significantly, however, with 5 weeks of exercises we observed an increase on VO_2max, what could indicate an adaptation to the activity and an improvement on mice aerobic capacity.

We observed that both the 2 and 5 weeks protocol were able to increase distance, maximum speed and exhaustion time significantly when compared to its respective sedentary groups, indicating that there was an improvement on mice physical capacity, what has already been seen in other studies [11]. Ayachi et al. observed that treadmill exercise

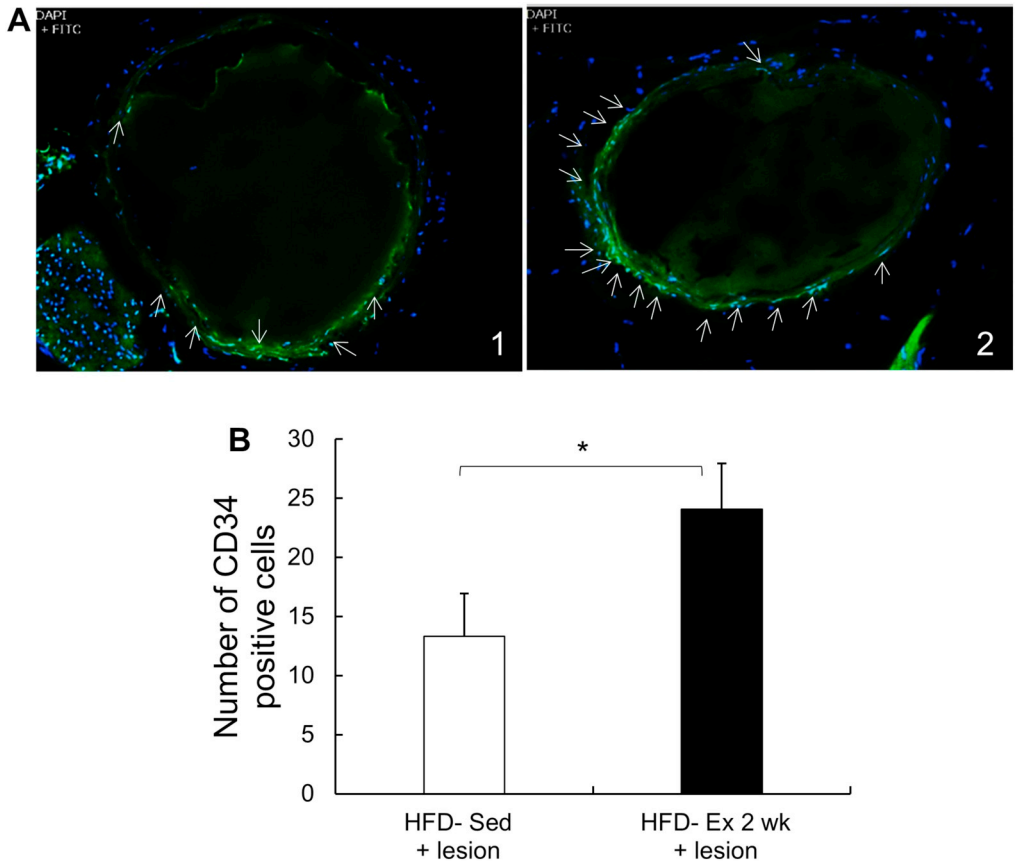

Fig. 7. Effect of exercises on progenitor cells mobilization. Progenitor cells were identified using immunohistochemistry with CD 34 antibody labeled with FITC, and the positive cells were counted using Image J program. (A) Immunohistochemistry for CD34 in LDLr−/− mice carotid artery. 1 - HFD sed lesion (animals LDLr−/− 12 weeks-old HFD fed six weeks and lesioned); 2 - HFD ex 2 wks + lesion (animals LDLr−/− 12 weeks-old and 6 weeks and exercised the last 2 weeks), white arrows indicate labeled cells. (B) Number of CD34+ cells. $n = 5$, $^{*}p < 0.05$ and $^{**}p < 0.01$.

