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TOTAL PARTICULATE MATTER FROM COMBUSTION OF CASHEW NUT SHELL EXACERBATES LUNG INJURY IN OVA-INDUCED ASTHMA MODEL

***[1,] Daniel Silveira Serra, [2]Adenilson Pereira Escossio, [3]Gardilene Monteiro da Silva, [4]Patricia Freitas Brasil, [5]Rinaldo Santos Araújo, [6]Mona Lisa Moura de Oliveira, [7]José Henrique Leal-Cardoso and [8]Francisco Sales Ávila Cavalcante**

[*1,2,3,7]Institute of Biomedical Sciences, State University of Ceará, Ceará, Brazil. Av. Dr. Silas Munguba, 1700, zip: 60714-903, Fortaleza-Ceará, Brazil
[4,6,8]Science and Technology Center, State University of Ceará, Ceará, Brazil
[5]Department of Chemistry and Environment, Federal Institute of Ceará, Ceará, Brazil

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ABSTRACT

Air pollution, even at levels below that allowed by official agencies, can significantly affect the environment and health. Our hypothesis is that exposure to atmospheric pollutants, specifically total particulate matter (TPM) from the combustion of cashew nut shell (CNS), in the individuals at risk group (previous respiratory disease) may exacerbate the harmful effects to the respiratory system. Accordingly, we performed analyses of respiratory mechanics and micromechanics and histopathology and morphometry of lung parenchyma. BALB/c mice were used and divided into four groups that were subjected to the positive or negative protocol of OVA-induced asthma model, and they were exposed to 15 µg TPM from CNS combustion or saline. Our results demonstrated significant changes in all analyzed values of the OVA and OVA+TPM groups compared to the control group, and in the most of the OVA+TPM group values compared to the OVA group. We conclude that exposure to air pollutants, specifically TPM from the combustion of CNS, can exacerbate the respiratory system injury in mice with previous lung disease (OVA-induced asthma model).

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INTRODUCTION

Air pollution represents one of the greatest public health issues in the world and is associated with a number of harmful health effects, even within levels considered safe according to environmental laws (De Castro *et al.,* 2003). About 25% of deaths caused by air pollution in the world are due to respiratory diseases (Lelieveld *et al.,* 2015). Exposure to air pollutants has been reported to be an important factor for increased hospitalizations, school absence, low birth weight and poor congenital formation among children and women of reproductive age (Lin *et al.,* 2004; Medeiros and Gouveia, 2005).

Corresponding author: **Daniel Silveira Serra**
Institute of Biomedical Sciences, State University of Ceará, Ceará, Brazil. Av. Dr. Silas Munguba, 1700, zip: 60714-903, Fortaleza-Ceará, Brazil.

The group most susceptible to the harmful effects of air pollution consists of children, the elderly, pregnant women and individuals with previous cardiovascular or respiratory diseases, where these individuals are part of the so-called risk group (Danida, 2000). Among the elderly, the susceptibility to air pollution may be increased due to physical weakness, low physiological resilience of the respiratory system and previous diseases (Ghosh *et al.,* 2007). Respiratory diseases, especially acute respiratory infections, asthma and bronchitis, are the most common causes of increased morbidity and mortality in children (Prietsch *et al.,* 2003; Braga*et al.,* 2007). Asthma is characterized by an increase in eosinophil infiltrates, bronchoconstriction, mucosal hypersecretion, airway inflammation and hyperresponsiveness (Ishmael,2011; Maddox and Schwartz, 2002). According to the global asthma report in 2014, about 334 million people had asthma, and their prevalence was rising rapidly.

The association between asthma and air pollution is the subject of investigation of several research studies in humans or animals (Kim *et al.,* 2016; Ierodiakonou *et al.,* 2016; Bowatte *et al.,* 2017). The decrease in atmospheric pollutants is closely related to the decrease in the burning of fossil fuels. Accordingly, the search for alternative sources of clean energy is increasing. Following this trend, the renewable energy sector grew between 15 and 55% per year between 2005 and 2012 (Zervos, 2013), focusing on the use of biomass as a source for energy production, with the highest growth potential in the coming years (WEC, 2010). Brazil is one of the largest agricultural producers in the world, with great potential for the production of residual biomass. According to the International Nut & Dried Fruit Coucil (2016), Brazil is the world's fifth largest producer of cashew nuts, and the energy utilization of residual products from cashew nut processing, such as cashew nut shell (CNS), is already a reality in some industries (Sanger *et al.,* 2011; Alcocer *et al.,* 2015). Given the above and due to the increasing use of biomass as an energy source in industries, there is a need for studies investigating the harmful effects of atmospheric pollutants generated by biomass burning on the health of mice with previous respiratory disease. The aim of the present study was to evaluate the effects of total particulate matter (TPM) from the combustion of CNS on the respiratory system of mice using the OVA-induced asthma model. Our hypothesis is that exposure to atmospheric pollutants, specifically TPM from the combustion of CNS, in the individuals at risk group (with pre-existing respiratory disease) may exacerbate the harmful effects to the respiratory system. Accordingly, we performed analyses of respiratory mechanics and micromechanics and histopathology and morphometry of lung parenchyma.

MATERIALS AND METHODS

TPM Collection system

A CNS combustion system was developed to collect TPM from its exhaust gases (Figure 1).

