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Protective effect of regular aerobic training on inflammatory and toxicity markers of lung tissue in L-NAME-induced hypertension

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ABSTRACT

Hypertension is a multi-factorial, life-threatening disease. The present study was designed to investigate protective effect of aerobic regular training on inflammatory and toxicity markers in lung tissue of male wistar rats exposed to chronic nitro-L-arginine-methyl ester(L-NAME)-induced hypertension. Thirty two adult male Wistar rats are randomly classified into 4 groups; aerobic training, L-NAME, saline and control groups. Hypertension was induced by administration of L-NAME (10 mg/kg) for 8 weeks and 6 sessions a week. Aerobic training was performed between 25 to 64 minutes and the speed 15 to 22 m/min for 8 weeks and 5 sessions a week. Chronically administration of L-NAME cause a significant increase in angiotensin converting enzyme (ACE), interleukin 6 (IL-6) levels, and a significant decrease in superoxide dismutase (SOD) and nitric oxide (NO) levels, as compared to saline and control groups. In contrast, aerobic training for 8 weeks caused a significant increase in SOD, NO and a significant decrease in IL-6 and protein carbonyl (PC), as compared to L-NAME and saline groups. Our study suggests aerobic regular exercise provided significant protection against L-NAME-induced toxicity in lung tissue by up-regulation of antioxidant systems and down-regulation of the inflammatory and vasoconstrictor factors in hypertensive rats. These results suggest that aerobic regular exercise may be considered as a potentially useful strategy to limit toxicity in lung tissue.

Keywords: Toxicity, Oxidative stress, Aerobic training, L-NAME, Inflammation, Hypertension.

INTRODUCTION

Hypertension is an over whelming global challenge, which ranks third as a means of reduction in disability-adjusted life-years. Hypertension and its related conditions such as coronary artery disease, stroke, heart failure, and chronic kidney disease is a growing public health issue for which successful treatment often remains inadequate (Mehmet *et al.*, 2012). Pulmonary arterial hypertension (PAH) is a multi-factorial and deadly disease with a mortality rate of 20 to 40%, 3 years after diagnosis (Reshma *et al.*, 2011, Harm *et al.*, 2009). PAH is characterized by elevated pulmonary arterial pressure (PAP) ≥ 25 mmHg at rest as assessed by right heart catheterization, is a fatal disease caused by small pulmonary artery obstruction by vascular proliferation and remodeling. It is caused by increased resistance at one of several sites in the pulmonary circulation, including the heart's left chambers and valves (Afshar *et al.*, 2012).

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PAH is characterized by increased pulmonary vascular resistance (PVR), frequently leading to right-sided heart failure (HF), endothelial dysfunction and death (Reshma *et al.*, 2011, Fukumoto *et al.*, 2011). The pathogenesis of PAH is complex and multi factorial, often resulting from interactions between genetic susceptibility and environmental or acquired factors, including hemodynamic stress, inflammation, hypoxia, and others (Abman *et al.*, 2011).

Inflammation is a feature of PAH and increased circulating levels of cytokines are reported in patients with PAH (Soon *et al.*, 2010). Animal models also support the role of inflammatory cytokines in the initiation and progression of PAH. For example, IL-6 is consistently elevated in animal models of experimental PAH. To our knowledge, there are few studies dealing with the effect of regular aerobic training on inflammation and oxidative stress in lung tissue in rats exposed to N-nitro L-arginine methylester (L-NAME). Furthermore, oxidant stress of the walls of pulmonary blood vessels is the important factor in the pathogenesis of PAH, since the inhibition of this free radical damage by antioxidants or by reactive oxygen species (ROS) scavengers prevented or attenuated the development of hypoxic pulmonary hypertension (Hodyc *et al.*, 2012). ROS are constantly produced in the cells, but under normal physiological conditions the enzymatic and non-enzymatic antioxidant mechanisms of the cell overcome the destructive potential of ROS. Over production of ROS or a decrease in antioxidants results in oxidative stress, and may cause cellular damage by peroxidation of membrane lipids, sulfhydryl enzyme inactivation, protein cross-linking and DNA breakdown. On the other hand, abnormalities in vasodilator substances and specifically nitric oxide (NO) have been implicated in the pathogenesis of PAH (Weiling *et al.*, 2004). NOS convert L-arginine to NO and L-citrulline in a reaction that requires oxygen that it inhibitors by replace arginine with its analogues such as L-NAME (Weiling *et al.*, 2004, Rafei *et al.*, 2010). L-NAME is a nonspecific inhibitor of NO synthase (NOS) and causes an increase of blood pressure in a dose dependent manner when administered to the experimental animals (Bernátová *et al.*, 2007, Hlavačková *et al.*, 2011). The protective role of regular aerobic exercise as an antioxidant factor on vascular function and oxidant/antioxidant process in lung during chronic hypertension has not been sufficiently studied.