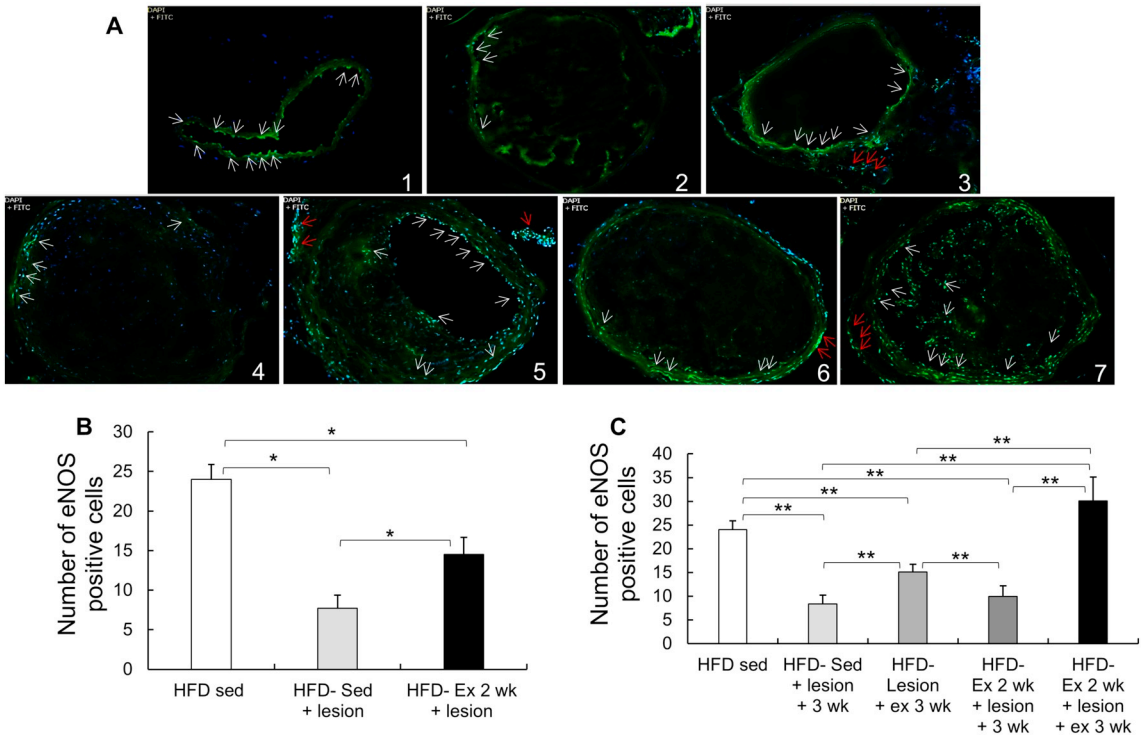

Fig. 8. Effect of the exercises on eNOS. eNOs was identified using immunohistochemistry with eNOS antibody labeled with FITC and the positive cells were counted using Image J program. (A) Immunohistochemistry for eNOS in LDLr−/− mice carotid artery. 1 - Sedentary group without lesion; 2 - Sedentary + 1 h after lesion; 3 - Exercise 2 weeks + 1 h after lesion; 4 - Sedentary + 21 days after lesion; 5 - Sedentary + lesion + 21 days of exercises after lesion; 6 - Exercise 2 weeks + lesion + 21 days after lesion and 7 - Exercise 2 weeks + lesion + exercise 21 days, white arrows indicate labeled cells. (B) Number of eNOS positive cells 1 h after lesion. (C) Number of eNOS positive cells 21 days after lesion. $n = 5$, $^{*}p < 0.05$ and $^{**}p < 0.01$.

resulted in linear increases in HR (Heart rate), maximum oxygen consumption ($\dot{V}o_{2\ max}$), and respiratory exchange ratio, similar to that seen in larger species. Their values of VO_2 max for sedentary old mice are similar to the one determined in our work [12]. It is already known that physical exercise can help on the maintenance or reduction of body weight [13]. On this study, we could observe that the two weeks exercise protocol was not able to reduce body weight significantly, but this value was reduced significantly with 5 weeks exercise when compared to the group of the same age.