For this collection, CNS (400 g) was first placed in a cylindrical stainless steel burner (Figure 1, A). Liquefied petroleum gas (LPG-Figure 1, B) and ambient air from an air compressor (Figure 1, C) were then supplied only for the initial ignition of CNS combustion. The process of CNS combustion was accompanied by thermocouples (Figure 1, D) and flow transducers (Figure 1, E) connected to a data acquisition system (FieldLogger-Figure 1, F) for the analysis and control of temperature and LPG and air flow, transferring the information to a notebook (Figure 1, G). The exhaust gases generated by CNS combustion were directed to a chimney (Figure 1, H). To collect the TPM filters, the flue gases were directed to a system containing 2 glass fiber filters (Figure 1, I), where a flow of 5 L/m was maintained with a suction pump (AirChek® XR5000- Figure 1, J).

Preparation of solutions for intranasal instillation of TPM

Before collecting the particulates (TPM) from the combustion exhaust gases from the CNS, the clear filters were heated in a thermal oven to 50°C for 24 h for desiccation and then weighed. Afterward, the CNS combustion process to collect TPM was performed. Subsequently, the filters were again placed in the thermal oven at 50°C for 24 h, and again weighed. The filters were then packed in a Becker containing saline and sonicated for 8 h (QUIMIS®- Q3350). After sonication, the filters were again placed in athermal oven at 50°C for 24 h and weighed. The efficiency of the particulate extraction was calculated from the difference between the weights of the filters before and after the collection process (Maatz *et al.,* 2009). The final particle:volume ratio (µg:µL) adopted in the study was 1:2.

Animals

BALB/c mice, females (7-8 weeks of age), with a body weight of 25 ± 5g, given water and feed *ad libitum*, were used in this study.

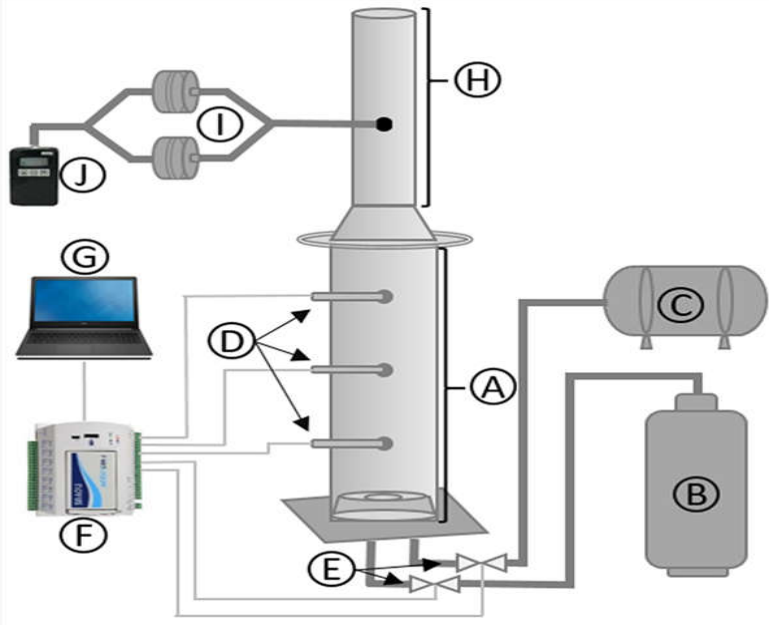

Figure 1. Combustion system and collection of filters with total particulate matter (TPM) from combustion exhaust gases from CNS. A - Biomass combustion reactor; B-LPG; C - Air compressor; D - Thermocouples; E - Flow transducers; F - Data acquisition system (Fieldlogger); G – Notebook; H - Chimney; I - System for collection of TPM; J - Suction pump

Mice were housed in plastic cages at 22°C, with a 12-h light (06:00–18:00 h) and 12-h dark (18:00–06:00 h) cycle. The animal ethics committee of the State University of Ceará (Protocol No. 3928428) previously approved all animal use and care procedures. Invasive procedures were performed under anesthesia and every effort was made to minimize suffering.

OVA-induced asthma model and experimental design

According to a modification of the method (Parreira *et al.,* 2012), mice were sensitized and challenged with ovalbumin (OVA; Sigma, MO, USA). In brief, BALB/c mice were subcutaneously sensitized with 100 µg OVA emulsified in 1 mg of aluminum hydroxide (Sigma, MO, America) as adjuvant in 200 µL of phosphate-buffered saline (PBS) on days 0, 7 and 14. The intranasal challenge was always performed under anesthesia with sevoflurane, for pulmonary aspiration of the OVA solution. The intranasal challenge was performed on days 25, 26 and 27 with OVA (100 µg, diluted in 50 µl of PBS). The negative control group received the same volume of saline subcutaneously (days 0, 7 and 14) and intranasally (days 25, 26 and 27). The experimental design is shown in Figure 2. We used 32 BALB/c mice, randomly divided into four groups. In the first group (Ctrl group, n=8), the mice were subjected to the negative asthma control protocol previously mentioned, and 24 h after protocol termination, they received intranasal instillation of 30 µL of solution from clean filter sonication in saline. In the second group (TPM group, n=8), the mice were subjected to the aforementioned negative asthma control protocol, and 24 h after protocol termination, they received intranasal instillation of 15 µg TPM from the CNS combustion exhaust gases, diluted in 30 µL of saline. In the third group (OVA group, n=8), the mice were sensitized and challenged with OVA as previously mentioned, and 24 h after protocol termination, theyreceived intranasal instillation of 30 µL of solution from clean filter sonication in saline.

In the fourth (OVA+TPM group, n=8), the mice were sensitized and challenged with OVA as previously mentioned, and 24 h after protocol termination,they received intranasal instillation of 15 µg TPM from the CNS combustion exhaust gases, diluted in 30 µL of saline.