The control of hypertension appears to be one of the major therapeutic goals. Physical activity has been shown to be a useful non-pharmacological strategy for the prevention and management of hypertension (Cardoso *et al.*, 2010). Wallace reported that exercise as the most promising non-pharmacological treatment of hypertension (Wallace. 2003). Several researchers have reported a single bout of physical exercise has been shown to induce formation of ROS and nitrogen species and the related oxidative damage. On the other hand, regular training is known to increase the resistance against ROS induced lipid peroxidation, and to decrease the accumulation of oxidative protein and DNA damage (Belviranl *et al.*, 2006). Researchers reported many patients with PAH continue to experience significant impairment in

physical function and quality of life (Fowler *et al.*, 2011). Grünig et al reported exercise training in PAH is an effective but not a completely harmless add-on therapy even in severely diseased patients and should be closely monitored (Grünig *et al.*, 2012). Also, Arena reported that exercise training is likewise beneficial in patients with interstitial lung disease and PAH (Arena *et al.*, 2011).

Although, a partial list of proposed mechanisms for exercise-induced protection, much controversy exists concerning the effects of aerobic training on the oxidative status and antioxidant defense systems of the lung tissue, particularly during chronic Nitro-L-arginine-methyl ester (L-NAME)-induced hypertension. Furthermore, despite the knowledge that hypertension can induce oxidative stress (Mahmoodi *et al.*, 2012), and the effects of these biomarkers on blood pressure in different tissues, there are few data available with respect to effects of regular aerobic training on lung tissue, particularly during chronic L-NAME-induced hypertension. Other property of our study is the application of non-pharmacological strategies, without undesirable serious toxic side effects on lung tissue. This method had been recommended as a useful tool for long term health care service programs for patients with chronic illness such as hypertension.

The hypothesis proposed was that if the hypertension and PAH pathogenesis involved various factors including ROS, stress oxidative, anti-oxidative and inflammatory biomarkers, and on the other hand, if hypertension is related to free radical formation, oxidative stress and inflammation, an enhancement in antioxidant/oxidation ratio after regular aerobic exercise may protect against L-NAME - induced hypertension in the lung. Thus, the purpose of our study was to determine the protective effect of 8 weeks of aerobic training on the inflammatory marker (interleukin 6, [IL-6]), vascular dysfunction (nitric oxide [NO] and angiotensin-converting enzyme [ACE]) and oxidative/antioxidant (superoxide dismutase [SOD] and protein carbonyl [PC]) markers of lung tissue in the rats exposed to chronic L-NAME-induced hypertension.

MATERIALS AND METHODS

Experimental Design and Experimental Environment

All experiments were performed in accordance with the guidelines outlined by the Experimental Animal Laboratory and approved by Department of Physiology, University of Mazandaran and were performed according to guiding procedures in the Care and Use of Animals, prepared by the Council of the American Physiological Society. The experiments were carried out with thirty two male Wistar rats, (8-week-old, initially weighing 240 ± 20 g), which were obtained from the Pasture Institute of Iran. Rats were housed in standard cages of polycarbonate ($20 \times 15 \times 15$ cm), a large air-conditioned room with a controlled temperature of $22 \pm 2^\circ\text{C}$, light- dark cycles of 12:12 hours and humidity of $50 \pm 5\%$. The pollutant standard index (PSI) was in the acceptable range as determined by the Iranian Meteorological Organization. Rats were fed with a standard rat chow provided by Pars Institute for animals and poultry with a daily regimen of 10 /100 gr of body weight for each rat. Water was available ad libitum.

