



Residual oil fly ash worsens pulmonary hyperreactivity in chronic allergic mice

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ABSTRACT

BALB/c mice received saline (SAL groups) or ovalbumin (OVA groups) intraperitoneally (days 1, 3, 5, 7, 9, 11 and 13). After 27 days, a burst of intratracheal OVA or SAL (days 40, 43 and 46) was performed. Animals were then divided into four groups ($N=8$, each) and intranasally instilled with saline (SAL-SAL and OVA-SAL) or residual oil fly ash (SAL-ROFA and OVA-ROFA). 24 h later, total, initial and difference resistances (R_{tot} , R_{init} , R_{diff}) and static elastance (Est) were measured. Lung responsiveness to methacholine was assessed as slope and sensitivity of Est , R_{tot} , R_{init} , and R_{diff} . Lung morphometry (collapsed and normal areas and bronchoconstriction index) and cellularity (polymorphonuclear, mononuclear and mast cells) were determined. OVA or ROFA similarly impaired lung mechanics and increased the amount of polymorphonuclear cells and collapsed areas. OVA-ROFA showed even higher hyperresponsiveness, bronchoconstriction and mast cell infiltration. Thus, we concluded that ROFA exposure may add an extra burden to hyperresponsive lungs.

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1. Introduction

Epidemiological studies report that the inhalation of particulate matter is associated with a decline in lung function, increased respiratory symptoms, morbidity and mortality, especially in susceptible populations (Atkinson et al., 2001; Darrow et al., 2011). Among these, asthmatic persons are particularly affected by air pollution with recurrent respiratory exacerbations (Peden, 2001). However, the mechanisms underlying the increased sensitivity related to air pollution exposure in asthmatics are not well understood.

Animal models of pulmonary allergic inflammation have been shedding some light onto the mechanisms of asthma worsening after exposure to particulate matter (PM). Saldiva et al. (1992a) observed that the chronic exposure of rodents to urban air pollution results in secretory cell hyperplasia and ultrastructural ciliary alterations of the airway epithelium. Furthermore, respiratory defenses are compromised after prolonged exposure to air pollution in rats (Lemos et al., 1994). Interestingly, even a short-term exposure to concentrated ambient particles induces vasoconstriction of small pulmonary arteries in normal rats and in

those with chronic bronchitis (Batalha et al., 2002). Ambient levels of particulate air pollution trigger pulmonary inflammation with increased proinflammatory mediator levels (Ishii et al., 2004). In this vein some components of urban PM, such as diesel exhaust particles, can enhance allergen-induced airway inflammation (Dong et al., 2005). Additionally, residual oil fly ash (ROFA), a PM collected in oil-burning power plants, has been used in experimental animal studies to investigate the responses to PM inhalation (Antonini et al., 2002; Arantes-Costa et al., 2008; Gavett et al., 1999). It should be stressed that ROFA exposure leads to increased susceptibility to lung infection (Antonini et al., 2002) and can exacerbate respiratory system inflammation in mice with chronic allergic pulmonary inflammation (Arantes-Costa et al., 2008).

Although the ROFA-induced impairment of lung structure and hyperresponsiveness has been described (Arantes-Costa et al., 2008; Gavett et al., 1999), a detailed mechanical explanation to these findings has not been reported yet. Hence, we aimed at evaluating whether acute exposure to ROFA impairs lung mechanics in a dose–response approach and how it associates with histological alterations, bronchoconstriction index and lung inflammatory cell content in a murine model of chronic allergic inflammation.

2. Materials and methods

This Project was approved by the Ethics Committee on the Use of Animals, Health Sciences Center, Federal University of Rio de

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Janeiro, under the code IBCCF 049. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences, USA.

