



Official Research Journal of  
the American Society of  
Exercise Physiologists

ISSN 1097-9751

**JEP**online

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## **Residual Oil Fly Ash (ROFA) Inhalation Promotes Lung and Heart Oxidative Stress without Hemodynamic Effects in Exercising Rats**

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### **ABSTRACT**

**Heck TG, Nunes RB, Petry MR, Maslinkiewicz A, Saldiva PHN, Dal Lago P, Rhoden CR.** ROFA Instillation Promotes Lung and Heart Oxidative Stress without Hemodynamic Effects in Exercising Rats. **JEPonline** 2014;17(1):78-89. The purpose of this study was to test the hypothesis that exercise effort could increase oxidative damage induced by residual oil fly ash (ROFA) inhalation that resulted in hemodynamic alterations during exercise. Wistar rats that were submitted to ROFA before 20 min of swimming exercise showed an increase in lipid peroxidation (MDA and Chemiluminescence) in the lungs. The ROFA treatment also increased lipid peroxidation in the heart. However, the treatment had no influence on heart rate or blood pressure responses during exercise. These findings indicate that particulate matter inhalation during exercise may exacerbate oxidative stress in the heart and lungs without producing significant alterations in the hemodynamic variables.

**Key Words:** Exercise, Oxidative Stress, Pollution, Particulate Matter

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## INTRODUCTION

Numerous epidemiological studies indicate that the exposure to particulate matter (PM) increases cardiovascular complication. In fact, there are several indications that the exposure is associated with the first cardiovascular event (22), the number of hospital readmissions (41), the increase in blood pressure (48), and the increased risk of death for cerebrovascular and coronary heart diseases (3). The mechanisms by which PM inhalation promotes cardiovascular injuries might be related to direct or indirect actions. Ultrafine inhaled particles accumulated in the lungs are capable of translocating from the airways into the bloodstream (23) causing systemic inflammation via oxidative stress that mediates endothelial dysfunction and atherosclerosis (44) and increases blood coagulability by activating platelets and coagulation factors such as fibrinogen. Furthermore, the interaction between pollutants and lung cells may engage the heart by activating pulmonary neural reflexes that initiate alterations in the autonomic nervous system and/or increase the levels of pro-inflammatory cytokines. These inflammatory mediators result in an increase of lipid peroxidation and antioxidants enzyme activity in the heart of rats (17,29) as well as dysfunction of the autonomic nervous system activity (15).

Particulate matter has the ability to induce oxidative stress, which is largely mediated by the transition metal adsorbed at its surface. These metals generate hydroxyl radicals that are able to induce cellular damage(15). Similar effects after instillation of residual oil fly ash (ROFA –ultrafine particles rich in transition metals) have been reported Rhoden et al. (29). These effects (i.e., the increase in inflammation and oxidative stress markers) can also be found in humans (4). There are published indications that pulmonary damage elicited by particle inhalation is augmented during exercise through increased lung deposition, which may act in synergism with increased oxygen transportation across the alveolar-capillary surface (5,32,39). Although the impact of PM on physical performance has not been well documented, systemic oxidative stress and inflammation induced by the polluting particles might have a negative impact on cardiovascular function and can be related to the decrease in physical performance (11). As an example, chronic exposure to outdoor traffic pollution has been suggested to be associated with the decrease in physical effort along with an increase in risk of abnormal cardiovascular functionality during exercise (39).

Despite the extensive literature on oxidative stress induced by exercise and PM, relatively few studies have investigated the cardiorespiratory effects of particles inhalation during exercise on oxidative parameters. Hence, the purpose of this study was to test the hypothesis that exercise effort could increase oxidative damage induced by residual oil fly ash (ROFA) instillation while not producing significant alterations in the hemodynamic responses during exercise.

## METHODS

### Animals

Fifteen male Wistar rats aged 90 days from the Animal Breeding Unit of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) were subjects in this study. The animals were kept in plastic cages (47 cm x 34 cm x 18 cm) under controlled humidity (75 to 85%) with a temperature of  $22 \pm 2^{\circ}\text{C}$ , a dark-light cycle of 12 hrs (lights on from 7 am to 7 pm). They were fed with a conventional laboratory diet (Supra-lab, Alisul Alimentos S.A, Brazil) and water ad libitum.