Familiarization with Exercise Training and Subjects

Classification

Rats in all groups were adapted to the treadmill by running for 5 days. The familiarization protocol was designed as once a day for 10 min/session at a speed of 10 m/min at a slope of 0°. An electric stimulus (30 V, 0.5 A) was manually turned on for less than 2 s when the animals stayed on the electric grid for longer than 10 s. Following this familiarization period, they were randomly assigned into four experimental groups. The groups were defined as follows:

Group 1– the control group; the animals were not exposed to any variable. Group 2– the saline group; these rats received NaCl solution that was injected with 0.1 mg/kg dosage, intraperitoneally, in the same manner and for the same duration of time as other groups. Group 3– N(ω)-nitro-L-arginine methyl ester (L-NAME); the animals were exposed to L-NAME at a concentration of 10 mg/kg in the form of a solution, intraperitoneally, 6 days weekly for 8 wks, in order to induce the hypertension (Selvi *et al.*, 2007, Therrien *et al.*, 2009). Group 4 – aerobic exercise; the rats in this group similarly received L-NAME, and in addition they performed progressive running exercise of 15 to 22 m/min for 25 to 64 min, 5 times a week. Due to the sloping surface can damage the body cell membranes (Dabidi Roshan *et al.*, 2012); the training program was conducted on the even surface.

L-NAME-induced hypertension

According to some researchers results induction of L-NAME causes hypertension in rats (Mohamed *et al.*, 2002, Sventek *et al.*, 1996), thus hypertension was induced by administration of the soluble analogue of L-arginine at a concentration of 10 mg/kg intraperitoneally, 6 days weekly for 8 wks.

Preparation of Lung tissue and Biochemical Analysis

All groups were anesthetized with ketamine and Xylazine and decapitated after 10 to 12 hours overnight fasting. Blood samples were collected 24 hours after the last dose of treatment. These blood samples were initially centrifuged by a refrigerated centrifuge at 3000 rpm for 15 minutes within 30 minutes of collection and then stored at -80°C for subsequent assay of angiotensin-converting enzyme (ACE) and nitric oxide (NO).

The Thoracic cavity was then opened and the lung tissues were quickly excised from the umbilical root. Lung tissue was weighed and was placed into Petri dishes containing cold isolation medium (0.1 mol/L K₂HPO₄, 0.15 mol/L NaCl, pH 7.4) to remove the blood and were frozen immediately in liquid nitrogen and stored at -80°C for subsequent analysis of protein carbonyl (PC), superoxide dismutase (SOD) and interleukin-6 (IL-6). Lung tissue was squashed in liquid nitrogen, homogenized in a lysis buffer (5 ml/g of tissue) with a protease inhibitor cocktail for mammalian cell and tissue extracts (Sigma Aldrich, St. Louis, U.S.A) 100 μ l/1 ml, and 10 mM Tris base (Sigma-Aldrich, St. Louis, U.S.A), pH 7.4 and centrifuged at 1600 g at 4 °C for 15 min. Lung tissue supernatant was diluted 1:30. Plasma was diluted 1:10. Protein carbonyls (PC) were analyzed according to Dabidi Roshan *et al*

(2011). Homogenate samples were centrifuged at 500 g and 4°C for 3 min. Aliquots containing 900 μ l of the resulting supernatant were incubated for 15 min with 0.9% streptomycin sulphate and 0.1% Triton X-100, and centrifuged at 12,000 g for 10 min at 4°C. Aliquots of 0.5 ml supernatant were incubated for 60 min in the dark, with 2 ml of 2.5 M HCl, or with 2 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl, and shaken every 10 min. Protein concentration in the samples was determined by measuring the absorbance at 280 nm using a bovine serum albumin standard curve in 6 M guanidine hydrochloride and 20 mM potassium phosphate buffer (pH 7.3).