2.1. Animal preparation

Thirty-two male BALB/c mice (25 ± 5 g) received intraperitoneal injections of saline (100 μ L, 0.9% NaCl, $N=16$) or ovalbumin (OVA, 10 μ g in 100 μ L, 0.9% NaCl, $N=16$) on each of seven alternate days (days 1, 3, 5, 7, 9, 11 and 13). Forty days after the first instillation, the mice were challenged three times with intratracheal instillations of ovalbumin (20 μ g, 20 μ L, 0.9% NaCl) or saline (20 μ L, days 41, 44 and 47). Immediately after the last challenge they were divided into four groups ($N=8$, each) and intranasally instilled with 10 μ L of saline (SAL-SAL and OVA-SAL, respectively) or 10 μ L of ROFA (20 μ g/mL, SAL-ROFA and OVA-ROFA). For the instillation, the mice were anesthetized with sevoflurane and solutions (saline or ROFA) were gently instilled into their snouts with the aid of a precision pipette. The animals recovered rapidly after instillation.

2.2. ROFA granulometry

Our ROFA was extracted from an incinerator located at the University Hospital, University of São Paulo, Brazil. The distribution of particle sizes was determined by laser diffraction (Long Bench Mastersizer S, Malvern Instruments Ltd, Malvern, Worcestershire, United Kingdom). The particulate matter was visualized by scanning electron microscopy (JEOL 5310, Tokyo, Japan).

2.3. Pulmonary mechanics

Twenty-four hours after the intranasal instillation of ROFA, the animals were sedated (diazepam, 1 mg i.p.), anesthetized (pentobarbital sodium, 20 mg/kg BW i.p.), tracheotomized, and a snugly fitting cannula (0.8 mm i.d.) was introduced into the trachea. The animals were then paralyzed (pancuronium bromide, 0.1 mg/kg) and the anterior chest wall was surgically removed; thus, the pressure measured in the airway represents transpulmonary pressure (PL). A constant-flow ventilator (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) provided artificial ventilation with a frequency of 100 breaths/min, a tidal volume of 0.2 mL, flow of 1 mL/s, and positive end-expiratory pressure amounting to 2 cmH₂O. For the determination of pulmonary mechanics a 5-s end-inspiratory pause could be generated by the ventilator. A pneumotachograph with 1.5 mm i.d., length of 4.2 cm and distance between side ports of 2.1 cm was connected to the tracheal cannula for the measurements of airflow (V'). Lung tidal volume (VT) was determined by V' signal integration. The pressure gradient across the pneumotachograph was determined by means of a differential pressure transducer (Validyne MP45-2, Engineering Corp., Northridge, CA, USA). The equipment resistance (R_{eq}) including the tracheal cannula was previously measured using different flow rates ($R_{eq}=0.12$ cmH₂O/mL/s) and subtracted from pulmonary resistive pressure so that the present results represent intrinsic values. PL was measured with a differential pressure transducer (Validyne MP-45, Engineering Corp., Northridge, CA, USA). All signals were conditioned and amplified in a Beckman type R Dynograph (Schiller Park, IL, USA). Flow and pressure signals were also passed through 8-pole Bessel filters (902LPF, Frequency Devices, Haverhill, MA, USA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA, USA), and stored on a microcomputer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, QC, Canada).

Lung initial (Rinit), difference (Rdiff) and total resistances (Rtot), and static elastance (Est) were computed by the end-inflation occlusion method (Bates et al., 1985, 1988). Briefly, after end-inspiratory occlusion, there is an initial fast drop in transpulmonary pressure (ΔP_1) from the pre-occlusion value down to an inflection point (P_i) followed by a slow pressure decay (ΔP_2), until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung (Pel). ΔP_1 selectively reflects airway resistance in normal animals and humans (Bates et al., 1985; Saldiva et al., 1992b); Newtonian resistance (Rinit) was computed by dividing ΔP_1 by the flow immediately preceding the occlusion. ΔP_2 reflects stress relaxation or viscoelastic properties of the lung, together with a small contribution of time constant inequalities; Rdiff was calculated as $\Delta P_2/V'$ immediately preceding the occlusion. Est was calculated by dividing Pel by VT (Bates et al., 1985). Rtot is the sum of Rinit and Rdiff.

