CELL BIOCHEMISTRY & FUNCTION

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Cell Biochem Funct 2013; **31**: 136–151.
Published online 7 September 2012 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/cbf.2868

Physical training prevents oxidative stress in L-NAME-induced hypertension rats

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The present study investigated the effects of a 6-week swimming training on blood pressure, nitric oxide (NO) levels and oxidative stress parameters such as protein and lipid oxidation, antioxidant enzyme activity and endogenous non-enzymatic antioxidant content in kidney and circulating fluids, as well as on serum biochemical parameters (cholesterol, triglycerides, urea and creatinine) from Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced hypertension treated rats. Animals were divided into four groups ($n = 10$): Control, Exercise, L-NAME and Exercise L-NAME. Results showed that exercise prevented a decrease in NO levels in hypertensive rats ($P < 0{\cdot}05$). An increase in protein and lipid oxidation observed in the L-NAME-treated group was reverted by physical training in serum from the Exercise L-NAME group ($P < 0{\cdot}05$). A decrease in the catalase (CAT) and superoxide dismutase (SOD) activities in the L-NAME group was observed when compared with normotensive groups ($P < 0{\cdot}05$). In kidney, exercise significantly augmented the CAT and SOD activities in the Exercise L-NAME group when compared with the L-NAME group ($P < 0{\cdot}05$). There was a decrease in the non-protein thiols (NPSH) levels in the L-NAME-treated group when compared with the normotensive groups ($P < 0{\cdot}05$). In the Exercise L-NAME group, there was an increase in NPSH levels when compared with the L-NAME group ($P < 0{\cdot}05$). The elevation in serum cholesterol, triglycerides, urea and creatinine levels observed in the L-NAME group were reverted to levels close to normal by exercise in the Exercise L-NAME group ($P < 0{\cdot}05$). Exercise training had hypotensive effect, reducing blood pressure in the Exercise L-NAME group ($P < 0{\cdot}05$). These findings suggest that physical training could have a protector effect against oxidative damage and renal injury caused by hypertension. Copyright © 2012 John Wiley & Sons, Ltd.

KEY WORDS—hypertension; physical training; oxidative stress; kidney

INTRODUCTION

Currently, hypertension has affected more than 1 billion adults worldwide and 90 to 95% of these patients have essential hypertension.[1] This disease is considered as an independent risk factor for stroke, myocardial infarction, heart failure, arterial aneurysm and it has been the leading cause of chronic renal failure.[2,1]

It is well established that nitric oxide (NO) produced in the vascular endothelial cells shows a potent vasodilator effect[3,4] and plays an important role in the local regulation of platelet-vessel wall interactions and in vascular resistance and growth.[5] On the basis of these effects, NO has been proposed to have antihypertensive, antithrombotic and antiatherosclerotic properties.[3,4]

Chronic inhibition of NO produces a volume-dependent elevation of blood pressure (BP) and its physiological and pathological characteristics resemble essential hypertension.[6–8] Several studies have administered *in vivo* an inhibitor of NO biosynthesis, the Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), an L-arginine analogue, to induce hypertension in rats.[9–15]

In hypertension, the production of reactive oxygen species (ROS) is increased by various means and it can also cause other vascular diseases and disorders.[16] However, it still remains unclear whether increased ROS levels are a cause or a consequence of high BP. There is probably a feed-forward system whereby oxidative stress induces BP elevation, which in turn promotes increased ROS generation and oxidative damage.[17,16] At first glance, a compelling

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Received 15 March 2012
Revised 26 June 2012
Accepted 30 July 2012

explanation to this link is that the increased ROS production, specially superoxide radical (O_2), oxidizes NO to peroxynitrite and subsequently nitrite and nitrate. This, plus an inhibition of NO synthesis (by L-NAME administration), results in a very low concentration of NO-mediated vasodilatation, an increase in vasoconstriction, and subsequently an increase in systemic vascular resistance, which contributes to BP elevation.[16]

The increased generation or decreased scavenging or metabolism of ROS is defined as oxidative stress.[17] ROS and free radicals are mediators of several forms of tissue damage, such as ischemic injuries to different organs, inflammatory response and injury resulting from intracellular metabolism of drugs and chemicals.[18,17] One of the biological molecules to suffer oxidative damage in cells are proteins; their side chains can be carbonylated by reactive compounds.[19] In addition, membranes are composed mostly of phospholipids and proteins. Increasing ROS lead to the peroxidation of lipid membranes and loss of membrane integrity, resulting in necrosis and cell death.[19,20]

The effect of ROS is balanced by the antioxidant action of non-enzymatic antioxidants as well as by antioxidant enzymes. Superoxide dismutase (SOD) and catalase (CAT) are part of the antioxidant enzyme system. SOD catalyses the dismutation of superoxide anion (O_2^-) to H_2O_2. Subsequently, H_2O_2 is reduced to H_2O and O_2 by peroxidases such as glutathione peroxidase or CAT.[19,21] Ascorbic acid and non-protein thiols (NPSH) are important non-enzymatic antioxidant defences. They present a variety of functions in bioreduction and detoxification processes, being extremely important to redox balance.[22–25]

Moderate aerobic exercise has been recognized as a coadjuvant on hypertension treatment or prevention.[26,13,27] This way, many people are engaged in organized group exercise rehabilitation programmes or pursue individual exercise with or without medication to maintain good cardiovascular health.[26,28] Acute exercise increases the utilization of oxygen in the body and seems to enhance the free radical formation in various tissues of animals[23,24] and in circulating fluids, such as serum, plasma and total blood in human.[29]

Exercises that generate free radicals seem to increase the activity of antioxidant enzymes in the body.[30,13,23] Also, it has already been demonstrated that physical training improves the antioxidant capacity in the aorta of hypertensive rats.[13] Exercise training also generates NO by the induction of nitric oxide synthase (NOS).[13,31] The benefit of regular physical activity has been reported to improve cardiovascular functions in patients with chronic heart failure and other cardiovascular diseases,[26,28] besides having an important and well documented effect on reducing BP in hypertensive subjects and animals.[32,30,33–37]

It is hypothesized that the chronic NO-deficient hypertension is associated with the depletion of antioxidants and oxidative injury to the vascular tissue and kidney, and exercise conditioning normalizes the hypertensive response by scavenging free radical/ROS through the up-regulation of the antioxidant system in circulating fluids and kidney of rat.

As mentioned, moderate aerobic exercise has been related to reduce BP and improve antioxidant defence, and oxidative stress has been associated with the pathogenesis of hypertension. Therefore, this study was aimed to investigate the effect of exercise training on BP, kidney NO levels and oxidative stress parameters, such as protein and lipid oxidation, antioxidant enzyme activity and endogenous non-enzymatic antioxidant content in serum, plasma, total blood and kidney from L-NAME-induced hypertension treated rats.

MATERIALS AND METHODS

Chemicals

The L-NAME, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), tris (hydroxymethyl)-aminomethane GR, thiobarbituric acid (TBA) and Coomassie Brilliant Blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA). The bovine serum albumin was obtained from Reagen. All the other chemicals used in this experiment were of the highest purity.

Animals

Adult male Wistar rats (70–90 days; 220–300 g) from the Central Animal House of the Federal University of Santa Maria were used in this experiment. The animals were maintained at a constant temperature ($23 \pm 1\,^{\circ}C$) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol under number: 029/2011). All protocols are in accordance with the guidelines of the Colégio Brasileiro de Experimentação Animal, based on the Guide for the Care and Use of Laboratory Animals (National Research Council), and all efforts were made to minimize the number of animals used in this study and their suffering.

Experimental protocol

Rats were randomly divided into four groups, normotensive (Control), normotensive plus exercise (Exercise), hypertensive (L-NAME) and hypertensive plus exercise (Exercise L-NAME). In the hypertensive groups, hypertension was induced by the oral administration of the NOS inhibitor (L-NAME). L-NAME administration includes via drinking water, gavage and subcutaneous injection. Although most of the works induce hypertension through drinking water,[38,6,9–12,8,15] Ribeiro *et al.*[8] observed that average dairy water intake per box was 26% lower in L-NAME-treated rats than in controls. This way, we chose gavage administration to be sure on the dose ingested by rats and to be sure all rats were receiving the same dose. In addition, despite of hypertension induction, high doses[38,8] or long-term administration[6] of L-NAME may induce severe proteinuria and renal injury. This way, we decided to use a medium dose as used by Furstenau *et al.* ($30\,mg\,kg^{-1}\,day^{-1}$)[10] and at relative medium time, understanding that our rats would become hypertensive but the renal injury would happens and not be too deleterious. In the normotensive groups, the animals received water by gavage throughout the entire experiment to be submitted to the same stress (control groups). These rats

were euthanized 24 h after the last exercise session.[33,14] Blood was collected by cardiac puncture and kidneys were carefully removed.