We also analyzed lipid profile and observed significant increase on total cholesterol and triglycerides on sedentary LDL receptor-deficient animals fed with high-fat diet when compared to the standard diet sedentary group, in values similar to the observed in the literature [14]. This increase was not observed with the animals fed with a standard diet. We observed a significant reduction only in the cholesterol levels of 19.8% on the exercised group, while with triglycerides this reduction was not significant. It was previously described that LDLr−/− mice submitted to treadmill exercises at 15 m/min for 30 min and 5 times per weeks, presented a reduction of 15% on total cholesterol and of 40% on the atherosclerotic plaques present in the aorta artery [15]. Our animals presented a significant decrease of the body weight and cholesterol levels only after 5 weeks of exercises. However, we decided to use the 2 weeks exercise protocol previous to arterial lesion, since animals fed for 6 weeks and then exercised for 2 weeks, are already 12 weeks old, and an increase in the exercise protocol could add up more two variables in our findings, like the animal age and elevated levels of cholesterol and triglycerides. Also, analyzing the animals with 5 weeks of exercises was also a form to verify if the continuous exercise after the arterial injury can improve vascular recovery when compared with the animals exercised only before the injury. The decrease in the levels of cholesterol and triglycerides are not directly connected to a decrease in the atherosclerotic plaques. Analyzing the formation of atherosclerotic plaques in the aortic root, we observed a significant reduction of 49.6% and 43.2% in the plaque area on animals exercised 2 and 5 weeks, respectively (Supplemental material). These decrease in the atherosclerotic plaques on these animals led us to believe that other mechanisms aside the decrease in the lipid profile may be involved, like an increase in plasma myeloperoxidase activity, eNOS activity, and antioxidative stress. Chirico et al., observed in old mice exercised in voluntary wheel running that this kind leads to longer exercise distances than treadmilling and with less stress response to the animals, these exercises decreased inflammation, oxidative stress and metabolic parameters, decreasing aortic plaque size, increasing plaque stability and prolonging the animals lifespan [16]. Also, recent works have shown the involvement of metalloproteinases on plaque stability due to physical exercise. MMPs, mainly MMP2 presented decreased amount in atherosclerotic plaques in exercised old ApoE deficient mice, also in these animals the levels of TIMPs were increased promoting a decrease in the ratio MMP2/TIMP2 [17,18].

We used a chemical injury induced by ferric chloride, a well-established method performed in previous work from several groups [19,20]. The relation of thrombosis and physical exercise, either in the prevention or treatment, is not clarified on literature, being one of the goals of our study. We found that physical exercise realized before the injury was able to prolong thrombosis time significantly, inhibiting arterial thrombus formation. Observing thrombus size through histological analysis of carotid artery 1 h after lesion on exercised animals, we found a reduction of 28.1% of thrombus in the vessel lumen when compared to HFD fed sedentary animals. It can indicate that physical activity can reduce thrombus formation, immediately after lesion, and it might reflect vascular remodeling later on. To determine the extension of our arterial lesion, we analyzed the vessels 1 h after a lesion in animals exercised or not, and we observed that our lesion induces a loss of 67.7% of the number of CD31 positive cells in the endothelial layer after injury and that exercises can protect the vessel wall against this loss.