### Animal Care

The animals were handled humanely in the performance of this project to minimize the use of animals and to prevent animal distress. All protocols were approved by the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) Ethical Committee for Research (CPA 017/053).

## Adaptation to the Water

All rats were adapted to the water before the beginning of the study. The adaptation protocol consisted of 3 consecutive days of keeping the animals 10 min in shallow water at 30°C to reduce the rat behavior stress related to water contact. Physical training adaptations were not allowed with this intervention.

## Experimental Design

### ***Heart Oxidative Stress and Hemodynamic Alterations Induced by Intratracheal ROFA***

**Inhalation:** One day after the water adaptation period, the carotid of 15 additional rats was cannulated (as described later). Twenty-four hrs after the cannulation, resting hemodynamic data were recorded and blood samples were obtained for lactate concentration measurement. Later, the rats were quickly and lightly anesthetized with ether for intratracheal instillation and divided into 2 groups: (a) Exercise + Saline 100  $\mu\text{L}$  saline (EX-S,  $n = 8$ ); and (b) Exercise + ROFA 500  $\mu\text{G}/100\mu\text{L}$  ROFA suspension (EX-RF,  $n = 7$ ). After 10 min of the instillation, the rats from both groups were able to perform 20 min of a swimming exercise in a chamber filled 45 cm with water at 30°C. The hemodynamic parameters were recorded simultaneously during exercise. At the end of the exercise session, blood samples were obtained for lactate concentration determinations and, then immediately after, the rats were decapitated for heart extraction and posteriorly stored at -80°C for oxidative stress analysis.

## Characterization of the ROFA

The ROFA consisted of particles retained in the electrostatic precipitator installed in one of the chimneys of a large steel plant localized in São Paulo city, Brazil. The element composition of ROFA suspension particles was determined by neutron activation analysis that involved short irradiations of 5 min for the determination of chlorine (Cl), potassium (K), manganese (Mn), sodium (Na), and strontium (Sr) using a pneumatic transfer system facility under a thermal neutron of  $1.4 \times 10^{12} \text{ ncm}^{-2}\text{s}^{-1}$ . Longer irradiations of 16 hrs under a thermal neutron flux of about  $10^{12} \text{ ncm}^{-2}\text{s}^{-1}$  were performed for determinations of arsenic (As), barium (Ba), calcium (Ca), cerium (Ce), chromium (Cr), cobalt (Co), potassium (K), lanthanum (La), manganese (Mn), sodium (Na), rubidium (Rb), antimony (Sb), scandium (Sc), thorium (Th), vanadium (V), and zinc (Zn). After adequate decay times, the irradiated samples and standards were measured using a hyperpure detector Model GX2020 coupled to Model 1510 (Perkin Elmer Ortec, Oak Ridge TN, USA) integrated signal processor, both from Canberra Corporate Headquarters (Meriden CT, USA). Counting times from 200 to 50,000 sec were used, depending on the half-lives or activities of radioisotopes considered. The radioisotopes measured were identified according to their half-lives and  $\gamma$ -ray energies. The concentrations of the elements were calculated using a comparative method. Measurements of the diameters of the particles (largest diameter) were made using an integrating eyepiece with polarized light microscopy. Each line in the eyepiece represented a 10  $\mu\text{m}$  length at a magnification of 1000 x. About 230 events for each particle were measured. ROFA presented 44.6+0.1% of iron; bromine, 1.482+19  $\mu\text{g g}^{-1}$ ; cesium, 16.3+0.3  $\mu\text{g g}^{-1}$ ; cobalt, 9.90+0.25  $\mu\text{g g}^{-1}$ ; chromium, 107.7+1.4  $\mu\text{g g}^{-1}$ ; lanthanum, 10.3+0.1  $\mu\text{g g}^{-1}$ ; manganese, 3,884+24  $\mu\text{g g}^{-1}$ ; rubidium, 719.7+1.0  $\mu\text{g g}^{-1}$ ; antimony, 2.27+0.9  $\mu\text{g g}^{-1}$ ; arsenic, 154.4+0.8  $\mu\text{g g}^{-1}$ ; vanadium, 35+4  $\mu\text{g g}^{-1}$ ; and zinc, 491.9+3.1  $\mu\text{g g}^{-1}$ . Almost all particles had a diameter of less than 10  $\mu\text{m}$ ; the means and standard deviations were 1.7+2.56  $\mu\text{m}$  for carbon; 1.2+2.24  $\mu\text{m}$  for transition metals particles. About 78% of carbon particles and 98% of transition metals particles had a diameter less than 2.5  $\mu\text{m}$ . This preparation was used in previous studies (46,47).