Exercise protocol

Swimming was the exercise chosen for this study. The use of swimming rats as a model of exercise presents advantages over treadmill running, because swimming is a natural ability of the rats and it avoids the selection of animals, which is necessary in experimental protocols using treadmill running. The protocol used was according to Souza *et al.*[39] as follows:

Swimming protocol

All rats were adapted to water before the beginning of the training. The adaptation was to keep the animals in shallow water at $31 \pm 1\,^{\circ}C$[39] for 5 days, with duration of 1 h. This procedure was performed always at the same time, between 10:00 h and 00:00 h. The adjustment reduces stress, without, however, promoting adaptations to the training.

Animals were trained five times per week in an adapted swimming system with water heated to $31 \pm 1\,^{\circ}C$ for 6 weeks with duration of 60 min, performed always between 10:00 h and 00:00 h. The training tank used for this study was 80 cm in length, 50 cm in width and 90 cm in depth.[39] The workload (weight on the back) was gradually increased up to 5% of the animal's body weight (Table 1).

Sedentary animals were placed in shallow water (5 cm in depth), heated to $31 \pm 1\,^{\circ}C$, for 60 min, 5 days a week without the work load (5% of body weight) to be subjected to the same stress, however, without being submitted to the effects of physical training.

Hemodynamic parameter determination

In all rats, systolic blood pressure (SBP) and heart rate (HR) were measured in awake animals, by tail-cuff plethysmography (Kent Scientific; RTBP1001 Rat Tail Blood Pressure System for rats and mice, Litchfield, USA). Rats were conditioned with the apparatus before measurements were taken. During the measurements, the animals were kept for approximately 20 min in an acrylic retainer within an enclosure that maintains the temperature between 30–31 °C. SBP was recorded at the end of experiment (last treatment week). Heart rate values were derived from the pulsations detected by SBP.

Table 1. Swimming protocol, with training time from 1st week to 6th week, held from Monday to Friday

Week	Monday	Tuesday	Wednesday	Thursday	Friday
1st	20 min	30 min	40 min	50 min	60 min
	wo	wo	wo	wo	wo
2nd	40 min	50 min	60 min	60 min	60 min
	2% bw	2% bw	2% bw	2% bw	2% bw
3rd	40 min	50 min	60 min	60 min	60 min
	5% bw	5% bw	5% bw	5% bw	5% bw
4th, 5th, 6th	60 min	60 min	60 min	60 min	60 min
	5% bw	5% bw	5% bw	5% bw	5% bw

wo, without overload; bw, body weight ($n = 10$ to each group).

Blood and tissue preparation

Twenty-four hours after the last treatment, animals were previously anesthetized with halothane and submitted to euthanasia. Halothane was administered by the closed technique in a dose of 0·5%, according to Halothane bull (Tanohalo 1:1 ml; CRISTÁLIA Produtos Químicos Farmacêuticos LTDA) adapted to rats. Animals were kept in a closed chamber, having an environment saturated with anaesthetic, for approximately 2 min. The exact time spent in the chamber depended on the clinical signs of each animal. Immediately after collection, rats were killed by decapitation.

Blood was collected by cardiac puncture in tubes with and without anticoagulant system to determine oxidative stress parameters and to perform biochemical analysis. In tubes without an anticoagulant system, blood was centrifuged at 1800 g for 10 min, the precipitate was discarded, and the serum was used to determine substances reactive to thiobarbituric acid (TBARS), protein carbonyl, levels of cholesterol, triglycerides, urea and creatinine. CAT and SOD activities were determined using whole blood collected in citrated tubes and diluted to a ratio of 1:10 in saline solution Plasma was separated from tubes with ethylenediaminetetraacetic acid (EDTA) anticoagulant to determine NPSH content. The samples of kidneys were quickly removed, placed on ice and homogenized within 10 min in cold 50 mM Tris-HCl pH 7·4 (1/10, w/v). The homogenate was centrifuged at 2000 g, at 4 °C, for 10 min to yield the low-speed supernatant (S1) that was used immediately for TBARS, vitamin C and NPSH groups. Furthermore, during all procedures, S1 was maintained on ice. It is important to note that the immediate use of sample can prevent a possible alteration in the sample caused by storage time and by freezing process. All the procedures for the test samples and control sample were carried out together. In order to perform SOD and CAT assay, kidneys were diluted and homogenized as described further.

Biochemical analysis

The levels of urea and creatinine were determined using standard methods on Cobas MIRA® (Roche Diagnostics, Basel, Switzerland) automated analyzer. In addition, serum total cholesterol and triglyceride concentrations were measured using standard enzymatic methods using Ortho-Clinical Diagnostics® reagents on the fully automated analyzer (Vitros 950® dry chemistry system; Johnson & Johnson, Rochester, NY, USA).

NO determination

NO content in kidney supernatant was estimated in a medium containing 400 µl of 2% VCl_3 (in 5% HCl), 200 µl of 0·1% N-(l-naphthyl) ethylene-diamine dihydrochloride, 200 µl of 2% sulfanilamide (in 5% HCl). After incubating at 37 °C for 60 min, nitrite levels, which corresponds to an estimative of levels of NO, were determined spectrophotometrically at 540 nm, based on the reduction of nitrato to nitrite

by VCl_3.[40] Kidney nitrite and nitrate levels were expressed as nanomole of NO/milligram of protein.

Carbonylation of serum protein

The carbonylation of serum proteins was determined by a modified Levine method.[41] Firstly, from 1 ml of serum, proteins were precipitated using 0·5 ml of 10% trichloroacetic acid (TCA) and centrifuged at 1800 g for 5 min, discarding the supernatant. One half millilitre of 10 mmol l^{-1} 2,4-dinitrophenylhydrazine (DNPH) in 2 mol l^{-1} HCl was added to this protein precipitate and incubated at room temperature for 30 min. During incubation, the samples were mixed vigorously every 15 min. After incubation, 0·5 ml of 10% TCA was added to the protein precipitate and centrifuged at 1800 g for 5 min. After discarding the supernatant, the precipitate was washed twice with 1 ml of ethanol/ethylacetate (1:1), centrifuging out the supernatant in order to remove the free DNPH. The precipitate was dissolved in 1·5 ml of protein dissolving solution (2 g sodium dodecyl sulfate and 50 mg EDTA in 100 ml 80 mmol l^{-1} phosphate buffer, pH 8·0) and incubated at 37 ºC for 10 min. The colour intensity of the supernatant was measured using a spectrophotometer at 370 nm against 2 mol l^{-1} HCl. Carbonyl content was calculated by using the molar extinction coefficient (21×10^3 1 mol^{-1} cm^{-1}), and results were expressed as nanomoles per milligram protein.

Determination of lipid peroxidation

Lipid peroxidation was estimated by measuring TBARS in serum samples according to a modified method of Jentzsch *et al*.[42] Briefly, 0·2 ml of serum was added to the reaction mixture containing 1 ml of 1% ortho-phosphoric acid and 0·25 ml alkaline solution of thiobarbituric acid (final volume 2·0 ml), followed by 45 min heating at 95 ºC. After cooling, samples and standards of malondialdehyde (MDA) were read at 532 nm against the blank of the standard curve. Results were expressed as nanomoles MDA per millilitre of serum.

Lipid peroxidation in kidney was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the method described previously by Ohkawa *et al*.[43] In short, the reaction mixture contained 200 µl of samples of S1 from liver and kidney or standard (MDA 0·03 mM), 200 µl of 8·1% sodium dodecylsulfate, 750 µl of acetic acid solution (2·5 M HCl, pH 3·5) and 750 µl of 0·8% TBA. Mixtures were heated at 95 ºC for 90 min. TBARS tissue levels were expressed as nmol MDA/mg protein.

Catalase and superoxide dismutase activities

The determination of CAT activity in total blood was carried out in accordance with a modified method of Nelson and Kiesow.[44] This assay involves the change in absorbance at 240 nm due to CAT-dependent decomposition of hydrogen peroxide. An aliquot (0·02 ml) of blood was homogenized in potassium phosphate buffer, pH 7·0. The spectrophotometric determination was initiated by the addition of 0·07 ml in an aqueous solution of 0·3 mol l^{-1} hydrogen peroxide. The change in absorbance at 240 nm was measured for 2 min. Catalase activity was calculated using the molar extinction coefficient (0·0436 cm^2 µmol), and results were expressed as nanomoles per milligram protein.