Respiratory system mechanics

Twenty-four hours after intranasal instillation of TPM (TPM and OVA+TPM groups) or saline (Ctrl and OVA groups), the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p., 3% Hypnol®, Syntect, Brazil) and tracheotomized. The animals were intubated with a 14-gauge cannula (Eastern Medikit, Delhi, India) that was then connected to a computer-controlled ventilator for small animals (Scirec©-*flexVent*®, Montreal, QC, Canada). The animals were ventilated at baseline settings: respiratory frequency of 120 breaths/min, tidal volume of 10 mL/kg, limiting pressure of 30 cmH_2O, and positive end-expiratory pressure (PEEP) of 3 cmH_2O. Micewere then paralyzed with pancuronium bromide (0.5 mL/kg, i.p., Cristália, Brazil).
Initially, we standardized the mechanical history of the respiratory system with two deep inflations (DI, 6-s long, peak pressure: 30 cmH_2O) and the animals were then ventilated for 5 minat baseline settings. Soon after, the impedance of the respiratory system (Zrs) was measured with the forced oscillation technique (3-s duration) (Hantos *et al.,* 1992), in 12 sequential 30-s sampling intervals, for a total of 6 min (Bates *et al.,* 2009).

The experimental $Z_{rs}$was fitted to the constant phase model as previously described (22):

$$Z_{rs} = R_N + I(2\pi f)i + \frac{G-Hi}{(2\pi f)^{\alpha}} \quad \text{Eq. (1)}$$

$$\alpha = \frac{2}{\pi}\tan^{-1}\left(\frac{H}{G}\right) \quad \text{Eq. (2)}$$

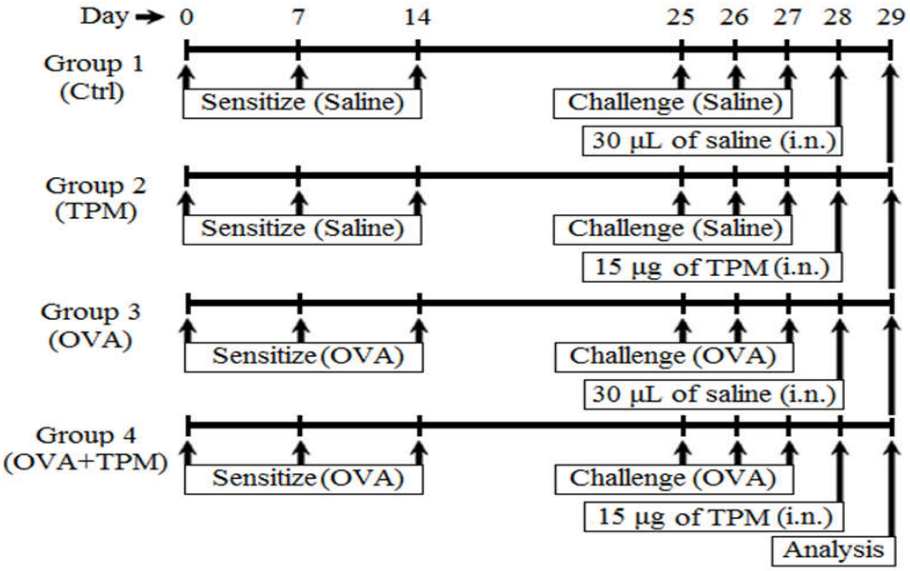

Figure 2. Schematic diagram of the experimental protocol. In group 1 (Ctrl), mice were sensitized with saline on days 0, 7 and 14, challenged with saline on days 25, 26 and 27, and exposed to 30 µL saline via intranasal route on day 28. In group 2 (TPM), mice were sensitized with saline on days 0, 7 and 14, challenged with saline on days 25, 26 and 27, and exposed to 15 µg total particulate matter (TPM)diluted in 30 µL saline via intranasal route on day 28. In group 3 (OVA), mice were sensitized with 100 µg OVA emulsified in 1 mg aluminum hydroxide on days 0, 7 and 14, challenged with 100 µg OVA diluted in 50 µl of DPBS on days 25, 26 and 27, and exposed to 30 µL saline via intranasal route on day 28. In group 4 (OVA+TPM), mice were sensitized with 100 µg OVA emulsified in 1 mg aluminum hydroxide on days 0, 7 and 14, challenged with 100 µg OVA diluted in 50 µl of DPBS on days 25, 26 and 27, and exposed to 15 µg TPMdiluted in 30 µL saline via intranasal route on day 28. All analyses were performed on day 29.

where R_N is the Newtonian resistance, which represents the central airway resistance, $i = \sqrt{-1}$, f is the frequency (Hz), I represents airway inertance, and G and H are respectively the dissipative and elastic properties of lung tissue (Hantos *et al.,* 1992). Thereafter, starting at the functional residual capacity (FRC), the flexiVent delivered 7 inspiratory pressure steps for a total pressure of 30 cmH_2O, followed by 7 expiratory steps, pausing at each step for 1 s. At each step, plateau pressure (P) was recorded and related to the total volume (V) delivered to produce a quasi-static PV (pressure-volume) curve. Static compliance (C_{ST})was calculated as the slope of the curve (Salazar and Knowles, 1964). Two quasi-static PV curves were obtained to measure C_{ST}, an estimate of inspiratory capacity (IC), and PV loop area.

Methacholine challenge

Immediately after measurements of respiratory system mechanics, two DIs (deep inflation) were performed, followed by 5 min of ventilation at baseline settings. Airway smooth muscle hyperresponsiveness was evaluated by inhalation of methacholine (MCh) (Sigma-Aldrich, St. Louis, MI, USA) delivered by aerosol produced by an ultrasonic nebulizer (Inalasonic, NS, São Paulo, Brazil) coupled to the inspiratory line of the ventilator. For such purpose, 4 mL of MCh solution (30 mg/mL) were added to the nebulizer container. Nebulization was carried out for 30 s under mechanical ventilation (Xue *et al.,* 2008) and the average amount delivered to the animal was 1.2 mg/kg of MCh solution. After nebulization, the same previous analysis was repeated (forced oscillation, 30-s sequential intervals for 6 min). Data regarding airway hyperresponsiveness collected after nebulization of MCh are presented as ΔR_N, where Δ means the parameter after nebulization minus its value before MCh challenge. All Δ values were normalized by the pre-nebulization values.