Superoxide dismutase (SOD) activity was determined spectrophotometrically using the method described by Dabidi Roshan *et al* (2011). In brief, for total SOD (tSOD) activity the adequate amount of protein (2 mg tissue wet weight) was incubated at 25 °C with 1 mM N,N'-bis(2-(bis(carboxymethyl)amino)-ethyl)glycine (DTPA) in 50 mM Tris-HCl, pH 8.2, in 1 ml final volume. Reaction was started with 0.3 mM pyrogallol, in which the auto-oxidation rate was recorded at 420 nm. Also, IL-6 was measured by a high sensitive Quantikine assay, as described by Bruunsgaard *et al* (1997). All samples to be statistically compared were processed in the same assay to avoid interassay variations. The serum NO concentration was determined by first reducing the nitrate to nitrite using nitrate reductase (Sigma). Plasma levels of angiotensin-converting enzyme (ACE) were measured using a sandwich enzyme-linked immune sorbent assay (ELISA).

Statistical Analysis

All data have been expressed as mean \pm standard deviation (SD). Statistical analysis was performed using a commercial software package (SPSS version 16.0 for Windows). One-way ANOVA and Tukey post hoc testing (Statistica software, Stat Soft, Inc., Tulsa, OK.) was performed to identify differences between groups. Differences were considered statistically significant at p-value < 0.05.

RESULTS

Changes in biomarkers related to oxidant/antioxidant system

Changes in biomarkers related to oxidant/antioxidant system consisting of superoxide dismutase (SOD) and protein carbonyl (PC) in rats exposed to N(ω)-nitro-L-arginine methyl ester (L-NAME)-induced hypertension and rats in control group are summarized in figures 1 and 2, respectively. Intra-peritoneal administration of L-NAME (10 mg/kg) caused an insignificant increase (12%) in level of PC in lung tissue and a significant decrease (25%) in SOD level, as compared to saline group. In contrast, aerobic exercise for 8 weeks resulted in a significant increase (18%) in levels of SOD in lung tissue, as compared to the saline group. Moreover, an insignificant difference was detected in PC level between rats in the aerobic exercise group with those of rats in the saline group (25%). On the other hand, aerobic exercise for 8 weeks cause a significant increase (58%) in levels of SOD and an significant decrease (33%) in PC levels, as compared to L-NAME group (figure 1,2). However, no significant differences

were detected in SOD and PC levels between rats in the control and saline groups.

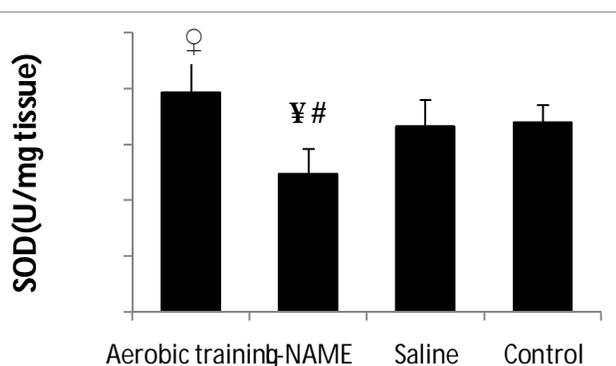


Fig. 1: Changes of superoxide dismutase (SOD) levels following 8 weeks of aerobic training in rats during chronic exposure to L-NAME. Data are presented as the mean \pm SD for 10 Rats. Abbreviation; N(ω)-nitro-L-arginine methyl ester (L-NAME), ¥ significant different from saline group ($P < 0.001$), ♀ significant different from L-NAME group ($P < 0.001$), # significant different from control group ($P < 0.001$).