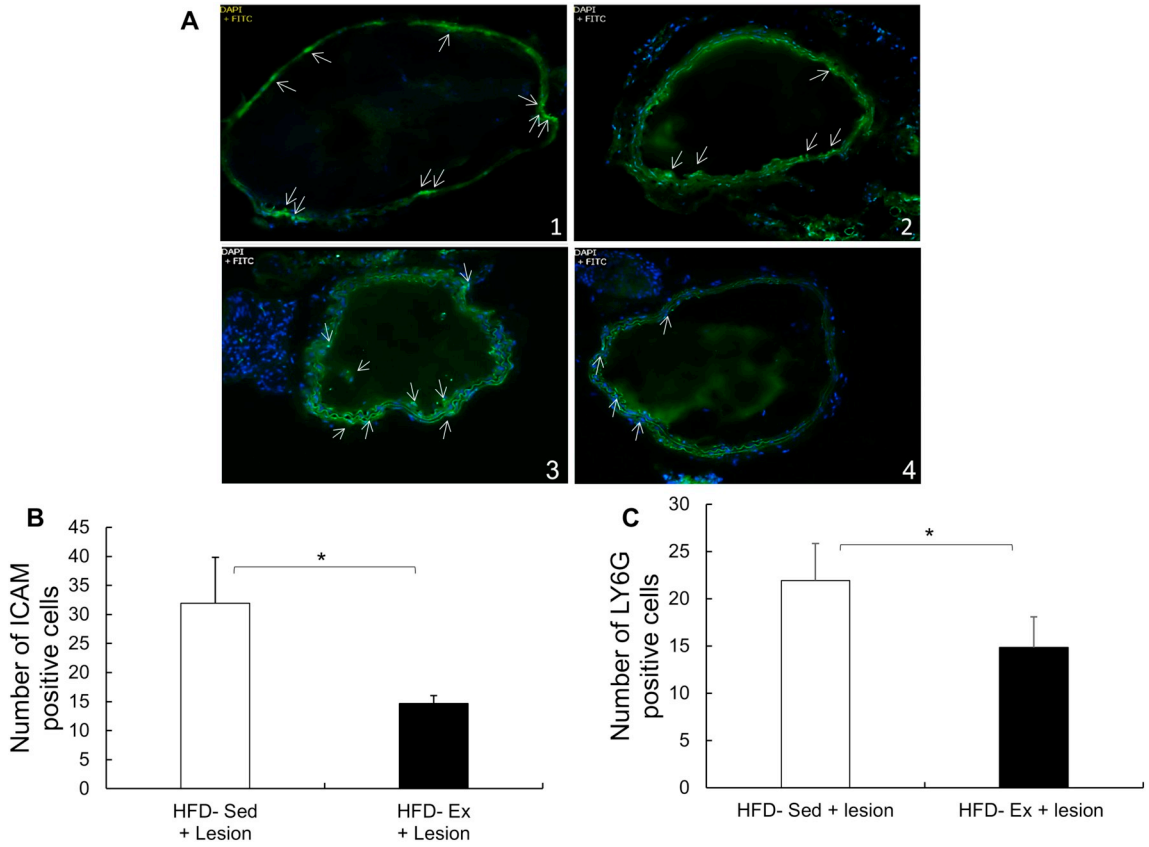

Fig. 9. Effect of the exercise on inflammation on the vessel. ICAM and LY6G antibodies labeled with FITC identified the inflammatory process, and the number of positive cells was counted using Image J program. (A) Immunohistochemistry for ICAM in LDLr−/− mice carotid artery. 1 - HFD sed lesion (animals LDLr−/− 12 weeks-old HFD fed six weeks and lesioned) and 2 - HFD ex 2 wks + lesion (animals LDLr−/− 12 weeks-old and 6 weeks and exercised the last 2 weeks); Immunohistochemistry for LY6G in LDLr−/− mice carotid artery. 3 - HFD sed lesion (animals LDLr−/− 12 weeks-old HFD fed six weeks and lesioned) and 4 - HFD ex 2 wks + lesion (animals LDLr−/− 12 weeks-old and 6 weeks and exercised the last 2 weeks), white arrows indicated labeled cells. (B) Number of ICAM positive cells 1 h after lesion. (C) Number of LY6G positive cells 1 h after lesion. $n = 5$, $^{*}p < 0.05$.