Lung tissue micromechanics

After the aforementioned measurements, the animals were euthanized with an extra bolus of sodium pentobarbital (120 mg/kg, i.p., 3% Hypnol®). The left lung was removed for histological analyses. Right lung subpleural lung parenchyma strips (approximately 2 × 2 × 6 mm) were obtained and maintained in Krebs-Henseleith (K–H) solution. Lung micromechanics was analyzed in an organ bath using an actuator (300B-LR model, Aurora Scientific Inc, Aurora, ON, Canada) able to apply up to 1 Newton (N) in uniaxial deformation tests, over the course of up to 8 mm. Its length and force accuracies are 1 μm and 0.3 mN, respectively, with a step change response time of 1.3 ms. Data acquisition was achieved with a dedicated computer. One of the ends of the lung parenchyma strips was attached to the actuator with cyanoacrylate glue, and the other end was secured to a fixed base. The tissue strip was immersed in an organ bath with K–H solution, aerated with carbogen mixture (95% O_2 and 5% CO_2), and the bath temperature controlled at 37°C. The length of the sample was then slowly adjusted until baseline force reached 1 gram-force. To minimize any tension history and return the tissue to a standard and stable point, each strip was preconditioned for 10 min under sinusoidal oscillations with an amplitude amounting to 10% of the strip resting length (L_o) and frequency of 1 Hz, so that a stable stress-strain loop was observed (Leite-júnior *et al.,* 2003). Soon after, uniaxial mechanical measurements consisting of dynamic or quasi-static stress-strain curves were made by applying sinusoidal deformations to the lung strips. The dynamic stress-strains data were fitted to a viscoelastic model (Cavalcante *et al.,* 2005). It is assumed that stress is proportional to strain and to strain-time ratio. Elastance and resistance are the factors of proportionality for strain and strain-time ratio, respectively. Hysteresivity was estimated as the ratio between dissipative and elastic forces in the tissue strip. The initial length (L_i) was set to 15% of $L_o$and the samples were oscillated at an amplitude of 2.5% of $L_i$at frequencies of 0.1, 0.3, 1, 3 and 10 Hz, 20 cycles each. Stress was measured as force normalized by the cross-sectional area of the sample. Strain was determined as instantaneous length divided by L_o.

Histological study

Slides containing left lung sections were stained with hematoxylin and eosin (HE) and examined with a light microscopy according to their qualitative and quantitative aspects. An investigator, who was unaware of the origin of the coded material, examined the samples microscopically. Quantitative analysis was performed using an integrated eyepiece with a coherent system consisting of a 100-point and 50-line grid coupled to a conventional light microscope. The fraction area of collapsed alveoli (alveolar morphometry), and the amount of polymorphonuclear (PMN) cells, as well as pulmonary tissue were determined by the point-counting technique (Weibel, 1990). Cellularity was assessed at 1000× magnification across 10-15 random non-coincident microscopic fields in each animal. The air-space enlargement was quantified by the mean linear intercept length of the distal air spaces (L_m) in 30 randomly chosen fields of tissue sections per group (Knudsen *et al.,* 2010). The bronchoconstriction index (BCI) was determined in 10 non-coincident microscopic fields per animal by counting the number of points in the airway lumen (NP) and intercepts through the airway wall (NI) using a reticulum and applying the equation: $BCI = NI/\sqrt{NP}$. Only airways in which the long diameter did not exceed the short diameter by more than 20% were accepted for measurement (Sakae *et al.,* 1994). Alveolar morphometric analysis and determination of bronchoconstriction index were done at 400× magnification.

Statistical Analysis

Data normal distribution and homogeneities of variances were tested with Kolmogorov-Smirnov (with Lilliefors's correction) and Levene median tests, respectively. For multiple comparisons, if both conditions were satisfied, Student's t-test was used. If any conditions werenot met, the Mann-Whitney non-parametric test was used instead. The multiple comparisons were then corrected with the Bonferroni's test (Benjamini and Hochberg, 1995). A difference was considered significant if $p < 0.05$.

RESULTS

Table 1 lists the mechanical data of therespiratory system of mice subjectted to the negative asthma control protocol (Ctrl and TPM groups) or asthma induction protocol (OVA and OVA+TPM groups). The groups Ctrl and OVA received intranasal instillation of 30 μL of solution from clean filter sonication in saline,and the groups TPM and OVA+TPM received intranasal instillation of 15 μg TPM from the CNS combustion exhaust gases, suspended in 30 μL solution saline. Figure 3 shows the variations in ΔR_N after administration of

MCh (30 mg/mL) in the groups. We observed an increase (ΔR_N) in the groups TPM, OVA and OVA+TPM in comparison to the Ctrl group. Figure 4 shows lung tissue micromechanical data.

At any oscillatory frequency, elastance, resistance and hysteresivity were higher in the animals of the OVA and OVA+TPM groups compared to the Ctrl group.