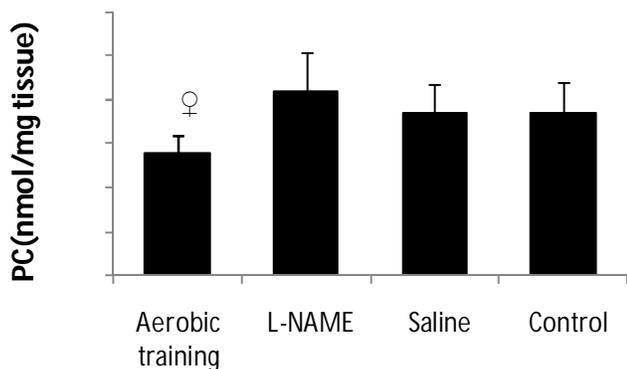


Fig. 2: Changes of protein carbonyl (PC) levels following 8 weeks of aerobic training in rats during chronic exposure to L-NAME. Data are presented as the mean \pm SD for 10 Rats. Abbreviation; N(ω)-nitro-L-arginine methyl ester (L-NAME), ♀ significant different from L-NAME group ($P < 0.001$).

Changes in inflammatory marker (IL-6)

Figure 3 shows changes in interleukin-6 (IL-6) level in the rats exposed to N(ω)-nitro-L-arginine methyl ester (L-NAME)-induced hypertension and rats in control group. The administration of L-NAME (10 mg/kg) for 8 weeks resulted in a significant increase (63%) in IL-6 level, as compared to the saline group (figure 3). Despite no significant differences were observed in IL-6 levels between rats in the control group with those of saline group, the aerobic regular exercise for 8 weeks significantly decrease in IL-6 level, in comparison with those of the L-NAME group (36%) (figure 3).

Changes in biomarker related to vascular dysfunction

Figures 4 and 5 shows changes in biomarkers related to vascular function including Nitric oxide (NO) and angiotensin-converting enzyme (ACE) in the rats exposed to N(ω)-nitro-L-arginine methyl ester (L-NAME)-induced hypertension and rats in control group. Intra-peritoneal administration of L-NAME (10 mg/kg) for 8 weeks lead to a significant increase in ACE level (28%) and significant decrease in NO level (25%), as compared to saline group.

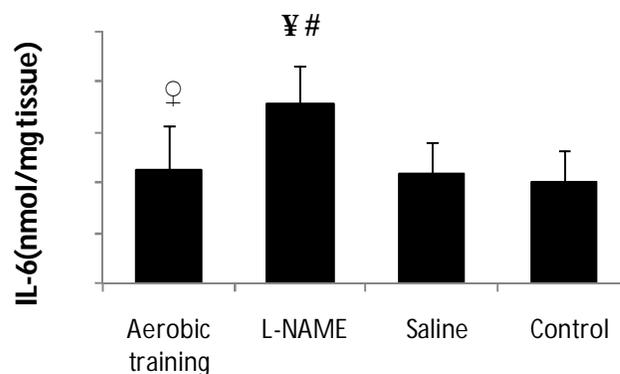


Fig. 3: Changes of interleukin 6 (IL-6) levels after 8 weeks of aerobic training in rats during chronic exposure to L-NAME. Data are presented as the mean \pm SD for 10 Rats. Abbreviation; N(ω)-nitro-L-arginine methyl ester (L-NAME), ¥ significant different from saline group ($P < 0.001$), ♀ significant different from L-NAME group ($P < 0.001$), # significant different from control group ($P < 0.001$).

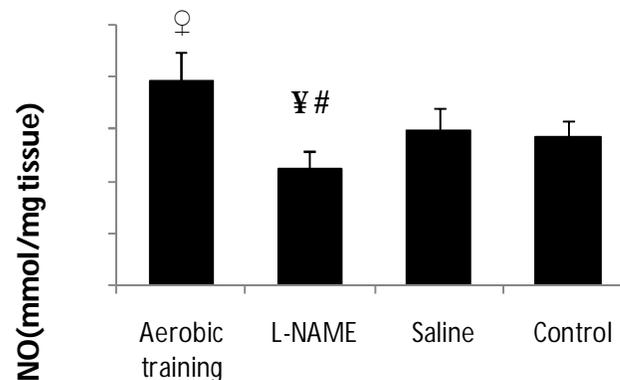


Fig. 4: Changes of nitric oxide (NO) levels following 8 weeks of aerobic training in rats during chronic exposure to L-NAME. Data are presented as the mean \pm SD for 10 Rats. Abbreviation; N(ω)-nitro-L-arginine methyl ester (L-NAME), ¥ significant different from saline group ($P < 0.001$), ♀ significant different from L-NAME group ($P < 0.001$), # significant different from control group ($P < 0.001$).