## Hemodynamic Data Acquisition and Analysis

One catheter (PE-10) filled with 0.06 mL saline was implanted in anesthetized rats (85  $\text{mg}\cdot\text{kg}^{-1}$  ketamine and 15  $\text{mg}\cdot\text{kg}^{-1}$  xylazine) into the carotid artery for direct arterial pressure measurements.

After surgery the rats were separated in individual cages and received food and water ad libitum. At the day of experiment the arterial cannula was connected to a strain-gauge transducer (P23Db, Gould-Statham), and blood pressure signals were recorded over 20 min at rest by a microcomputer equipped with an analog-to-digital converter board (CODAS, 2-kHz sampling frequency; Dataq Instruments, Inc). The recorded data were analyzed on a beat-to-beat basis to quantify changes in systolic arterial pressure (SAP), diastolic arterial pressure (DAP), mean arterial pressure (MAP), and heart rate (HR) (24). The hemodynamic data were also evaluated during the 20 min of exercise. We used the following surgery procedure: Dopalen® (Ketamine 1.16 g·10 mL<sup>-1</sup>, Agribands Ltda, Brazil) and Anasedan® (Xylazine 2.3 g·100 mL<sup>-1</sup>, Agribands Ltda, Brazil).

### **Measurement of Exercise Intensity by Blood Lactate Concentration**

Caudal venous lactate concentrations were determined before and after exercise by Lactate Analyzer (Accutrend®Plus System, Roche). The results were expressed as mmol·L<sup>-1</sup>.

### **Determination of Heart and Lung Oxidative Stress**

**Tissue Preparation:** The heart and lungs from each rat was excised, washed in saline solution and quickly frozen in liquid nitrogen. For determination of oxidative stress parameters, the heart and lung samples were homogenized (1:5 and 1:7 w/v) in 120 mmol KCl, 30 mmol sodium phosphate buffer (pH = 7.4) added with protease inhibitor 0.5 mmol PMSF (Phenylmethanesulfonyl Fluoride) at 0 to 4°C. The suspensions were centrifuged at 600 g for 10 min at 0 to 4°C to remove nuclei and cell debris. The pellets discarded and the supernatants were used as homogenates (24,27).

**Thiobarbituric Acid Reactive Substances Method (TBARS), Chemiluminescence, and Catalase Enzyme Activity:** Homogenates were precipitated with 10% TCA, centrifuged, and incubated with thiobarbituric acid (T5500-Sigma) for 60 min at 100°C. TBARS were extracted using butanol (1:1 V/V). After centrifugation, the absorbance of the butanol layer was measured at 535 nm (6). The amount of TBARS formed was expressed in nanomoles of malondialdehyde per milligram of protein (nmol MDA/mg prot). Malondialdehyde standard was prepared from 1,1,3,3-tetramethoxypropane (Fluka, USA) (24,46,47).