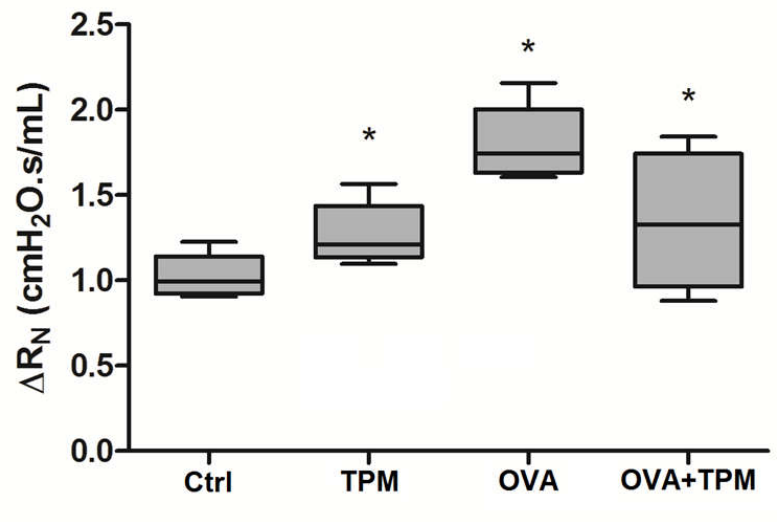

Figure 3. Methacoline challenge as a function of number of measurements after MCh nebulization (30 mg/mL for 30 s). In Ctrl and TPM groups, mice were sensitized and challenged with saline, and exposed by intranasal instillation to 30 µL of saline (Ctrl group) or 15 µg total particulate matter from combustion of cashew nut shell (TPM group),diluted in 30 µL of saline. In OVA and OVA+TPM groups, mice were sensitized and challenged with OVA, and exposed by intranasal instillation to 30 µL of saline (OVA group) or 15 µg TPM(OVA+TPM group),diluted in 30 µL of saline. *Represents statistically significant differences (p<0.05).

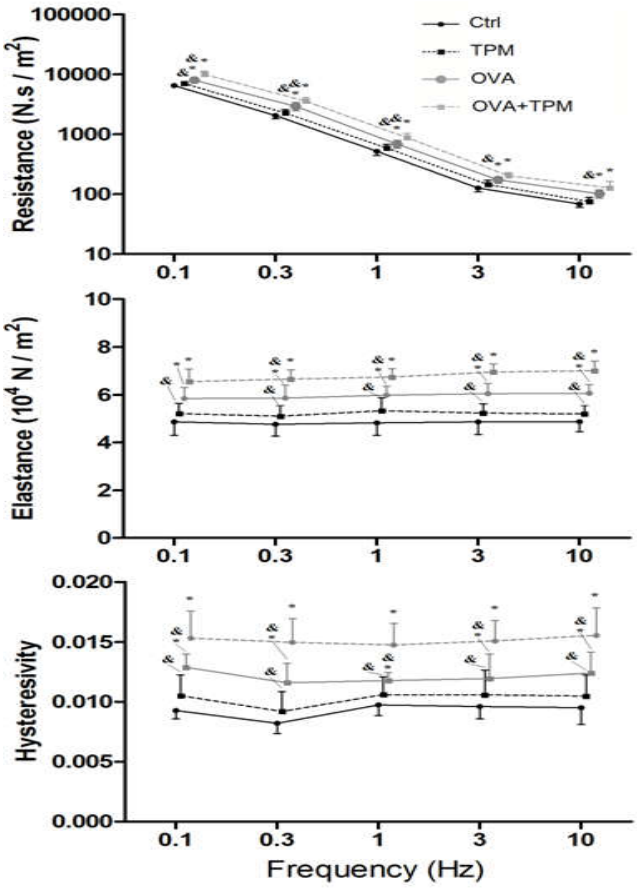

Figure 4. Tissue micromechanics as a function of oscillatory frequency. In Ctrl and TPM groups, mice were sensitized and challenged with saline, and exposed by intranasal instillation to 30 µL of saline (Ctrl group) or 15 µg total particulate matter from combustion of cashew nut shell (TPM group),diluted in 30 µL of saline. In OVA and OVA+TPM groups, mice were sensitized and challenged with OVA, and exposed by intranasal instillation to 30 µL or saline (OVA group) or 15 µg TPM (OVA+TPM group),diluted in 30 µL of saline. Resistance, elastance and histeresivity in Ctrl, TPM, OVA and OVAP+TPM groups expressed as mean ± SD, n=8. *Values significantly different from Ctrl group; &Values significantly different from OVA+TPM group.

We did not observe significant alterations in the animals of the TPM group compared ot those in the Ctrl group. Figure 5 shows the photomicrographs of the pulmonary parenchyma of the animals of the Ctrl, TPM, OVA and OVA+TPM group. The presence of alveolar collapse, thickened septa and cellular infiltration in the photomicrographs of the pulmonary parenchyma of TPM, OVA and OVA+TPM groups was demonstrated.

Table 2 displays that alveolar collapse, amount of PMN cells, the mean alveolar diameter and bronchoconstriction index. We observed an increase in alveolar collapse, mean alveolar diameter and bronchoconstriction index in the TPM, OVA and OVA+TPM groups when compared to the Ctrl group. Amount of PMN cells was only increased in the OVA and OVA+TPM groups compared to the Ctrl group, these findings suggested pulmonary inflammation and bronchoconstriction.