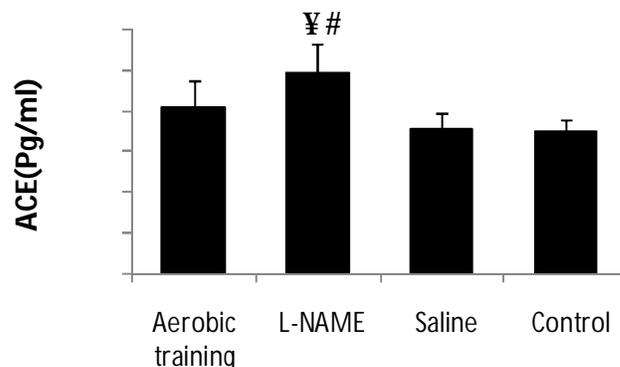


Fig. 5: Changes of angiotensin-converting enzyme (ACE) levels following 8 weeks of aerobic training in rats during chronic exposure to L-NAME. Data are presented as the mean \pm SD for 10 Rats. Abbreviation; N(ω)-nitro-L-arginine methyl ester (L-NAME), ¥ significant different from saline group ($P < 0.001$), # significant different from control group ($P < 0.001$).

Despite a significant increase in NO level following 8 weeks of aerobic exercise, as compared to saline and control groups (31%, 38%, respectively), an insignificant decrease were detected in the ACE levels between rats in the aerobic exercise with those of rats in the saline and control groups (31% and 38%, respectively)(figure 4, 5). On the other hand, no significant

differences were detected in NO and ACE levels between rats in the control and saline groups.

DISCUSSION

We investigated protective effects of 8 weeks of aerobic training on inflammatory (IL-6), vascular dysfunction (ACE and NO) and oxidant/antioxidant (SOD and PC) markers in lung tissue in male rats during chronic exposed to N(ω)-nitro-L-arginine methyl ester (L-NAME)-induced hypertension. The results of the present study indicate that intra-peritoneal administration of L-NAME (10 mg/kg) caused a significant increase in ACE, IL-6, an insignificant increase PC levels and a significant decrease in NO and SOD levels, as compared to saline and control groups. In contrast, the primary finding in the present study was that after 8 weeks of aerobic training a balance was detected in oxidants/antioxidants levels and markers related to vascular dysfunction, as compared to the saline and L-NAME groups.

Pulmonary arterial hypertension (PAH) is a severe and life-threatening disease with still largely unknown pathogenesis. The development of PAH is multi-factorial, with genetic background and environmental stress as two critical components. Oxidative stress is characterized by an increase in oxidants (e.g. hydrogen peroxide and superoxide) with or without a decrease in antioxidants or antioxidant enzymes. In cardiovascular tissue, oxidative stress has been implicated in the pathogenesis of conditions such as heart failure, ventricular hypertrophy and both systemic and pulmonary hypertension (Crosswhite and Sun, 2010). In this study, we found intra-peritoneal administration of L-NAME (10 mg/kg) caused a significantly increase in IL-6, an insignificantly increase in PC levels and a significantly decrease in NO and SOD levels, as compared to saline and control groups. Moreover, in this respect, we have previously shown that chronically administration of L-NAME resulted to an increased oxidative stress and Interlukine-6(IL-6) (Miguel Carrasco *et al.*, 2008). In addition, it reduced the level nitric oxide (NO), as compared to control group (Claudio *et al.*, 2011). These data suggest that the increased oxidative stress production by L-NAME could be blocked by treadmill regular exercise, with improve antioxidants and vascular dysfunction. Data of the current study provided additional support to understand how regular physical exercise, particularly treadmill running training, could contribute to augmentation of lung resistance against oxidative stress-based toxicity induced by L-NAME administration.