For CAT assay in kidney, the tissue was homogenized in 50 mM potassium phosphate buffer, pH 7·5, at a proportion of 1:9 (w/v) and 1:5 (w/v), respectively. The homogenate was centrifuged at 2000 g for 10 min to yield a supernatant that was used for the enzyme assay. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7), 10 mM H_2O_2 and 20 µl of the supernatant. The rate of H_2O_2 reaction was monitored at 240 nm for 2 min at room temperature, the same as in total blood assay. The enzymatic activity was expressed in units mg^{-1} protein (one unit of the enzyme is considered as the amount of CAT that decomposes 1 µmol of H_2O_2 per min at pH 7 at 25 °C.)

Superoxide dismutase activity measurement in total blood is based on the inhibition of the radical superoxide reaction with adrenalin as described by Misra and Fridovich.[45] In this method, SOD present in the sample competes with the detection system for superoxide anion. A unit of SOD is defined as the amount of enzyme that inhibits the rate of adrenalin oxidation by 50%. Adrenalin oxidation leads to the formation of the coloured product, adrenochrome, which is detected by spectrophotometer. Superoxide dismutase activity is determined by measuring the rate of adrenochrome formation, observed at 480 nm, in a reaction medium containing glycine-NaOH (50 mM, pH 10) and adrenalin (1 mM).

With the purpose of performing the SOD assay in kidney,[45] the tissue was adequately diluted with Tris-HCl pH 7·4 at a proportion of 1:40 (w/v) and 1:60(w/v), respectively. Briefly, epinephrine undergoes auto-oxidation at pH 10·2 to produce adrenochrome, a coloured product that was detected at 480 nm. The addition of samples (10, 20, 30 µl) containing SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored for180 s. The amount of enzyme required to produce 50% inhibition was defined as one unit of enzyme activity, the same as in total blood assay.

NPSH determination

NPSH was measured spectrophotometrically with Ellman's reagent.[46] An aliquot of 200 µl for kidney in a final volume of 900 µl of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM DTNB (0·05 ml). A standard curve using cysteine was added to calculate the content of thiol groups in samples and was expressed as µmol SH/g tissue.

Aliquots (0·1 ml) of plasma were added to a phosphate buffer 0·3 mol L^{-1} (0·85 ml), pH 7·4 and the reaction was read at 412 nm after the addition of 10 mM DTNB (0·05 ml). Results were expressed as µmol ml^{-1} of plasma.

Ascorbic acid quantification

Renal vitamin C analysis was determined by the modified method described by Jacques-Silva *et al.*[3] Proteins of kidney were precipitated in a cold 10% TCA solution at a proportion of 1:1 (v/v) and submitted to centrifugation again. This supernatant was then used for analysis. A 300 µl aliquot of sample in a final volume of 575 µl of solution was incubated for 3 h at 37 °C, then 500 µl H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a colour reagent containing 4·5 mg ml^{-1} dinitrophenyl hydrazine (DNPH) and $CuSO_4$ (0·075 mg ml^{-1}). Vitamin C levels are expressed as µg ascorbic acid/g tissue.

Protein determination

Protein was measured by the method of Bradford[47] using bovine serum albumin as standard.

Statistical analysis

Data were analysed statistically using analysis of variance followed by the Duncan multiple test using SPSS 8·0 for Windows (SPSS, Chicago, IL, USA). Some data were analysed by Pearson's correlation. Differences were considered significant when $P < 0·05$. Variables are presented as mean ± SD.

RESULTS

Systolic blood pressure

In the present study, the oral administration of L-NAME (an inhibitor of NOS) by gavage was associated with a significant increase in SBP when compared with control groups, validating the hypertensive model. On the other hand, we observed that exercise clearly presented hypotensive effect, reducing SBP in Exercise L-NAME group ($P < 0·05$) (Figure 1).

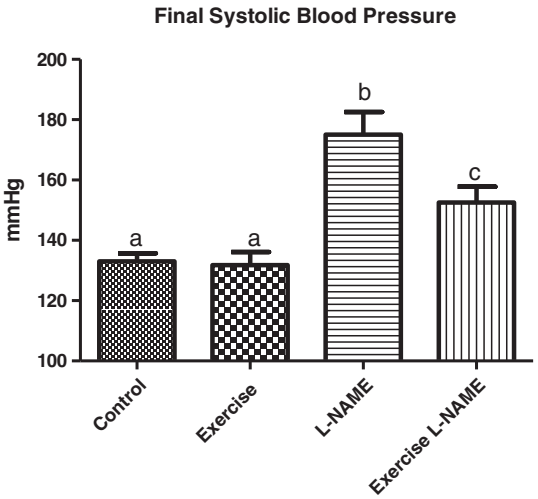

Figure 1. Final systolic blood pressure (SBP) measurements of Control group, Exercise group, L-NAME group and Exercise L-NAME group. SBP was followed as described in materials. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0·05$, $n = 10$ to each group). ANOVA–Duncan's Test

NO levels

Levels of NO content in kidney are shown in Figure 2(A), which displays a significant decrease in NO levels in the L-NAME group when compared with other groups ($P < 0·05$). As can be observed, NO levels in the Exercise L-NAME group remained close to the values found in normotensive groups. Figure 2(B) shows a negative Pearson's correlation between kidney NO levels and SBP ($r = -0·819$, $P < 0·05$), indicating that the NO levels decrease as the augment of BP.

Biochemical analysis

Table 2 shows lipid profile and renal markers of Control, Exercise, L-NAME and Exercise L-NAME groups. Cholesterol and triglyceride levels were significantly increased in the L-NAME group when compared with the other groups ($P < 0·05$). As can be observed, exercise training prevented the increase of cholesterol and triglyceride levels in hypertensive rats ($P < 0·05$). Exercise *per se* did not alter significantly the cholesterol or triglyceride levels when compared with the control group, although the values were lower than other groups.

Levels of renal function markers, urea and creatinine also presented a significant increase in the L-NAME-treated group when compared with other groups ($P < 0·05$) (Table 2). However, exercise training prevented this increase of urea and creatinine levels in hypertensive rats ($P < 0·05$). Exercise *per se* showed no effects in urea or creatinine levels in the Exercise group when compared with the control group.

Body weight and heart rate

No difference was observed in the food and water consumption after the administration of L-NAME in the experimental groups (data not shown). The same results were observed to body weight (data not shown). Moreover, the heart rate, expressed as cycles minute^{-1} (cpm), remained unchanged in the L-NAME-treated group and in the exercise groups compared with the respective control groups. However, although not statistically significant, we could observe that in the exercise groups, heart rate values were lower than control and L-NAME-treated groups, which was an exercise expected result (Figure 3).

Protein oxidation

Regarding the oxidative stress parameters, Figure 4(A) shows the protein oxidation, determined by protein carbonyl content in serum. As can be observed, there was a statistically significant increase in protein oxidation in the L-NAME-treated group ($P < 0·05$) when compared with the control and exercise groups. In the Exercise L-NAME group, it is clear that exercise has the potential to reduce