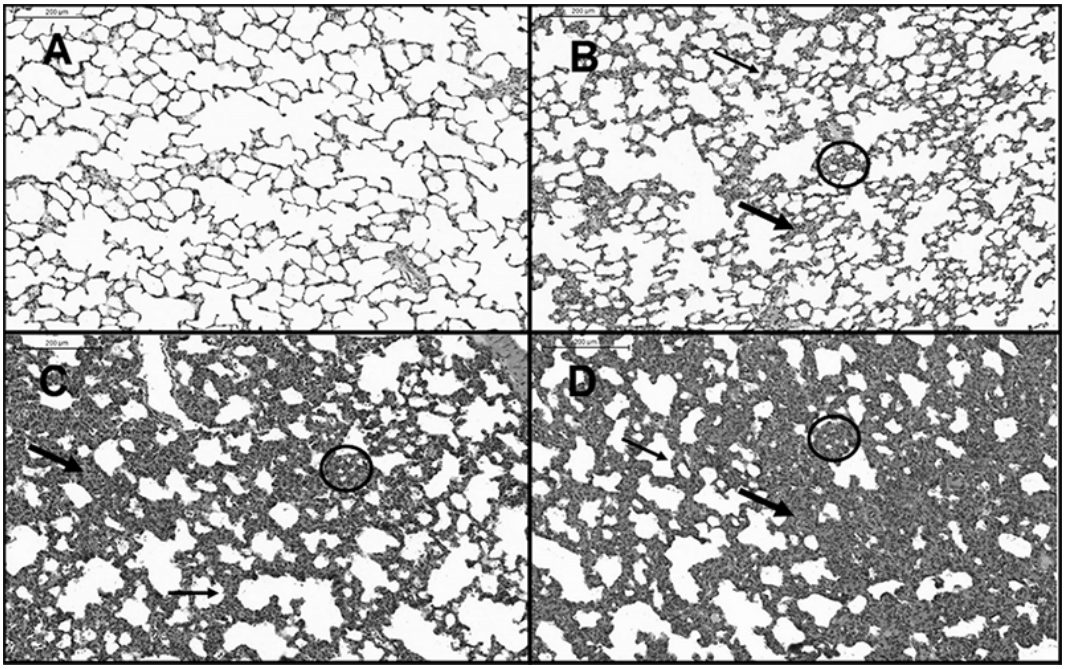

Figure 5. Photomicrographs of lung parenchyma stained with hematoxylin–eosin. A- Ctrl group; B- TPM **group; C- OVA group**; D-OVA+**TPM** group. **In Ctrl and TPM groups, mice were sensitized and challenged with saline, and exposed by intranasal instillation to 30 µL of saline (Ctrl group) or 15 µg total particulate matter from combustion of cashew nut shell (TPM group),suspended in 30 µL of saline. In OVA and OVA+TPM groups, mice were sensitized and challenged with OVA, and exposed by intranasal instillation to 30 µL of saline (OVA group) or 15 µg TPM (OVA+TPM group),suspended in 30 µL of saline. Thin arrows: thickened septa; thick arrows: cellular infiltrate; and circles: alveolar collapse.**

Table 1. Differences in lung function parameters between groups. In Ctrl and TPM groups, mice were sensitized and challenged with saline, and exposed by intranasal instillation to 30 µL of saline (Ctrl group) or 15 µg total particulate matter from combustion of cashew nut shell (TPM group),diluted in 30 µL of saline. In OVA and OVA+TPM groups, mice were sensitized and challenged with OVA, and exposed by intranasal instillation to 30 µL of saline (OVA group) or 15 µg TMP(OVA+TPM group),diluted in 30 µL of saline. Student t-test was used for statistical analysis. *P*<0.05 was considered statistically significant. Data are presented as mean±SD. *Values significantly different from Ctrl group;[&]values significantly different from OVA+TPM group.

	Measure	Group	Value
Forced Oscillation Technique	Airway resistance (R_N) ($cmH_2O.s/mL$)	Ctrl	0.137 ± 0.020
		TPM	$0.158 \pm 0.020^{\&}$
		OVA	$0.199 \pm 0.039^{*\&}$
		OVA+TPM	$0.257 \pm 0.047^{*}$
	Tissue damping (G) (cmH_2O/mL)	Ctrl	4.06 ± 0.47
		TPM	$4.84 \pm 1.11^{\&}$
		OVA	$5.97 \pm 1.28^{*}$
		OVA+TPM	$6.97 \pm 0.94^{*}$
	Tissue elasticity (H) (cmH_2O/mL)	Ctrl	16.74 ± 1.45
		TPM	$18.58 \pm 2.10^{\&}$
		OVA	$21.28 \pm 2.90^{*\&}$
		OVA+TPM	$26.27 \pm 5.43^{*}$
PV-Curve	Static compliance (C_{ST})(mL/cmH_2O)	Ctrl	0.103 ± 0.011
		TPM	$0.091 \pm 0.011^{\&}$
		OVA	$0.079 \pm 0.011^{*\&}$
		OVA+TPM	$0.060 \pm 0.014^{*}$
	Inspiratory capacity (IC) (mL)	Ctrl	1.00 ± 0.04
		TPM	$0.94 \pm 0.06^{\&}$
		OVA	$0.85 \pm 0.10^{*\&}$
		OVA+TPM	$0.69 \pm 0.12^{*}$
	PV loop area (mL)	Ctrl	2.25 ± 0.33
		TPM	$2.61 \pm 0.56^{\&}$
		OVA	$3.14 \pm 0.44^{*\&}$
		OVA+TPM	$4.06 \pm 0.79^{*}$

Table 2. Morphometric parameters. In Ctrl and TPM groups, mice were sensitized and challenged with saline, and exposed by intranasal instillation to 30 µL of saline (Ctrl group) or 15 µg total particulate matter from combustion of cashew nut shell (TPM group),diluted in 30 µL of saline. In OVA and OVA+TPM groups, mice were sensitized and challenged with OVA, and exposed by intranasal instillation to 30 µL of saline (OVA group) or 15 µg TPM(OVA+TPM group),diluted in 30 µL of saline. Values are mean ± SD. PMN, polymorphonuclear; BCI, bronchoconstriction index. *Values significantly different from Ctrl group; &values significantly different from OVA+TPM group