Chemiluminescence (CL) was measured in a liquid scintillation counter in the out-of-coincidence mode (LKB Rack Beta Liquid Scintillation Spectrometer 1215, LKB - Produkter AB, Sweden). Heart homogenates were placed in low-potassium vials at a protein concentration of 0.5 to 1.0 mg of protein·mL<sup>-1</sup> in a reaction medium consisting of 120 mmol·L<sup>-1</sup> KCl, 30 mmol·L<sup>-1</sup> sodium phosphate buffer (pH = 7.4). Measurements were started by the addition of 3 mmol·L<sup>-1</sup> tert-butyl hydroperoxide. The data were expressed as counts per sec per milligram of protein. Catalase (CAT) activity was determined by following the decrease at 240 nm-absorbance in a reaction medium containing 50 mmol·L<sup>-1</sup> sodium phosphate buffer (pH = 7.2), and 10 mmol·L<sup>-1</sup> hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (1). It was expressed as picomole of H<sub>2</sub>O<sub>2</sub> reduced per sec per milligram of protein (pmol of H<sub>2</sub>O<sub>2</sub>/sec/mg prot). Protein was measured by the method of Bradford (2), using bovine serum albumin (1 mg·mL<sup>-1</sup> to 0.1 mg·mL<sup>-1</sup> curve) as standard. The results were expressed in mg of protein/mL of homogenized tissue.

### **Statistical Analyses**

The Student's *t*-Test was used for the statistical analysis in blood lactate concentration and all oxidative stress parameters (TBARS, CL, and CAT). The hemodynamic data (HR, MAP, DAP, and SAP) were also analyzed by the Student's *t*-Test and Paired *t*-Test to compare rest and exercise hemodynamic data in the same group. The results were expressed in mean ± standard deviation. The level of statistical significance was set at 5%. All statistical analyses were conducted using the SPSS (V.18) Software for Windows.

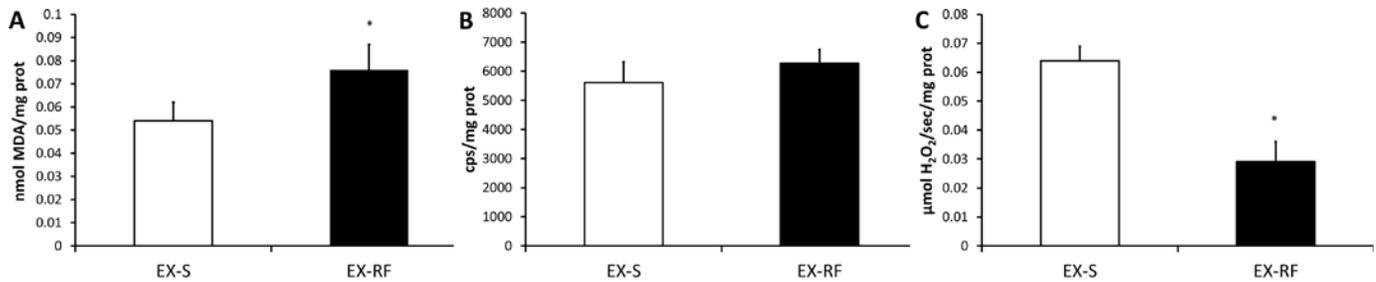
## RESULTS

### ROFA Inhalation did not Influence the Blood Lactate Concentration during Exercise in Rats

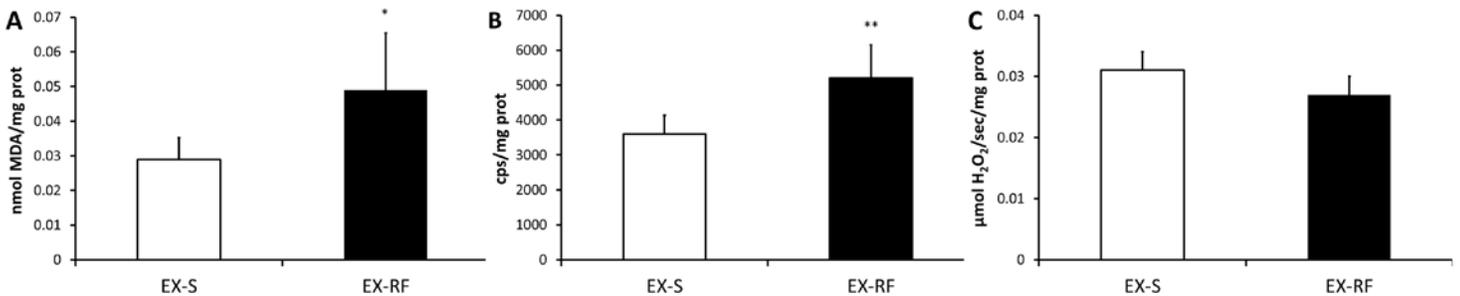
Swimming exercise for 20 min promoted an increase in blood lactate levels ( $\text{mmol}\cdot\text{L}^{-1}$ ) in both Saline (EX-S =  $1.82 \pm 0.41$  at rest vs.  $4.01 \pm 0.83$  after exercise) and ROFA groups (EX-RF =  $1.78 \pm 0.63$  at rest vs.  $4.08 \pm 0.76$  after exercise) ( $P < 0.05$ ). The ROFA instillation did not promote modifications in the blood lactate concentration.