After arterial lesion, the vessel wall tends to recover. Impaired vascular remodeling can lead to the formation of neointima with vessel restenosis due to the formation of a layer of smooth muscle cells inside the vessel lumen. It was previously observed that 21 days after arterial lesion the neointima formed could occlude vessel lumen and is formed by smooth muscle cells. It was also observed that treatment with glycosaminoglycans immediately after injury could prevent this growth, indicating that using anticoagulants to prevent thrombosis can inhibit neointima growth later on [10]. We observed a significant reduction in the neointima formation of 9.2% on exercised animals after injury and 24.6% in animals exercised before and after injury. Pynn et al., also observed a decrease in neointima formation after arterial injury using $FeCl_3$, a decrease in the presence inflammatory cells in the vessel wall 3 weeks after injury with no alteration in the thrombosis time. In their assays, they used ApoE deficient animals not fed with HDF, older than the animals we used [21]. The increased thrombosis time formed in our work may be related to the higher inflammatory profile of the vessel wall induced by the higher cholesterol and triglycerides levels in our HFD animals, different that was observed in the study cited above.

CD 34 is not the only marker for EPCs, but it is one of the main markers of EPC [22], and the presence of CD34 positive cells in the vessel wall may be indicating an increase in the mobilization of this progenitor from the bone marrow. We observed that 2 weeks of exercises increased the number of labeled cells significantly in 111.4% in the intima layer. Laufs et al. also analyzed the influence of physical exercise on neointima and endothelial progenitor cells (EPCs) participation. They observed an increase in circulating EPCs number on exercised animals, as well as a reduction in neointima formation and involvement of nitric oxide in this process. The increased presence of these cells could be involved in vascular repair and re-endothelization after endothelial injury [2].

We analyzed eNOS activity by immunohistochemistry, due to its importance on vascular tonus and platelets functions. NO presence is important in thrombotic events mediated by platelets. NOS inhibition or loss in thrombotic events affecting vascular components induces a powerful thrombotic effect as observed in our thrombotic model that induces vessel wall disorganization with significant loss of the endothelial layer [23]. There was a reduction on the number of labeled cells both 1 h and 21 days after lesion on sedentary and exercised, when compared to sedentary without lesion group, indicating that a lesion on the endothelium leads to a decrease in eNOS presence [15]. On the other hand, we observed that 2 weeks exercise recovered 88% of eNOS presence, in sedentary injured animals, while on 21 days groups only the animals exercised before and after injury presented a significant recovery when compared to control.

Metalloproteinases are enzymes that promoted the degradation of extracellular matrix, being able to act, in atherosclerotic plaques instability, vascular remodeling on thrombus resolution, and remodeling of the vessel wall [24,25]. We analyzed gelatinases activities in the vessel 1 h after the lesion in, and we observed a significant increase on gelatinases activity on the animals exercised 2 weeks before arterial lesion when compared to the same sedentary group. On the animals analyzed 21 days after lesion, we observed a significant increase of gelatinases activities only on animals exercised after injury and exercised before and after that, when compared to sedentary and exercised only before lesion animals.