Group	Alveolar collapse (%)	PMN cells ($x10^{-3}/\mu m^2$)	Mean alveolar diameter (µm)	BCI
Ctrl	4.34±3.20	8.99±3.06	47.75±3.23	3.12±0.15
TPM	9.77±3.70*&	12.36±4.71&	41.43±5.96*	3.36±0.10*&
OVA	23.98±7.21*&	28.29±6.89*	32.59±4.81*	3.97±0.61*
OVA+TPM	31.56±6.56*	32.66±7.96*	28.59±4.99*	3.89±0.33*

DISCUSSION

The use of alternative sources or biofuels in the energy matrix has been listed as a worldwide solution to reduce air pollution, because they can do less harm to the environment. The use of biofuels has been growing, providing more efficient technologies and promoting social and environmental improvements, such as: reduction of pollution levels, increase in quality of life, and generation of jobs and income (Guardabassi, 2006). Brazil is one of the largest agricultural producers in the world and has great potential for the production of residual biomass. The energy utilization of residual products from the processing of cashew nuts, such as CNS, is already a reality in some industries. However, the adverse health effects of exposure to exhaust gases from the CNS combustion process are not completely known, so this was investigated in this study. In relation to exposure to air pollutants, special attention should be given to children, the elderly, pregnant women and individuals with previous cardiovascular or respiratory diseases, because these individuals are part of the so-called risk group (Danida, 2000), and may be more susceptible to the harmful effects of these pollutants. Several studies have been specifically aimed at individuals in this group. Previous studies suggest that regulatory policies for current air pollution control are inadequate and that these pollutants may be associated with the development and morbidity of asthma among children (Patel and Miller, 2009).

In a study of ten European cities, 14% of the cases of incident asthma in children and 15% of all exacerbations of childhood asthma were related to exposure to pollutants related to road traffic (Perez *et al.,* 2013). In a study reviewing the adverse effects of outdoor pollution in the elderly (Simoni *et al.,* 2015), the authors noted that there are few studies specifically targeting the elderly, and that specific respiratory morbidity and most of the data analyzed have shown increased risks in the elderly, compared with the rest of the population. In a previous study, the authors studied exposure to air pollution during pregnancy and found that exposure in the prenatal period to traffic-related air pollution could hamper fetal growth (Leem *et al.,* 2006). Alterations in respiratory mechanics were found with exposure to $PM_{4.0}$ from the combustion of CNS in animals subjected to a cigarette smoke-induced emphysema model (Gondim *et al.,* 2017). Our work aimed to demonstrate the harmful effects on the respiratory system caused by exposure to TPM from the combustion of CSN in mice previously subjected to the OVA-induced asthma model, simulating the exposure of this pollutant from biomass burning in individuals with previous respiratory disease. These individuals who participate in the processing of cashew nuts, either artisanal or industrial, as well as the population living near the cashew processing industries, using CNS as the energy matrix, are exposed to particles. A n acute exposure to particles from CNS combustion exhaust gases was used in the exposure model, where animals were given a single intranasal instillation of 15 µg TPM (TPM and OVA+TPM groups) suspended in 30 µl of saline. Assuming that the mean TPM concentration in a cashew nut processing environment can be on average 500 $\mu g/m^3$ (Galvão, 2016), and that the total volume of air inhaled by mice in 24 h is about 0.06 m^3, the exposure to a 15 µg dose is representative of 48 h exposure to this environment. An earlier study evaluated the respiratory toxicity of a similar dose with repeated exposure to particles produced by traffic and sugarcane burning (Mazzoli-Rocha *et al.,* 2014). In other studies, a dose of 15 µg of diesel exhaust particles was used to analyze effects of eugenol on the acute pulmonary toxicity of these particles (Zin *et al.,* 2009), and in the analysis of the protective effect of curcumin against pulmonary and cardiovascular effects induced by repeated exposure of these particles in mice (Nemmar *et al.,* 2012). In relation to our results, we assessed respiratory mechanics by the forced oscillation technique using constant phase model, and quasi-static PV curve. Our results demonstrated a small change, but not significant, between the TPM and Ctrl groups (Table 1), which could be explained by the small concentration and short period of exposure to TPM. However, there were significant changes among all values of the OVA and OVA+TPM groups compared to Ctrl group, and most values in the OVA group compared to the OVA+TPM group (Table 1).

Newtonian resistance (R_N) has been used as a good estimate of total central airway resistance (Bates, 2009). Therefore, we can assume that the significantly higher values in the OVA and OVA+TPM groups might have represented greater narrowing of airway lumen or increased stiffness of smooth muscle of the airways in this group, compared to the Ctrl group. In fact, this hypothesis was supported by morphometric data (Table 2, BCI) confirming narrower airways in both OVA groups. The increase in R_N in the OVA and OVA+TPM groups was expected; the OVA-induced asthma model provides an inflammatory process in the airways dominated by eosinophils (Wagers *et al.,* 2004), and this cellular increase was observed in these groups (Table 2-PMN Cells). Tissue resistance (G) and tissue elastance (H) are related to the intrinsic properties of the tissue. We believe that the changes in the OVA and OVA+TPM groups could have been due to changes in tissue rheological properties (Bates, 2009). This could result from thickening of the alveolar septum and presence of atelectatic areas, as observed in our histological analysis (Figure 5, panel C-D), showing increased amount of collapsed alveoli, reduced alveolar diameter, and an increased number of PMN cells (Table 2). Altogether, these findings would suggest lung parenchyma inflammation.