2.4. Measurement of airway responsiveness

Different progressive doses (3–10,000 μ g/mL) of methacholine (MCh, acetyl- β -methylcholine chloride; Sigma-Aldrich, St. Louis, MO, USA) were administered via a silastic catheter indwelled into the jugular vein. Data were sampled at 30 s, 1 min and 3 min after the injection of the agonist (Lima et al., 2002). During off-line data processing, the sample with the highest PL in each dose was analyzed. The lung responsiveness to methacholine was assessed as reactivity and sensitivity of Est, Rtot, Rinit and Rdiff. Sensitivity represents 50% of the maximal variation between the baseline and the highest values of each mechanical parameter; reactivity was measured as the slope of the linear regression associating mechanical variables and MCh concentrations.

2.5. Lung histology

Immediately after the measurements of lung mechanics, a laparotomy was performed, and heparin (1000 IU) was intravenously injected. The abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage and quick death. The trachea was clamped at end-expiration. The right lungs were removed *en bloc*, quick-frozen by immersion in liquid nitrogen, and fixed with Carnoy's solution. The lungs were, then, embedded in paraffin, and 4- μ m thick slices were cut and stained with hematoxylin/eosin or alcian-blue. Morphometric 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 (Axioplan, Zeiss, Oberkochen, Germany). The fraction area of collapsed alveoli or normal pulmonary areas, and the amount of polymorpho- (PMN) and mononuclear (MN) cells, as well as pulmonary tissue were determined by the point-counting technique (Weibel et al., 1966). Morphometric analysis and bronchoconstriction index were performed at 400 $\times$ magnification and the cellularity was assessed at 1000 $\times$ magnification across 10–15 random non-coincident microscopic fields in each animal. The bronchoconstriction index (BCI) was determined in 10 non-coincident microscopic fields per animal by counting the number of point into the airway lumen (NP) and intercepts through the airway wall (NI) using a reticulum and applying the equation: $BCI = NI/\sqrt{NP}$ (Sakae et al., 1994).

2.6. Statistical analysis

Statistical analyses were performed with SigmaStat 3.11 statistical software (SYSTAT, Chicago, IL, USA). The normality of the data (Kolmogorov–Smirnov test with Lilliefors' correction) and the homogeneity of variances (Levene median test) were evaluated. Then, two-way ANOVA test followed by Tukey test was used to