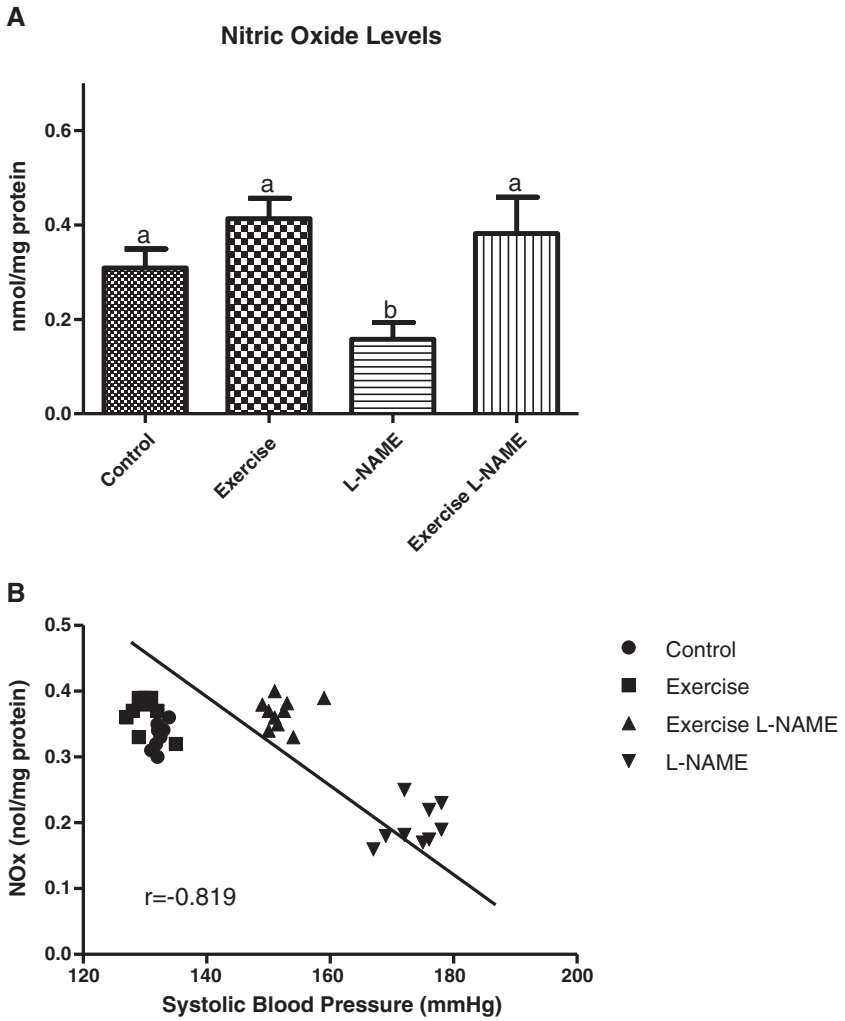

Figure 2. (A) Nitric oxide levels in the kidney of Control group, Exercise group, L-NAME group and Exercise L-NAME group. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0{\cdot}05$, $n = 10$ to each group). ANOVA–Duncan's Test. (B) Pearson's correlation between kidney levels of nitric oxide and systolic blood pressure ($r = -0{\cdot}819$, $P < 0{\cdot}05$)

protein oxidation in hypertensive rats ($P < 0{\cdot}05$). Figure 4(B) shows a positive Pearson's correlation between protein oxidation and SBP ($r = 0{\cdot}841$, $P < 0{\cdot}05$). As can be observed, the protein oxidation increases as BP augments.

Lipid peroxidation

Figure 5 shows TBARS values from serum (Figure 5A) and kidney (Figure 5B). In both cases, a rise in the lipid oxidation in hypertensive and sedentary rats ($P < 0{\cdot}05$) was observed when compared with normotensive groups. Moreover, the exercise training in the hypertensive group reduced the TBARS values in serum when compared with the L-NAME group ($P < 0{\cdot}05$); however, these results were not observed in TBARS from kidney. Figure 5(C) shows a positive Pearson's correlation between kidney TBARS values and SBP ($r = 0{\cdot}799$, $P < 0{\cdot}05$). As can be observed, the lipid peroxidation increases as the BP augments.

CAT and SOD activities

CAT determined in total blood and kidney is shown in Figure 6 (A,B). We observed a statistically significant decrease in the CAT activity in L-NAME-treated group when compared with the control and exercise groups ($P < 0{\cdot}05$) in samples of total blood and kidney. In the Exercise L-NAME group, although with no statistical significance, it was clearly that exercise had the potential to increase this enzyme activity in total blood of hypertensive rats when compared with the hypertensive sedentary group. In kidney, exercise had the ability to augment significantly the CAT activity in the exercise L-NAME group when compared with the L-NAME group ($P < 0{\cdot}05$).

The same results were found concerning the activity of SOD enzyme, as shown in Figure 6. In total blood and in kidney (Figure 7A,B), we observed a statistically significant decrease in the SOD activity in hypertensive sedentary group when compared with the control and exercise groups ($P < 0{\cdot}05$). In the Exercise L-NAME group, although with no statistical

Table 2. Lipid profile and renal markers of Control, Exercise, L-NAME and Exercise L-NAME groups

	Control	Exercise	L-NAME	Exercise L-NAME
Cholesterol ($mmol\,l^{-1}$)	$1{\cdot}51 \pm 0{\cdot}17^{a}$	$1{\cdot}39 \pm 0{\cdot}21^{a}$	$2{\cdot}21 \pm 0{\cdot}13^{b}$	$1{\cdot}65 \pm 0{\cdot}20^{a}$
Triglycerides ($mmol\,l^{-1}$)	$0{\cdot}83 \pm 0{\cdot}05^{a}$	$0{\cdot}78 \pm 0{\cdot}07^{a}$	$1{\cdot}17 \pm 0{\cdot}06^{b}$	$0{\cdot}94 \pm 0{\cdot}04^{c}$
Urea ($mg\,dl^{-1}$)	$51{\cdot}2 \pm 9{\cdot}7^{a}$	$47{\cdot}1 \pm 4{\cdot}5^{a}$	$87{\cdot}2 \pm 7{\cdot}5^{b}$	$59{\cdot}9 \pm 8{\cdot}2^{a}$
Creatinine ($mg\,dl^{-1}$)	$0{\cdot}43 \pm 0{\cdot}07^{a}$	$0{\cdot}38 \pm 0{\cdot}09^{a}$	$0{\cdot}59 \pm 0{\cdot}03^{b}$	$0{\cdot}48 \pm 0{\cdot}05^{a}$

Data are expressed as means ± SD. Different letters in the same line indicate differences among the groups and same letter in the same line indicates groups are not statistically different. ($P < 0{\cdot}05$; $n = 10$ animals per group). ANOVA–Duncan's test.

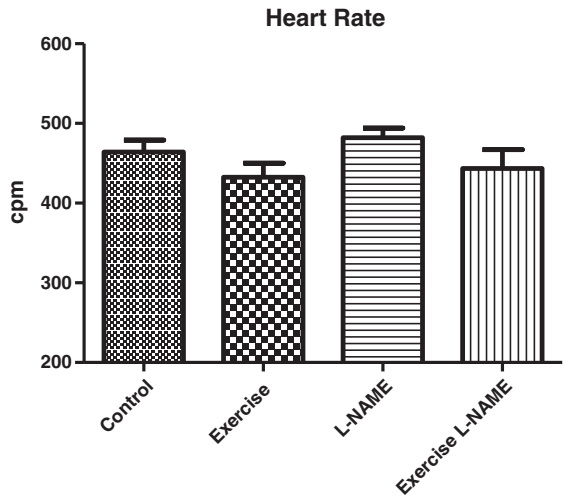

Figure 3. Heart rate values of Control group, Exercise group, L-NAME group and Exercise L-NAME group. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0{\cdot}05$, $n = 10$ to each group). ANOVA–Duncan's Test

significance, it was clear that exercise had the potential to increase this enzyme activity in total blood of hypertensive rats when compared with the hypertensive sedentary group. In kidney, exercise was able to significantly improve the CAT activity in the exercise L-NAME group when compared with the L-NAME group ($P < 0{\cdot}05$).

Figures 6(C) and 7(C) show a negative Pearson's correlation between kidney CAT and SOD activities and SBP ($r = -0{\cdot}723$ and $r = -0{\cdot}791$, respectively; $P < 0{\cdot}05$). As can be observed, both enzyme activities decrease as the BP increases.

NPSH levels

NPSH levels measured in plasma and kidney are shown in Figure 8(A,B). There was a decrease in the NPSH levels in the L-NAME-treated group in both plasma and kidney when compared with normotensive groups ($P < 0{\cdot}05$). In the Exercise L-NAME group, there was a statistically significant increase in the NPSH levels when compared with the L-NAME group ($P < 0{\cdot}05$). Plasma and kidney had the same behaviour in this measurement.

Ascorbic acid content

Figure 9 shows the ascorbic acid content in kidney. As observed in the figure, the groups studied did not present statistical differences. However, we can see an indicative of a decrease in the ascorbic acid levels in hypertensive sedentary rats.

DISCUSSION

Several studies have shown the beneficial effects of regular physical activity in reducing the elevated BP in both human[26,2] and animal hypertension models. Regarding to the experimental models of hypertension, physical training was efficient to reduce BP in spontaneously hypertensive rats,[37] Dahl salt-sensitive and salt-resistant rats,[35] deoxycorticosterone acetate (DOCA)-induced hypertension,[32] hypertension due to the manipulation of kidney arteries[34] and hypertension induced by L-NAME administration.[13,33,14]

Regular physical exercises have been recommended by health professionals to maintain good cardiovascular fitness and prevent or treat hypertension. For the evaluation of training-related effects in hypertension models, several kinds of exercise protocols have been applied. This way, it has become evident that regularly performed aerobic exercise significantly reduces the high BP in rats with spontaneous hypertension[32,36] and in rats with hypertension induced by L-NAME administration[13,33,14] as in the present study, where rats performed 6 weeks of swimming protocol, corresponding to moderate aerobic exercise.