Hypertension is a complex disease process in which multiple mechanisms disrupt homeostatic maintenance of normal blood pressure, therefore, several mechanisms may be involved in the hypotensive effects of treadmill regular training (Sue, 2009). The negative impact of hypertension on health status is clear, especially taking into account the disability, decreased quality of life, and mortality associated with stroke and cardiovascular disease (Sue, 2009). Oxidative stress and homocysteine levels, which because arterial endothelium dysfunction resulting in increased vascular tone, have been implicated in the pathogenesis of essential hypertension (Sue,

2009). Characteristics of blood flow mediate the process Smooth and consistent, or laminar, blood flow is associated with nitric oxide production and increased antioxidant expression which protect against oxidative vascular injury while turbulent flow and shear are associated with the production of reactive oxygen species (ROS) leading to oxidative damage, as occurs in hypertension (Sue, 2009). Pulmonary vasoconstriction and altered reactivity is often considered as one of the earliest components of PAH, followed over time with alterations of vascular structure. Increased vasoconstriction is likely related to an imbalance between the impaired production of endogenous vasodilators [including NO, prostacyclin, and others] and excessive production of vasoconstrictors. These changes reflect endothelial cell dysfunction, which results from injury due to several mechanisms, including hypoxia, hemodynamic stress, inflammation, oxidative stress, and altered growth factor production (Abman *et al.*, 2011). In our study, treadmill regular training induced a significant increases in NO levels in male rats during chronic exposed to L-NAME. Acute inflammation acts as part of the host's innate protective response to infection or tissue injury. Endothelial cell injury or microbial infection causes changes in vascular permeability, local edema, and in the distribution of chemo attractants. Animal models also support the role of inflammatory cytokines in the initiation and progression of PAH. For example, IL-6 is consistently elevated in animal models of experimental PAH (Soon *et al.*, 2010). Interleukin-6 (IL-6) is a pleiotropic cytokine with a wide range of biologic activities in immune regulation, hematopoiesis, inflammation, and oncogenesis. Recent accumulating evidence indicates a pathologic role for IL-6 in promoting proliferation of both smooth muscle and endothelial cells in the pulmonary arterioles, resulting in development of pulmonary arterial hypertension (PAH) (Furuya *et al.*, 2010). Oxidative stress is a condition in which the delicate balance that exists between prooxidant (free radicals) production and their subsequent amelioration *via* the antioxidant defense system (ADS) becomes skewed in favor of free radical expression. An increasing body of evidence suggests that oxidative stress is involved in the pathogenesis of many cardiovascular diseases, including hypertension, hypercholesterolaemia, atherosclerosis, diabetes and heart (Sue, 2009). The implication of these pathophysiological mechanisms is that essential hypertension is primarily an inflammatory response to damage to the vascular endothelium (Sue, 2009). These results demonstrate that minimal increases in physical activity may decrease blood pressure making the adjustment to adding beneficial amounts of exercise more feasible for sedentary hypertensive patients (Sue, 2009). In present study, the observations showed that treadmill exercise resulted in a significant increase in NO and SOD and caused a significant decrease in IL-6, PC and ACE, as compared to saline and control groups.

CONCLUSION

In summary, our study suggests that chronic administration of L-NAME cause extension inflammation and

imbalance in markers related to oxidative damage and vascular function. In addition, this study shows for the first time that the treatment with aerobic regular exercise provided significant protection against L-NAME-induced toxicity in lung tissue by up-regulation of antioxidant systems and down-regulation of the inflammatory and vasoconstrictor factors in hypertensive rats. Overall, these results suggest that aerobic regular exercise during administration of L-NAME may be considered as a potentially useful strategy to limit toxicity in lung tissue.

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