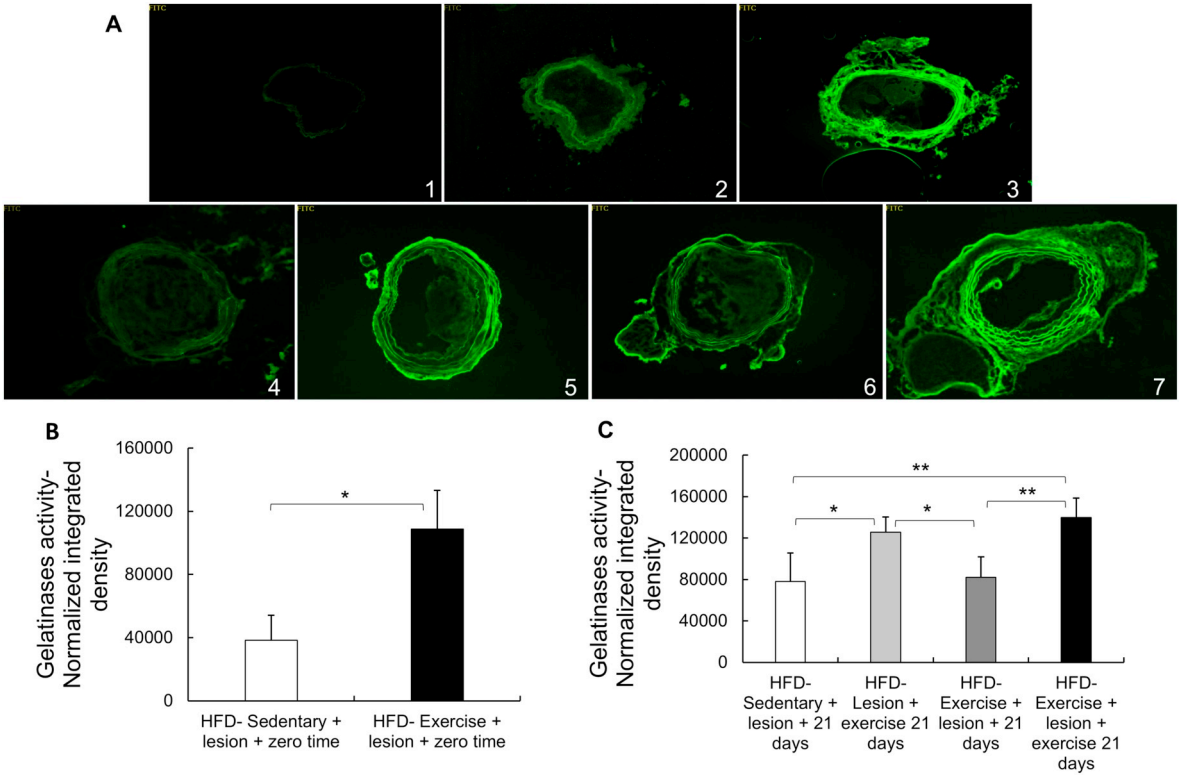

Fig. 10. Effect of the exercises on gelatinases activities. (A) *In situ* zymography using DQ gelatin, a FITC gelatinase chromogenic substrate 1 h and 21 days after lesion. Quantification was done measuring fluorescence intensity with ImageJ and comparing to control. 1 - Blank (no chromogenic substrate); 2 - Sedentary + 1 h after lesion; 3 - Exercise 2 weeks + 1 h after lesion; 4 - Sedentary + 21 days after lesion; 5 - Sedentary + lesion + 21 days of exercises after lesion; 6 - Exercise 2 weeks + lesion + 21 days after lesion and 7 - Exercise 2 weeks + lesion + exercise 21 days. (B) Quantification of the Gelatinases activity 1 h after lesion. (C) Quantification of gelatinases activities 21 days after lesion. $n = 5$, $^{*}p < 0.05$ and $^{**}p < 0.01$.