Chronic administration of L-NAME, besides the induction of dose-dependent and time-dependent sustained elevation in BP,[6] is associated with a renal damage such as increased renal vascular resistance and proteinuria, coincident with the appearance of renal histopathology as glomerular sclerotic injury and arteriolar narrowing.[6,48] These damages in kidneys are associated with the increased production of ROS and decreased levels of NO,[48–50] as found in this work.

In the present study, the administration of L-NAME significantly decreased the levels of NO in kidney and displayed a negative correlation with the BP corroborating the literature[48,50] and indicating a probable vasoconstriction that induced hypertension by the absence of bioactive NO.

However, our data showed that 6 weeks of swimming training significantly prevented the decrease of kidney NO

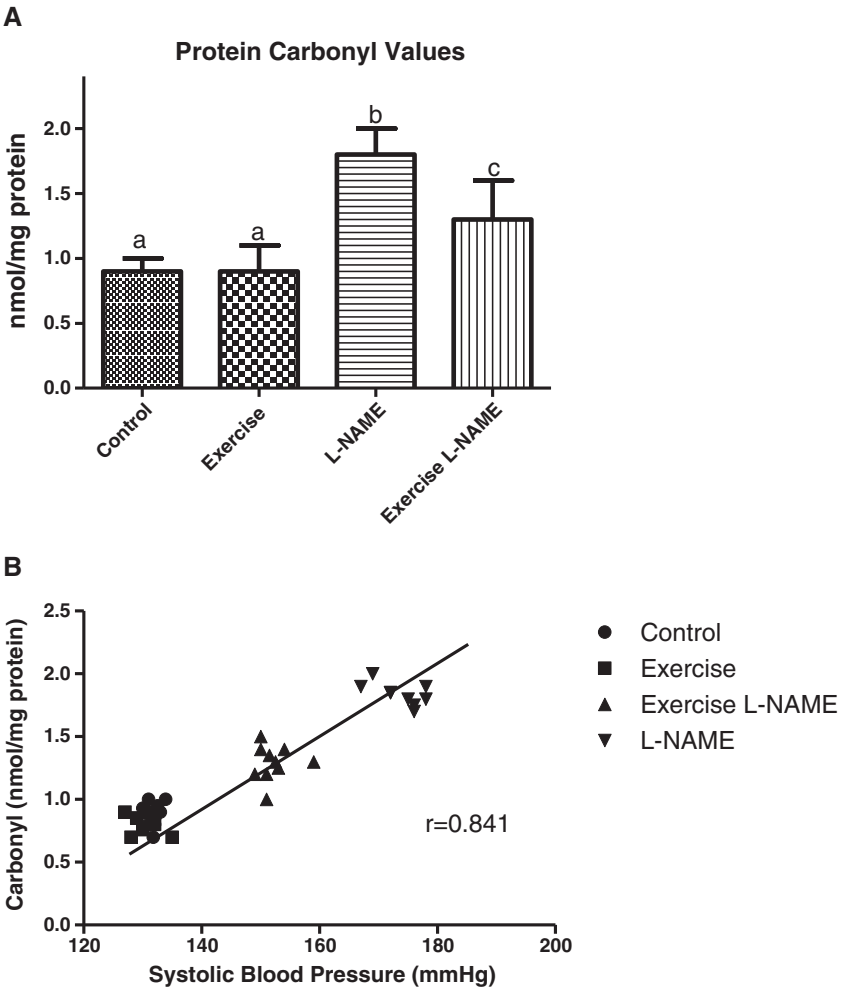

Figure 4. (A) Protein carbonyl values content in serum of Control group, Exercise group, L-NAME group and Exercise L-NAME group. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0{\cdot}05$; $n = 10$ to each group). ANOVA–Duncan's Test. (B) Pearson's correlation between protein carbonyl values content in serum and systolic blood pressure ($r = 0{\cdot}841$, $P < 0{\cdot}05$)

levels in hypertensive rats, indicating beneficial role of exercise conditioning in NO levels. Exercise conditioning is well related to the improvement of NO levels in L-NAME-treated rats.[9] NO has cardioprotector and vasodilatador effects, being extremely important to the regulation of BP. Increased levels of NO, as evidenced also by our study, have been considered the main mechanism for lowering BP due to physical exercise.[51,13,31,52]

Hypertension is a disease characterized by multiple alterations in the structure and function of the cell membrane, and it is often associated with important metabolic abnormalities including those concerning lipid metabolism.[53] Dyslipidemia accompanying essential hypertension consists of elevated plasma triglycerides, low high-density lipoprotein (HDL) cholesterol and increased levels of atherogenic low-density lipoprotein (LDL) cholesterol particles.[54,13,55] The altered membrane microviscosity seen in hypertensive subjects reflects the changes of membrane lipid composition resulting from the intensive exchange between circulating and membrane lipids, as well as from abnormal cellular lipid synthesis and metabolism, which includes oxidative stress and lipid peroxidation[54] as verified in our study by TBARS levels and the other biomarkers of tissue damage that discussed further.

Our results show a significant rise in serum cholesterol and triglyceride levels in L-NAME-treated rats. However, exercise training was efficient to prevent lipid alterations. This prevention may be explained by the increased demand of the working muscle for fatty acids as an energy-yielding substrate as well as the replenishment of fatty acid containing stores for the regeneration of damaged muscle fibres.[56,57] Lower cholesterol and triglyceride levels can also be explained through the biochemistry of exercises because there is an increased activity of lipoprotein lipase, which augments the degradation of triglycerides from very low-density lipoproteins and causes the lipoprotein particles to shrink.[56,57]

According to Herzberg,[56] aerobic exercise has been shown to reduce the risk of cardiovascular disease, and this reduction is at least partially mediated by changes in circulating lipoproteins resulting from adaptive changes in enzymes involved in