Analysis of the quasi-static PV curve was based on the respiratory model (Salazar and Knowles, 1964). The reduction of C_{ST} in the OVA and OVA+TPM grups compared to Ctrl group was consistent with the distortion of the pulmonary parenchyma with closure of small airways, constituting an effectively smaller lung (Wagers *et al.,* 2004). The higher PV loop area in the OVA and OVA+TPM groups could be attributed to the elastic component of impedance, which is supported by the lower C_{ST} found in these groups (Table 1). However, the higher PV loop area might be attributed to tissue changes (increase in collagen fibers, edema, alveolar collapse, presence of PMN cells) and possibly to a surfactant-associated mechanism (Muller *et al.,* 1998; Wagers *et al.,* 2001; Manali *et al.,* 2011). The results in Table 1, when compared to the Ctrl group, showed an increase in the values of R_N, G and H, respectively, of 15.37, 19.12 and 10.95% in the TPM group, 44.90, 46.97 and 27.11% in the OVA group, and 86.75, 71.38 and 56.86 in the OVA+TPM group. Similar findings were observed with the PV curve results. When compared to the Ctrl group, we identified a decrease in C_{ST} and IC and increase in PV loop area, respectively, of 11.28, 5.38 and 16.15% in the TPM group, 23.33, 15.10 and 39.65% in the OVA group, and 41.52, 30.89 and 80.53% in the OVA+TPM group. The pulmonary response to M Ch challenge was assessed by ΔR_N (Figure 3), also called airway hyperresponsiveness (AHR). After challenge with MCh, we observed significant alterations in the TPM, OVA and OVA+TPM groups when compared to the Ctrl group, demonstrating AHR in the animals in these groups. The use of a bronchoconstrictor in the smooth musculature of the airways may promote a reduction in lumen size(42, 46) or closure (Evans *et al.,* 2003), leading to an increase in ΔR_N.AHR is associated with airway inflammation, featuring eosinophilia, an increase in smooth muscle area, and goblet cell hyperplasia (Southam *et al.,* 2007).

Previous studies have reported AHR after exposure to pollutants in models of acute and chronic allergic inflammation with or without OVA (Southam *et al.,* 2007; Wang *et al.,* 2008; Zosky *et al.,* 2008; Serra *et al.,* 2017). Significant changes in ΔR_N(Figure 3) are expected to be found along with changes in $R_N$values (Table 1), as we observed in the OVA and OVA+TPM groups. However, in the TPM group, we did not observe changes inR_N, but we did see changes in ΔR_N. It is likely that the apparent (but not significant) increase in the R_N in the animals in the TPM group represented the presence of a mild inflammatory process at the airway level, which may be exacerbated when challenged with MCh, leading to significant changes in ΔR_N. The analysis of results concerning the micromechanics of the pulmonary parenchyma (Figure 4), provide us with additional information on the results obtained in *in vivo* analyses, as this approach reduces some of the mechanisms that contribute to hysteresis *in vivo*: surfactant and shear stress (Leite-júnior *et al.,* 2003). Similarly to the *in vivo* respiratory mechanics data, we did not obtain changes in resistance (R), elastance (E) and hysteresivity values between the TPM and Ctrl groups (p>0.05). However, R, E and hysteresivity showed statistically higher mean values (p<0.05) in the OVA and OVA+TPM groups in comparison to Ctrl group and OVA group in comparison to OVA+TPM group, at all frequencies analyzed (0.1, 0.3, 1, 3, 10 Hz). Similar results (OVA and OVA+TPM groups) were obtained in a study of rats exposed (13 weeks) to exhaust gases from glycerol combustion (Serra *et al.,* 2017). In this study, the increased R and E*in vitro* (Figure 4) might have been related to the presence of pulmonary parenchymal heterogeneities caused by factors such as alveolar edema, extracellular matrix remodeling and inflammation. Regarding the changes in hysteresis, the structural damping hypothesis states that changes in hysteresivity in the face of acute lung injury must reflect modifications in the kinetics of the stress-bearing process, for example, the extracellular fiber matrix, the surface lining layer, and the contractile apparatus (Fredberg and Stamenovic, 1989). Putatively, a significant modification of collagen–elastin fiber network might have been the main determinant of increased hysteresivity (Rocco *et al.,* 2003) in the OVA and OVA+TPM groups.

To confirm our hypothesis that exposure to air pollutants, specifically TPM from the combustion of CNS, in the individuals at risk group (with respiratory disease) can exacerbate the harmful effects to the respiratory system, we analyzed the percentage change in variables of the respiratory system that were analyzed. Results with significant differences in the comparisons between groups OVA and OVA+TPM (Table 1 and Figure 4), may be indicative of exacerbation of asthma, due to exposure to the particles. Specifically, the values of R_N, G,and Hin the OVA group were increased by 28.88, 16.61, 23.41%, respectively, compared to the OVA+TPM group. In addition, there was a decrease in $C_{ST}$and IC, and an increase in the PV loop area by 23.72, 18.60 and 29.27%, respectively. These results supportedour hypothesis that exposure to a pollutant, may exacerbate the effects of prior lung disease. Our study had limitations; the use of glass fiber filter for collection and subsequent removal of particles from the combustion of CNS mightnot have been the most appropriate for animal exposure. In the methods used to remove particles from fiberglass filters, even on clean filters (Ctrl group), fiberglass particles could be eventually released, and could cause irritation or inflammation in the lungs. However, similar methods were usedin a study with repeated exposure to particles produced by traffic and sugarcane burning (Zin *et al.,* 2009), which may reinforce our findings.

Conclusion

In conclusion, we suggest that exposure to air pollutants, specifically TPM from the combustion of CNS, can exacerbate the respiratory system injury in mice with previous lung disease.

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