### ROFA Inhalation Increased Oxidative Stress in Heart and Lungs of Rats Submitted to Exercise

As indicated in Figure 1, rats treated with the ROFA presented an increase in MDA levels (Figure 1A) ( $P = 0.0047$ ), and a significant decrease in CAT activity in lung tissue (Figure 1C) ( $P = 0.0007$ ). The Chemiluminescence of lung homogenates was not influenced by the ROFA treatment (refer to Figure 1B) ( $P = 0.3505$ ). In the heart, rats treated with the ROFA presented an increase in MDA (Figure 2A) ( $P = 0.03$ ) and Chemiluminescence levels (Figure 2B) ( $P = 0.015$ ). However, CAT activity (Figure 2C) was not significantly different between the groups ( $P = 0.087$ ).



**Figure 1. Lung Oxidative Stress of Rats Submitted to Exercise after ROFA Instillation.** Lipid peroxidation levels measured by: (A) MDA concentration; (B) chemiluminescence; and (c) CAT activity of lung homogenates from rats submitted to 20 min of swimming exercise after Saline (EX-S,  $n = 8$ ) or ROFA (EX-RF,  $n = 7$ ) intratracheal instillation. Results are expressed as mean  $\pm$  standard deviation. \*Significant differences ( $P < 0.05$ ) between groups.



**Figure 2. Heart Oxidative Stress of Rats Submitted to Exercise after ROFA Instillation.** Lipid peroxidation levels measured by: (A) MDA concentration; (B) chemiluminescence; and (c) CAT activity of heart homogenates from rats submitted to 20 min of swimming exercise after Saline (EX-S,  $n = 8$ ) or ROFA (EX-RF,  $n = 7$ ) intratracheal instillation. Results are expressed as mean  $\pm$  standard deviation. \*Significant differences ( $P < 0.05$ ) between groups.

### ROFA Inhalation Had No Influence on Hemodynamic Response during Exercise in Rats

All the hemodynamic data are demonstrated in Table 1. The HR and DAP values and the SAP and MAP values were similar between the groups during the rest condition. During exercise, there were no significant differences in the hemodynamic data between the treatment groups. When the rest condition was compared to the exercise condition, the HR values increased during the exercise condition, while diastolic, systolic and mean arterial pressure decreased independently of treatment (Saline or ROFA). The magnitude of the increase in HR and the decrease of the arterial pressure were similar for the Saline and ROFA groups during the exercise.

**Table 1. Hemodynamic Response during Exercise after ROFA Instillation.**

	Saline (n = 8)	Rofa (n = 7)
<b>DAP (mmHg)</b>		
Rest	101.1 ± 6.2	103.5 ± 7.8
Exercise	87.9 ± 8.3*	87.3 ± 11.3*
delta	-13.2 ± 8.2	-16.2 ± 7.5
<b>SAP (mmHg)</b>		
Rest	136.3 ± 10.4	136.7 ± 9.8
Exercise	127.3 ± 9.7*	128 ± 11*
delta	-9.0 ± 9.2	-8.7 ± 3.9
<b>MAP (mmHg)</b>		
Rest	120.7 ± 6.4	121.7 ± 6.8
Exercise	109.3 ± 9.7*	109 ± 11.4*
delta	-11.4 ± 8.7	-12.7 ± 5.8
<b>HR (beats·min<sup>-1</sup>)</b>		
Rest	370 ± 38	348 ± 14
Exercise	432 ± 15*	416 ± 36*
delta	61 ± 43	68 ± 24