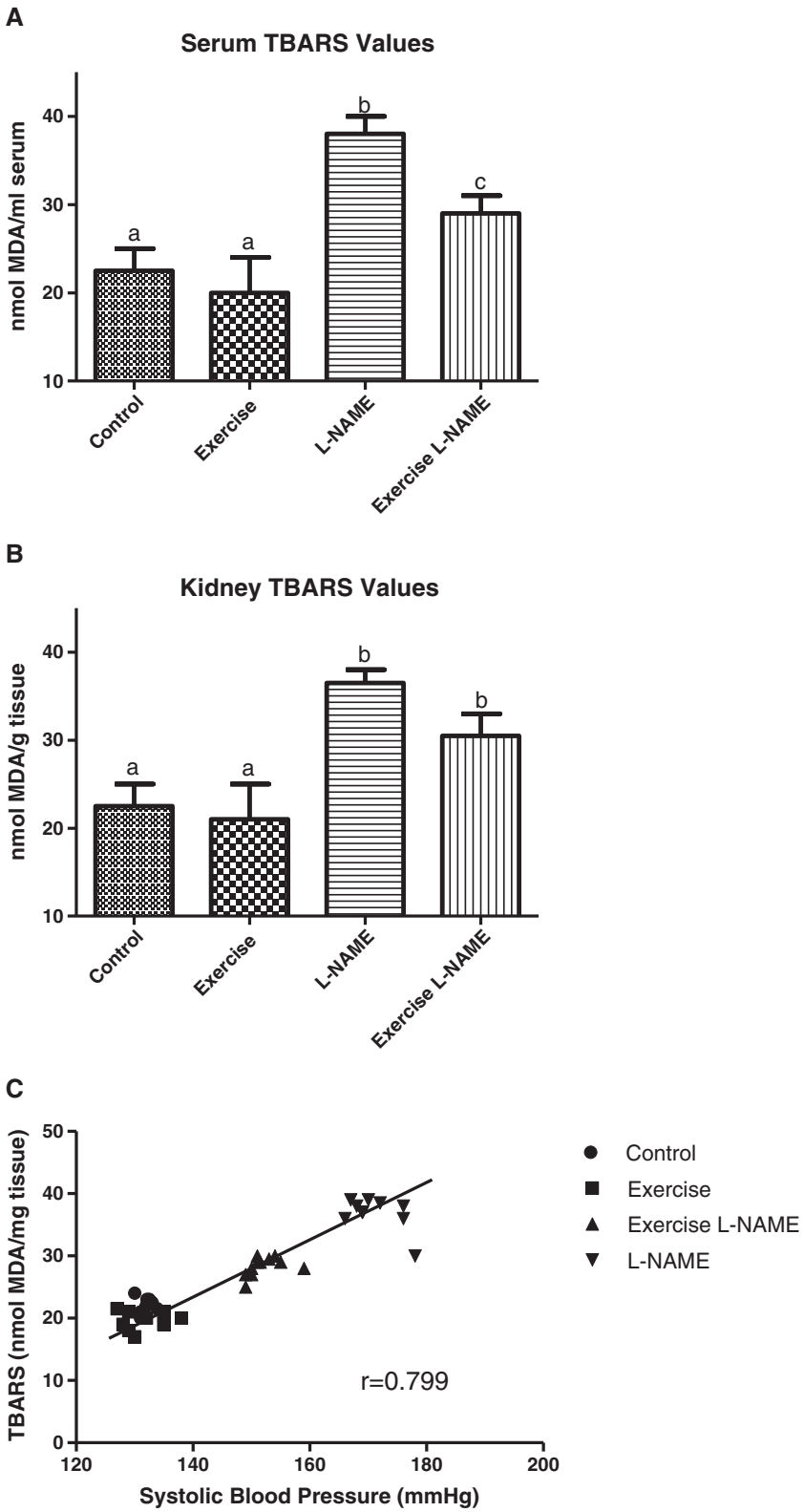

Figure 5. Levels of thiobarbituric acid reactive substances (TBARS) in serum (A) and kidney (B) of Control group, Exercise group, L-NAME group and Exercise L-NAME group. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0{\cdot}05$; $n = 10$ to each group). ANOVA–Duncan's Test. (C) Pearson's correlation between kidney TBARS levels and systolic blood pressure ($r = 0{\cdot}799$, $P < 0{\cdot}05$)

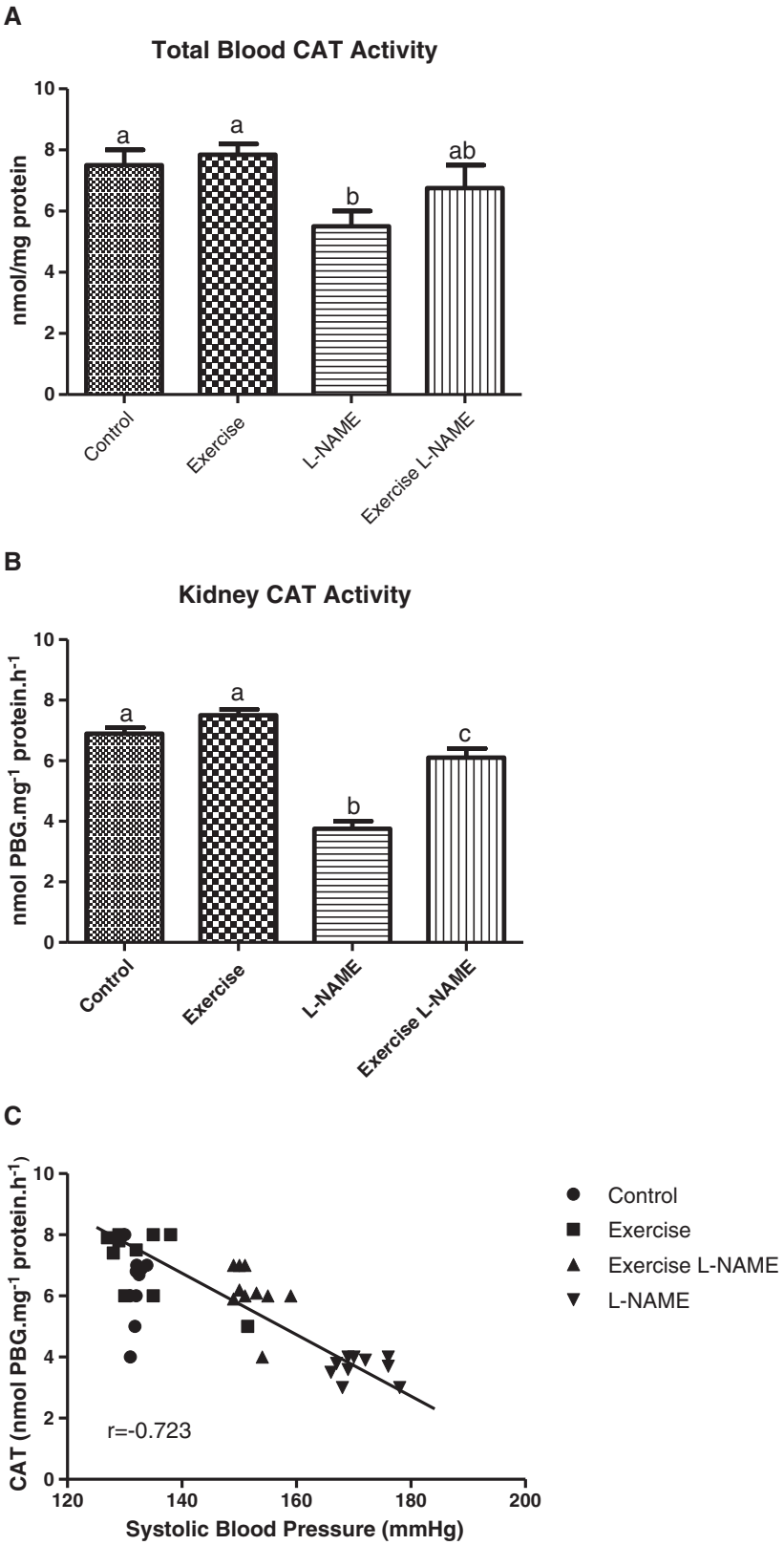

Figure 6. Catalase (CAT) activity in total blood (A) and kidney (B) of Control group, Exercise group, L-NAME group and Exercise L-NAME group. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0{\cdot}05$; $n = 10$ to each group). ANOVA–Duncan's Test. (C) Pearson's correlation between kidney CAT activity and systolic blood pressure ($r = -0{\cdot}723$, $P < 0{\cdot}05$)