Physical activity can promote vasodilatation due to an increase in blood flow, generating higher mechanical stress, leading to an increased expression of eNOS. Thereby, higher levels of nitric oxide and VEGF activates and increases MMP-2, and MMP-9 expression and all these molecules can mobilize EPCs to circulation, contributing to vascular repair and re-endothelization [3]. We also observed that MMPs activity occur predominantly on the elastic lamina, what can be explained due to its participation in vascular remodeling and arterial expansion when there is an increase in blood flow [26]. Analyzing the gel zymography in supplemental figures, we observed that 1 h after lesion there was an increase in MMP-2 activity on exercised animals, with a decrease in the MMP-9 activity of the same group. On the other hand, on 21 days after lesion, we noticed that MMP-2 did not differ among groups, and there was an increase on MMP-9 activity on the group with exercise only after lesion and with exercise before and after the injury. This increase in MMP-2 that we observed may be related to thrombus matrix remodeling. It was observed in MMP-2 deficient mice, a reduction on the intima layer of carotid artery after injury when compared to wild-type, and an increase on intima/media ratio, confirming the participation of this enzyme on intima layer hyperplasia and smooth muscle cells migration [27]. It was observer that platelet-associated MMPs activities might be related to increasing or decreasing thrombus formation. MMP2 increased activities may be related to increased thrombus formation and MMP9 with its decrease, with the balance between MMPs and TIMPs activities being directly related to thrombus formation and platelet activation [28]. Our MMP analysis was done 1 h after arterial lesion when platelet activation and thrombus formation was accelerated and later 21 days after lesion the presence of MMP9 was detected and may be related to the observed neointima inhibition promoted by the exercises. More studies analyzing vessel wall re-organization, accumulation of lipids in the carotid arteries, different MMPs and TIMP activities and ROS production in the vessel wall could be the next step to analyze and clarify the exercises mechanisms of action in the prevention of thrombosis and vessel wall positive remodeling after arterial lesion.

5. Conclusions

In conclusion, our study demonstrated that physical exercise could be used as a complementary treatment to prevent cardiovascular diseases and to stimulate vascular recovery after vascular injury. Exercises done before arterial injury can prepare the vascular wall to better recovery after the arterial injury even before the reduction of body weight and cholesterol levels. The physical activity performed before and after lesion can act by protecting the endothelial layer, promoting increased levels of eNOs, decreasing inflammation in the vessel wall and promoting thrombus resolution and vessel wall remodeling through increased extracellular matrix metalloproteinases activities mainly in the initial process of thrombus resolution and in the final process of vessel recovery after injury.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.01.003.

Disclosure of interest

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

Financial support

This work was supported by grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-PROEX 0487,

process number 1588684) to M.F.T., Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - process number 164052/2015-1) to D.G.P. and C.P.V., that was also supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2012/23640) and C.C.W. to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (308368/2016-9).

Authors' contribution

M.F.T. elaborated the project, performed all the assays with the animals, histology, immunofluorescence, and zymography. D.G.P. helped with histological assays. C.C.Z., assisted with VO_2max experiments. C.C.W. helped with the thrombosis assays and reviewed the article; C.P.V. was responsible for the overall project helped to write the article and with overall assays.