**DAP** = diastolic arterial pressure, **SAP** = systolic arterial pressure, **MAP** = mean arterial pressure, **HR** = heart rate at rest and during swimming exercise after Saline (EX-S, n = 8) or ROFA (EX-RF, n = 7) intratracheal instillation. Results are expressed as mean ± standard deviation. \*Significant differences (P<0.05) between exercise and rest values in the same treatment group using the Paired Student's *t*-Test.

## DISCUSSION

It is well-recognized that the majority of the experimental work regarding adverse effects of PM is conducted with rats in resting condition. In the present study we submitted rats to ROFA instillation during exercise. Specifically, the acute response of particles on the rats' hemodynamic response during a swimming exercise was studied to evaluate the toxic effects of PM. This study found that particles promoted heart and lung oxidative stress without modifying the hemodynamic response to swimming.

In our study it was necessary to measure the intensity of exercise practiced by the animal groups. As suggested by the literature, the blood lactate concentration was used to mark the intensity of exercise (14,18,20,40). Kramer et al. (18) and Gobbato et al. (14) determined that values of blood lactate concentration in rats around 2 mmol·L<sup>-1</sup> are consistent with rest conditions while values of 5.5 mmol·L<sup>-1</sup> indicate high intensity exercise. This study found an increase in lactate concentration after

exercise at values close to  $4 \text{ mmol}\cdot\text{L}^{-1}$ , indicating that the rats reached moderate intensity lactate levels independently of treatment groups (14,40).

The administration of particles in the lungs of rodents is a reasonable approach to assess the potential for and the profile of lung injury. In fact, it is common that a high dose of particles is used to investigate the mechanisms of pathophysiological lesions (7) and, in this regards, the dose of ROFA (500  $\mu\text{g}$ ) administered in the present study was well above the ambient concentration. In addition, it is likely that the composition of the ROFA used may not have reflected the urban particles that present a high contribution of mobile sources (17,28,46,47). Thus, we used ROFA to determine if the rats' exercise hemodynamic responses would be impaired by heart lipid peroxidation damage induced by particulate pollution.

Human studies have demonstrated that exposure to PM at rest produces changes in the subjects' HR, HR variability, blood pressure, and electrical conduction system of the heart in patients with preexisting cardiovascular disease. Since cardiorespiratory demand is increased during exercise, as observed by the increase in oxygen consumption ( $\text{VO}_2$ ), there is a significant increase in pulmonary ventilation and diffusion capacity. These physiological adjustments may be linked to an augmented risk of inhaling particles that result in damage to the cardiorespiratory system during exercise (32,33). This is an important point, given that the literature indicates that the total amount of ultrafine particles deposited in respiratory tract of humans during moderate exercise is approximately 5 times higher than at rest (9).

Given that the studies regarding heart oxidative stress and PM exposure were conducted with rats in the resting condition, the present study is novel in that it was conducted with rats during exercise. Thus, it was essential to determine the exercise intensity performed by the rats. Lactate concentration (in all experiments) and cardiovascular parameters during experiment 2 (Table 1) showed that the rats were submitted to a similar exercise intensity throughout the study. The findings support an increase in oxidative stress in the rats' heart that was induced by PM inhalation. Specifically, it was observed that ROFA instillation promoted an increase of lipid peroxidation in heart tissue of rats submitted to acute exercise. The fact that PM induced lipid peroxidation at rest is not novel. Previous studies (10,27,28,30) have demonstrated that pulmonary and cardiac adverse effects of PM might be mediated by mechanism dependent on oxidants and autonomic unbalance (13,29). But, the increased oxidative risk without hemodynamic alterations during exercise after particles exposure is a new and an important finding.