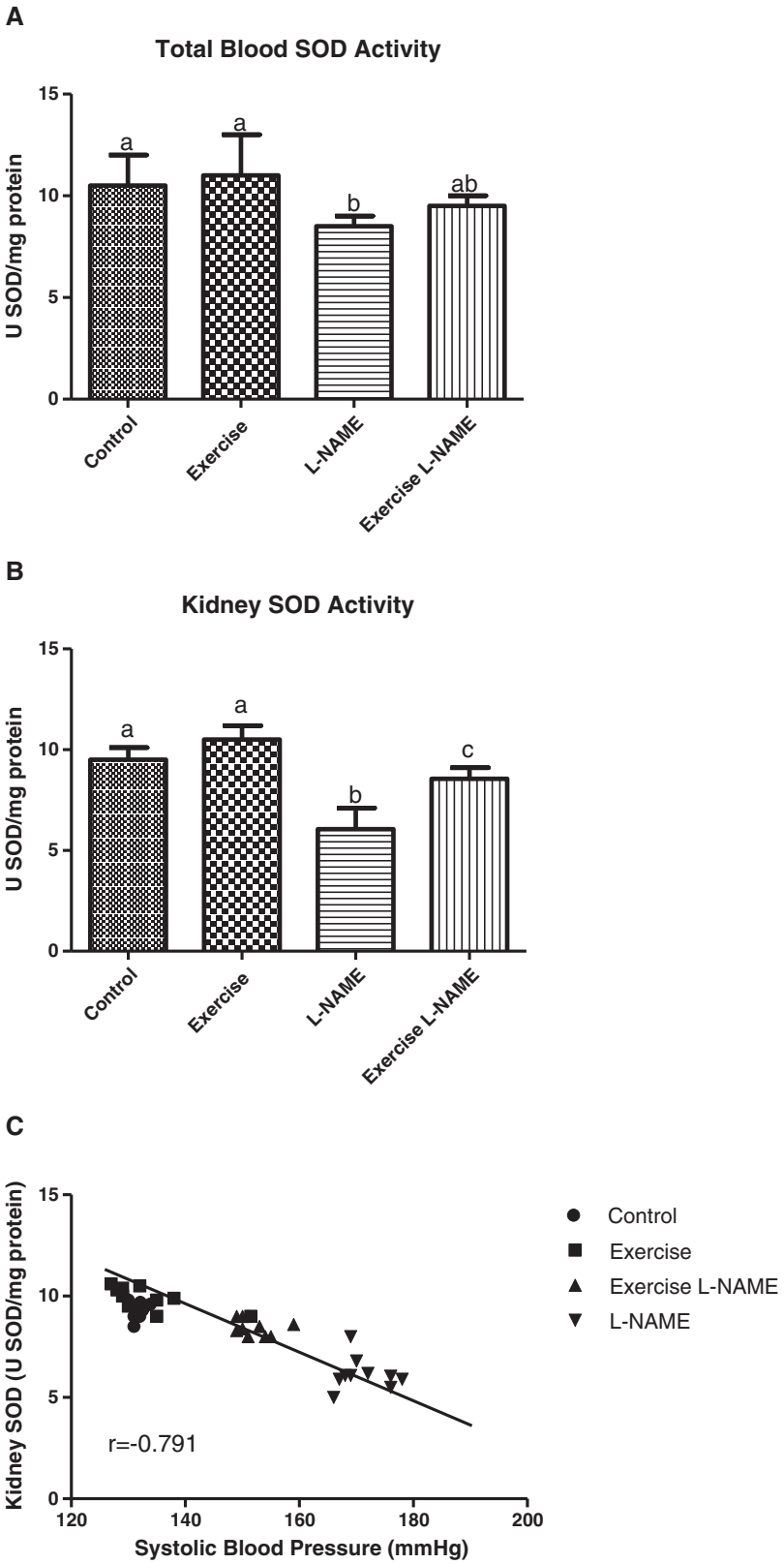

Figure 7. Superoxide dismutase (SOD) activity in total blood (A) and kidney (B) of Control group, Exercise group, L-NAME group and Exercise L-NAME group. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0{\cdot}05$; $n = 10$ to each group). ANOVA–Duncan's Test. (C) Pearson's correlation between kidney SOD activity and systolic blood pressure ($r = -0{\cdot}791$, $P < 0{\cdot}05$)

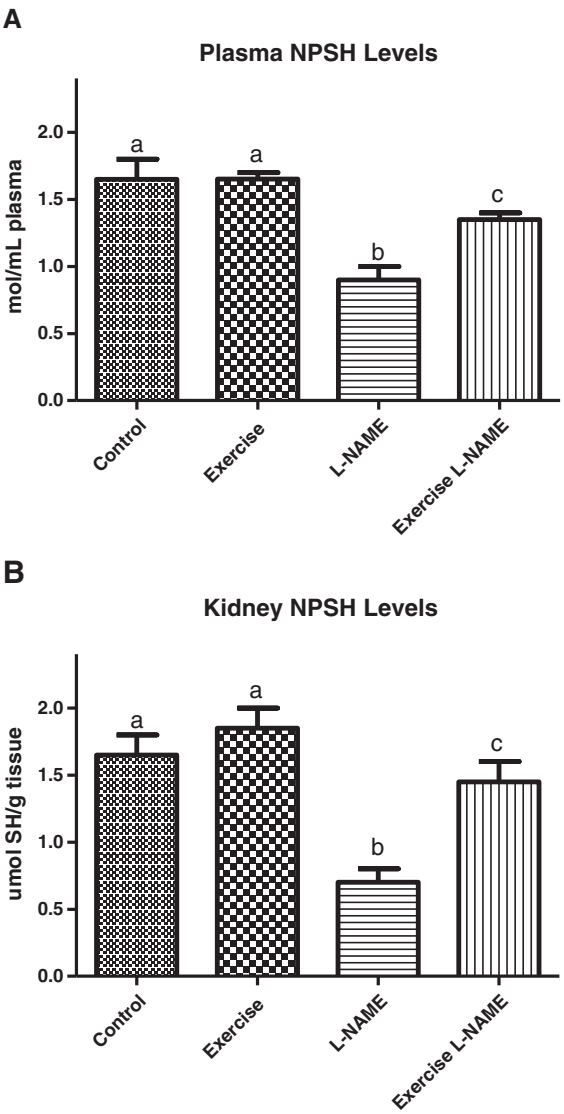

Figure 8. Non-protein thiols (NPSH) levels in plasma (A) and kidney (B) of Control group, Exercise group, L-NAME group and Exercise L-NAME group. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0{\cdot}05$; $n = 10$ to each group). ANOVA–Duncan's Test

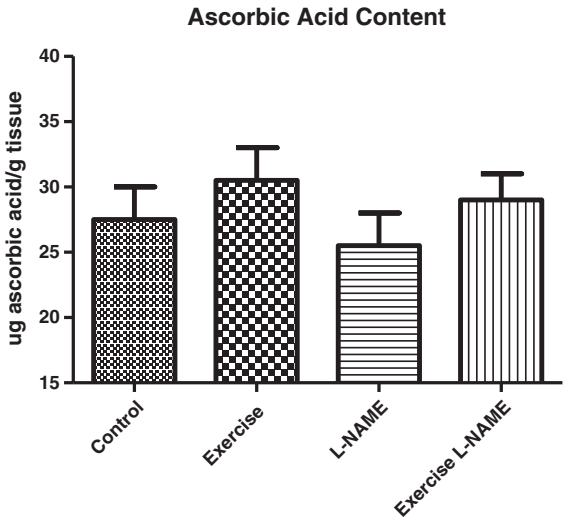

Figure 9. Ascorbic acid in kidney of Control group, Exercise group, L-NAME group and Exercise L-NAME group. Data are presented as mean ± SD. Groups with different letters are statistically different ($P < 0{\cdot}05$; $n = 10$ to each group). ANOVA–Duncan's Test

their metabolism. Moreover, aerobic exercise is associated with reductions in LDL, total cholesterol and triacylglycerol and increases in HDL. Exposure to oxygen can oxidatively damage LDL.[56,57] Oxidized LDL is a risk factor for atherosclerosis. Although aerobic exercise can cause oxidative damage when performed acutely, there are adaptive changes resulting from chronic exercise, as in our study, which can result in the improvement of the lipid profile.[56,57]

In addition to dyslipidemia, in the current study, hypertensive rats also presented renal damages that were evidenced by the elevation in serum urea and creatinine levels, which are considered as significant markers of renal dysfunction.[58,59] Of great importance, physical training prevented the increase in the levels of urea and creatinine in L-NAME-treated rats. These findings suggest that moderate aerobic exercise possesses the potential to attenuate renal injury caused by hypertension. This can be associated directly with the effect of physical training on improving the body antioxidant capacity, which protects the kidneys against oxidative damage, as evidenced in this and other studies.[58–60] On the basis of these results, we could suggest that physical training improves the dyslipidemia while inhibiting the progression of renal dysfunction in L-NAME-induced hypertension in rats.

Changes in the lipid profile and the evidence of renal injury verified in this study in hypertensive rats can be linked to the high production of ROS and oxidative stress, which has been shown to accompany this disease.[17,16]

It is well established that there is a link between hypertension and oxidative stress.[16,13] The relationship between the development of hypertension and the increased bioavailability of ROS, decreased antioxidant capacity, or both, has been demonstrated in several experimental models of hypertension, as well as in human hypertension.[61,16,13,62] However, it still remains unclear whether increased ROS levels are a cause or a consequence of high BP.[17] Furthermore, growing evidence from animal studies suggests that oxidative stress in kidney could be a key factor in the development and persistence of hypertension.[49,50] For this reason, in this study, we focused the investigation on kidney alterations as well as on circulating fluids that reflect alterations in the whole organism. It is relevant to highlight the importance of this study on the investigation of the benefits of the physical training on kidney and circulating fluids mainly, because we face lack of information even nowadays when it is well established that exercise training improves the hypertensive subject health.

Results of the present study confirm the assertive that a great production of ROS is slightly linked to hypertension development. Proteins are one of the major targets for ROS

because of their high overall abundance in biological systems.[19] Oxidative damage to proteins can occur directly by the interaction of the protein with ROS or indirectly by the interaction of the protein with a secondary product (resulting from the interaction of the radical with lipid or sugar molecule).[19] Carbonylation of proteins is an irreversible oxidative damage, often leading to the loss of protein function, which is considered a widespread indicator of severe oxidative damage and disease-derived protein dysfunction.[19] This way, the formation and accumulation of protein carbonyls has been one of the most commonly used methods for assessing overall protein oxidation.[19]

In our study, hypertensive rats showed an increase in protein oxidation, which suggests a severe damage in several tissues.[19] This damage in overall protein can contribute to a major development and aggravation of this disease,[19,16] as shown by the positive correlation between protein oxidation and BP. However, it was clearly showed that physical training had the ability to prevent protein oxidation, and the mechanisms of this prevention are discussed hereinafter.