References

[1] < EIMFactSheet_2014.pdf > .
[2] U. Laufs, et al., Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis, Circulation 109 (2) (2004) 220–226.
[3] S. Gielen, G. Schuler, V. Adams, Cardiovascular effects of exercise training molecular mechanisms, Circulation 122 (12) (2010) 1221–1238.
[4] F.H. Epstein, R. Ross, Atherosclerosis - an inflammatory disease, N. Engl. J. Med. 340 (2) (1999) 115–126.
[5] Y. Nakashima, et al., Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse, Arterioscler. Thromb. Vasc. Biol. 18 (5) (1998) 842–851.
[6] F. Herranz, et al., Superparamagnetic nanoparticles for atherosclerosis imaging, Nano 4 (2) (2014) 408–438.
[7] W. Insull, The pathology of atherosclerosis: plaque development and plaque responses to medical treatment, Am. J. Med. 122 (1) (2009) S3–S14.
[8] B. Furie, B.C. Furie, Mechanisms of thrombus formation, N. Engl. J. Med. 359 (9) (2008) 938–949.
[9] Y. Shiba, et al., M-CSF accelerates neointimal formation in the early phase after vascular injury in mice the critical role of the SDF-1–CXCR4 system, Arterioscler. Thromb. Vasc. Biol. 27 (2) (2007) 283–289.
[10] C.P. Vicente, L. He, D.M. Tollefsen, Accelerated atherogenesis and neointima formation in heparin cofactor II-deficient mice, Blood 110 (13) (2007) 4261–4267.
[11] K. De Angelis, et al., Exercise training changes autonomic cardiovascular balance in mice, J. Appl. Physiol. 96 (6) (2004) 2174–2178.
[12] M. Ayachi, et al., Validation of a ramp running protocol for determination of the true VO_2max in mice, Front. Physiol. 7 (2016) 372.
[13] R.L. Bradley, et al., Voluntary exercise improves insulin sensitivity and adipose tissue inflammation in diet-induced obese mice, Am. J. Physiol. Endocrinol. Metab. 295 (3) (2008) E586–E594.
[14] T.T. Liu, et al., Dihydromyricetin ameliorates atherosclerosis in LDL receptor-deficient mice, Atherosclerosis 262 (2017) 39–50.
[15] O. Meilhac, et al., Role of arterial wall antioxidant defense in beneficial effects of exercise on atherosclerosis in mice, Arterioscler. Thromb. Vasc. Biol. 21 (10) (2001) 1681–1688.
[16] E.N. Chirico, et al., Magnetic resonance imaging biomarkers of exercise-induced improvement of oxidative stress and inflammation in the brain of old high-fat-fed $ApoE^{-/-}$ mice, J. Physiol. 594 (23) (2016) 6969–6985.
[17] X. Zhang, et al., Exercise training increases the stability of atherosclerotic plaques in apolipoprotein E (ApoE) gene-deficient mice, Int. J. Clin. Exp. Med. 9 (7) (2016) 13820–13827.
[18] N. Kadoglou, et al., The anti-inflammatory effects of exercise training promote atherosclerotic plaque stabilization in apolipoprotein E knockout mice with diabetic atherosclerosis, Eur. J. Histochem. 57 (1) (2013).
[19] T. Vassequi-Silva, et al., Losartan and captopril treatment rescue normal thrombus formation in microfibril associated glycoprotein-1 (MAGP1) deficient mice, Thromb. Res. 138 (2016) 7–15.
[20] W. Li, M. Nieman, A.S. Gupta, Ferric chloride-induced murine thrombosis models, J. Vis. Exp. 115 (2016).
[21] M. Pynn, et al., Exercise training reduces neointimal growth and stabilizes vascular lesions developing after injury in apolipoprotein E–deficient mice, Circulation 109 (3) (2004) 386–392.
[22] J.M. Yang, M. Ii, N. Kamei, A. Cantas, A.M. Kwon, A. Kawamoto, H. Akimaru, H. Masuda, Y. Sawa, T. Asahara, $CD34^+$ cells represent highly functional endothelial progenitor cells in murine bone marrow, PLoS One 6 (5) (2011) e20219.
[23] C. Moore, C. Tymvios, M. Emerson, Functional Regulation of Vascular and Platelet Activity During Thrombosis by Nitric Oxide and Endothelial Nitric Oxide Synthase, (2010).
[24] K. Morishige, et al., Overexpression of matrix metalloproteinase-9 promotes intravascular thrombus formation in porcine coronary arteries in vivo, Cardiovasc. Res. 57 (2) (2003) 572–585.
[25] K.B. Deatrick, et al., Vein wall remodeling after deep vein thrombosis involves matrix metalloproteinases and late fibrosis in a mouse model, J. Vasc. Surg. 42 (1) (2005) 140–148.
[26] E. Sho, et al., Arterial enlargement in response to high flow requires early expression of matrix metalloproteinases to degrade extracellular matrix, Exp. Mol. Pathol. 73 (2) (2002) 142–153.
[27] M. Kuzuya, et al., Deficiency of gelatinase a suppresses smooth muscle cell invasion and development of experimental intimal hyperplasia, Circulation 108 (11) (2003) 1375–1381.
[28] T.G. Mastenbroek, et al., Platelet-associated matrix metaloproteinases regulate thrombus formation and exert local collagenolytic activity, Arterioscler. Thromb. Vasc. Biol. 35 (2015) 2554–2561.