In the present study, the heart CAT activity of the rats was similar in the treated groups. This finding disagrees with authors who demonstrated a protective effect of CAT in cardiac myocytes damage induced by diesel exhaust (25). As an example, in an earlier study (10), we showed that 90 days of ROFA treatment promoted an increase in heart CAT. Also, Gurgueira and colleagues (17) reported a 21% increase in CAT activity in heart tissue of rats exposed to concentrated ambient particles (CAPs) for 5 hrs. In contrast, the explanation for the lack of an increase in CAT activity in the present study might be explained to some extent by the different models (i.e., period and pollution protocols). Also, the fact that acute exercise per se can increase mitochondrial CAT activity by 358% with a longer exercise duration and a higher exercise intensity than used in the present study as compared with rest condition (34). Therefore, it is reasonable to believe that the exercise may have masked the PM effect on CAT activity.

The idea that oxidants generated by PM alter cardiac function is well documented (13,15,17). In fact, it has been demonstrated that sympathetic and parasympathetic blockers prevent the CAPs-mediated cardiac adverse effects, thus showing the critical role for autonomic nervous system signaling in

preventing damage (29). Similarly, with respect to the published literature (31,42) and ROFA instillation, the present study indicates that the HR, SBP, DBP, and MAP responses are a function of the exercise condition that masks the PM effect.

There are some controversial data in the literature regarding hemodynamic parameters in animal models of exercise. For example, Kramer et al. (18) demonstrated an increase of HR in rats submitted to a swim test. Yeung et al. (45) found an increase in HR and SAP without alteration in DAP using a treadmill for 7 min. When evaluating cardiovascular responses during human exercise in water, Park et al. (26) and Cider et al. (6) found an increase in HR and blood pressure when compared to resting condition. During water immersion at 30° of temperature, Park et al. (26) showed a 50% increase in cardiac output, although HR decreased 15%. The water immersion modified other hemodynamic parameters such as an increase of 8% in SAP, 15% in DAP and decrease of 32% in total peripheral resistance. Other studies found a decrease in blood pressure (8) or no significant alterations in these parameters (6,12). Interestingly, a recent review showed that the HR response of diving and proposed that the HR response of exercise in mammals are variable because the suprabulbar control over this reflex behavior. Moreover, underwater submersion results in bradycardia mediated by parasympathetic neurons and the redistribution of blood flow away from muscle followed by reperfusion during emersion (21).

In the present study, we observed that rats submitted to short-term moderate exercise activity increased HR in both groups independently with or without the treatment with ROFA. However, interestingly, all of the measurements regarding blood pressure were reduced with exercise. Also, there were no differences between exposure groups. Similar changes in HR and PAM analysis in rats during the swim exercise suggest that these effects occurred in response to gradual parasympathetic influence that could be explained by the frequent submerges of the animal (19).

Although there are few studies in the literature associating air pollution adverse effects with exercise (11,16,39,43), Sharman et al. (32) published a recommendation to avoid exercise practice in parks or in recreational areas close to busy roadways or industrial sites, which is consistent with exercise guidelines for people with high blood pressure (33). These authors suggest that it may be desirable to limit the duration of the exercise session when air pollution is critical. Specifically, the PM may decrease cardiorespiratory fitness (36) by influencing lung function after exposure (35). However, it is also important to point out that the acute pollution exposure during a single exercise session is possibly questionable especially in light of the protective effect that results from chronic exercise (37,38).

## **CONCLUSIONS**

The findings in this study indicate that the exposure of Wistar rats to ROFA before 20 min of swimming exercise resulted in an increase in lipid peroxidation (MDA and Chemiluminescence) in the lungs. The ROFA treatment also increased lipid peroxidation in the heart. However, the treatment had no influence on rats' heart rate or blood pressure responses during exercise. Thus, it is reasonable to conclude that the particulate matter inhalation during exercise may exacerbate oxidative stress in the heart and lungs without producing significant alterations in the hemodynamic variables.

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## ACKNOWLEDGMENTS

This work was supported by Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). T.G. Heck and M. Petry were supported by a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Dr C.R. Rhoden, Dr. P. Dal Lago and Dr. P.H.N. Saldiva are supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors declare they have no conflict of interest.

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