Another common method to access oxidative damage in tissues is to verify the lipid peroxidation through the contents of TBARS, where MDA levels are the main substances. MDA is a physiologic ketoaldehyde produced by peroxidative decomposition of unsaturated lipids.[19] MDA can be produced as a result of a normal metabolism, but a high production of MDA has been reported as an indicative of oxidative stress.[19] In this study, L-NAME-treated showed a significant increase on lipid peroxidation in both serum and kidney, which indicates severe damage in tissues. To confirm the link between lipid peroxidation and hypertension, a positive correlation in these variables was observed, indicating that the oxidative injury becomes worse with increasing BP, as already documented in hypertension.[17,16,13] On the other hand, our data showed that exercise training depleted serum MDA levels indicating the adaptive response of the exercise conditioning against peroxidative injury to vascular tissue. Previous reports have also demonstrated the suppression of lipid peroxidation in other tissues as a result of exercise training.[63,64,13]

In addition, it is well established that there is an elevation in NO production after exercise training[31,52], and the elevated NO levels after training may also inhibit lipid peroxidation.[51,13] On the other hand, exercise training cannot prevent lipid peroxidation in kidney, but an indicative of this prevention was observed. Thus, in this line of reasoning, we could infer that probably if the period of training had been extended, the lipid peroxidation in kidney could be reverted by training.

Free radical-scavenging enzymes such as SOD and CAT are part of the first line of cellular defence against oxidative injury, decomposing $O_2^{\cdot-}$ and H_2O_2 before interacting to form the more reactive hydroxyl radical ($^{\cdot}OH$).[23] In our study, chronic L-NAME administration significantly decreased both total blood and kidney antioxidant enzyme activities, indicating the inability of these tissues to scavenge ROS in hypertensive rats. Furthermore, our results displayed a negative correlation between kidney CAT and SOD activities and BP, indicating that with the aggravation of the levels of BP, the enzymatic antioxidant capacity of the kidneys became worse. This enzyme suppression in L-NAME-treated rats could also be a result of protein oxidation, taking into account that the protein carbonyls were acutely elevated in hypertensive rats. Moreover, Alvarez *et al.*[65] have demonstrated that some ROS, such as peroxinitrite ($ONOO^-$) generated during oxidative stress, can nitrosate and inactivate SOD. In summary, there are several pathways to explain the suppression of antioxidant enzyme activities in hypertension.

Other reports have found the same suppression in SOD and CAT activities related to animal models of hypertension.[66,13,67] According to Panico *et al.*[67], a down-regulation of SOD and CAT probably contributes to the pro-oxidant state of kidneys in hypertension, which is important because redox balance influences proximal tubule fluid reabsorption in the hypertensive kidney.

This decrease in the CAT and SOD activities in kidney was prevented by exercise training in our study. Our findings indicate that the kidney antioxidant enzyme activities significantly enhanced after 6 weeks training to rats, indicating the oxidative conditioning of organism. Increased antioxidant enzyme activities have also been previously reported in animals after exercise training.[13,68,24,69] Furthermore, a study carried out by Claudino *et al.*[9] showed that exercise reversed the impairment on the SOD activity in L-NAME-treated rats, corroborating our results.

It was suggested that this improvement in the enzymatic antioxidant system may enhance vascular dilation by allowing for a more rapid elimination of superoxide anion that would prolong the half-life of NO in the cells.[13,50] In diaphragm muscle, short term (5-day) training of rats increased both the enzymatic and non-enzymatic antioxidant defence mechanisms of the tissue, with increases being recorded in CAT, glutathione peroxidase and SOD activities.[69]

Regarding exercise training effects in blood antioxidant enzymes, we observed that exercise improve partially the decrease of SOD and CAT activities in Exercise L-NAME group. According to Nikolaidis and Jamurtas,[70] the exact origin of reactive species and oxidative damage detected in blood because of exercises is largely unknown. Blood interacts with all organs and tissues and, consequently, with several possible sources of reactive species. It is known that the factors regulating the activity of blood antioxidant enzymes *in vivo* related to exercise are poorly understood.[70] This way, we encourage more studies aiming to elucidate these factors, enabling us to interpret more clearly the effects of exercise on blood antioxidant enzymes. However, our study makes clear that exercise training has the potential do avoid blood damage, although there is a lack of mechanisms as described earlier.

In our study, it is interesting to note that in kidney, exercise had the potential to improve enzyme activities, but TBARS levels remained high when compared with control groups. It indicates that kidney is greatly affected by ROS in hypertension and suggests that this up-regulation in the SOD and CAT activities could be a body compensatory mechanism trying to scavenge the high production of

ROS in kidney, besides being an exercise response. In opposite of these, our results from blood did not present the same relation, what could indicate that the damage in other body tissues were more easily reversed by exercise training when compared with kidney damage.

Literature shows that during exercise there is a great production of ROS by mainly the mitochondria because of moderate exercise, such as swimming, as in this study, seems to increase the oxygen consumption rate by 10–20-fold.[24] Furthermore, Hansford *et al.*[71] found an increase on H2O2 production by rat heart mitochondria with lower values of O2 in the respiratory chain and using inhibitors, which can be similar to what happens in an exercise session. Thus, the ROS generated during exercise act as signals to increase the production of enzymes relevant to the adaptation of cells to exercise[72,13,23] and improve the body antioxidant capacity.

Noting this effect of exercise training, we can discuss the redox changes in GSH (the major non-enzymatic endogenous antioxidant), measured by NPSH levels, which have been reported to be a good marker of hypertension both in animals and humans.[13,73,62] The chronic administration of L-NAME significantly decreased plasma and kidney NPSH levels probably by depleting the GSH and increasing glutathione disulfide (GSSG), which clearly demonstrate the oxidative stress response during hypertension. Interestingly, depletion of tissue GSH by buthionine sulfoximine in rats has been demonstrated to increase BP.[62] Moreover, central GSH has also been shown to regulate the BP in rats.[74] Husain[13] also found the same reduction on aorta GSH levels in L-NAME-treated rats.

The results of the present study show that exercise training significantly increased the plasma and kidney NPSH levels in Exercise L-NAME group when compared with L-NAME group, indicating the beneficial effects of the training in the body antioxidant capacity. There are evidences that the skeletal muscle adapts exercise training by increasing the GSH content and reducing GSSG.[23,75,24] The enhanced NPSH levels by physical conditioning seem to be essential in maintaining the redox state and to cope with the oxidative stress during trained exercise. The present data and the aforementioned reports ascertain the role of GSH in the regulation of BP and therapeutic importance using moderate exercise training as an antioxidant in hypertensive patients in the clinic.

Vitamin C, referred to as antioxidant, is considered the primary antioxidant in plasma and cells to be depleted under conditions of oxidative stress and has been suggested to limit oxidative damage in humans, thereby lowering the risk of certain chronic diseases, such as hypertension and cardiovascular diseases in general.[22,25] Under certain conditions, L-ascorbic acid can exhibit antioxidant properties and thus may reduce the formation of oxidized small molecules, proteins and lipids, which are a possible cause of cellular de-regulation.[25] In this line of reasoning, it is common to find the content of ascorbic acid reduced in several pathologies as a consequence of oxidative stress.[76–78] Although not reaching statistical relevance, our findings also showed a slight decrease in the vitamin C content in hypertensive rats, which could be due to the increasing utilization of vitamin C as an antioxidant defence against ROS. Six weeks of swimming training, although not significant, prevented this reduction in the vitamin C content in hypertensive rats. Previous reports that proposed to access the content of ascorbic acid related to exercise are generally performed with the supplementation of these antioxidants in order to prevent oxidative stress caused by acute exercise.[79,80] There are no studies reporting changes in content of ascorbic acid due to training adaptation, and this is the first one that had this aim.

The findings of the present work are of growing importance because all of the established or non-traditional cardiovascular risk factors have been associated in clinical or experimental studies with evidence of increased ROS, which may be linked not only to vasoconstriction, salt retention, and hypertension but also to several other adverse long-term consequences. With this, we can highlight the great potential of moderate exercise training in preventing or treating hypertension, because we found that exercise had the ability to improve kidney NO levels and prevent oxidative damage in kidney, serum and plasma of rats treated with L-NAME and exposed to swimming. Furthermore, it also improved the antioxidant defences in kidney, serum, plasma and whole blood of these rats.

These observations suggest that training up-regulated the antioxidant defence system, thereby scavenging free radicals/ROS, preserving good levels of lipid profile and preventing kidney damage. We can conclude that aerobic exercise conditioning provided significant protection against oxidative injury in L-NAME treated hypertensive rats by up-regulating the NO and antioxidant systems, scavenging free radical/ROS and preserving the BP.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

ACKNOWLEDGEMENTS

The authors wish to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Instituto Nacional de Ciência e Tecnologia (INCT) and Rede Instituto Brasileiro de Neurociência (IBN-Net). Furthermore, the authors would like to thank to the members of BioEx (Laboratório de Bioquímica do Exercício – UFSM) and to Professor Cristina Ribas Furstenau for the help during the experiment.

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