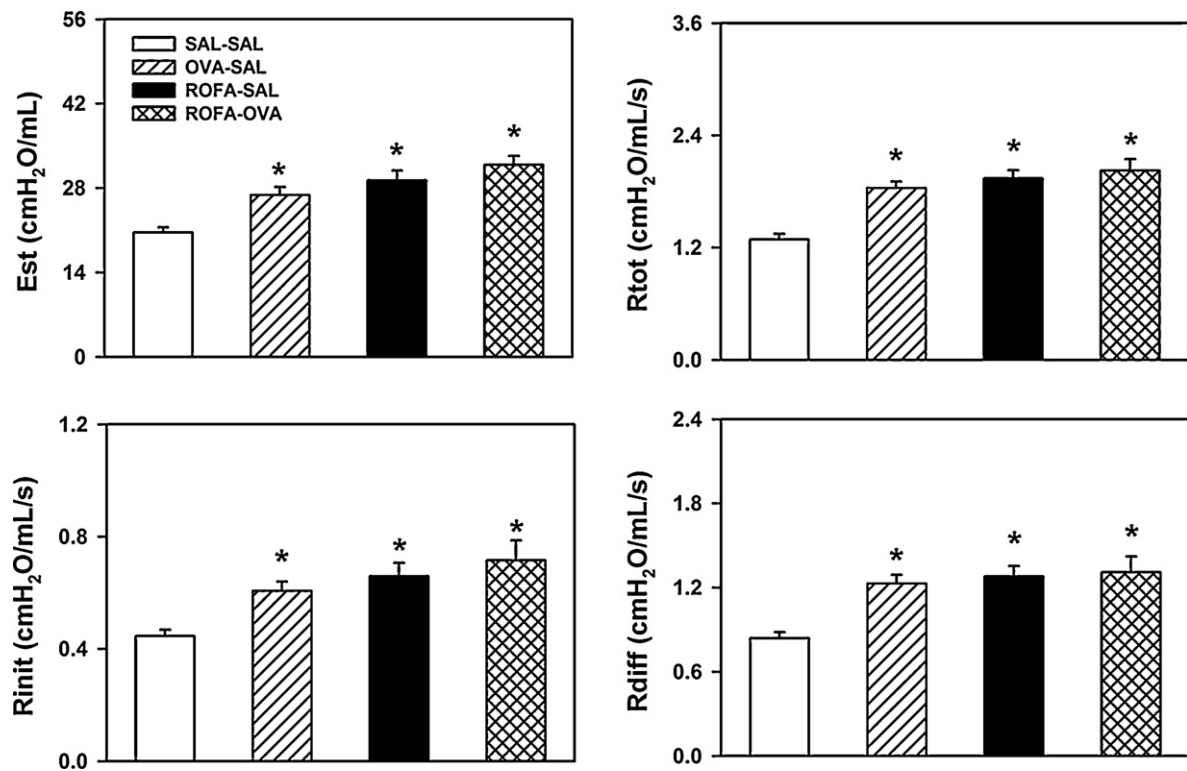


Fig. 1. Baseline pulmonary mechanics. Lung static elastance (Est), total (Rtot), initial (Rinit) and difference (Rdiff) resistances 24 h after acute exposure to residual oil fly ash (ROFA) or saline (SAL) in animals that had been exposed to saline (SAL) or ovalbumin (OVA). Columns represent the average of 10 mice, 10–15 determinations per animal. Bars represent SEM. *Significantly different from SAL-SAL. In all instances $p < 0.05$.

assess differences among groups. The significance level was set at 5%.

3. Results

Granulometry of our ROFA disclosed that the average particle diameter amounted to 66.5 μm . We observed that around 7.6% of ROFA particles presented an average diameter smaller than 10 μm and around 2.1% were smaller than 2.5 μm .

OVA-SAL, SAL-ROFA and OVA-ROFA presented similarly impaired lung mechanics at baseline, with higher Rinit, Rdiff, Rtot, and Est than SAL-SAL group (Fig. 1). Dose–response curves disclosed that OVA-SAL and SAL-ROFA presented higher slopes and sensitivity than SAL-SAL for Est, Rinit, Rdiff and Rtot. However, OVA-ROFA group showed even larger increases in slope and sensitivity for Rtot and Rinit compared with OVA-SAL and SAL-ROFA groups (Fig. 2).

OVA-SAL, SAL-ROFA and OVA-ROFA groups presented more PMN in the lung than SAL-SAL. A higher fraction of collapsed areas

was observed in OVA-SAL, SAL-ROFA and OVA-ROFA than in SAL-SAL. Additionally, the amount of collapsed areas was even higher in OVA-ROFA than in OVA-SAL mice. The bronchoconstriction index was significantly larger in the animals that received ovalbumin than in SAL-SAL (Table 1, Fig. 3). The number of mast cells was significantly higher in OVA-ROFA than in SAL-SAL and SAL-ROFA; in OVA-ROFA the amount of mast cells was about twice that in OVA-SAL (Table 1).

4. Discussion

4.1. Lung mechanics and histology after acute ROFA or chronic OVA exposures

Granulometry demonstrated that our ROFA was mainly composed by particles bigger than 10 μm , which would be less harmful than the smaller ones (Donaldson et al., 2001). In spite of this, we could observe an important inflammatory process induced by ROFA exposure (Table 1). Possibly, fine and ultrafine particles attached to

Table 1
Histological analyses.

	Cellularity		Mast cell (cell/total cell)	Morphometry		BCI
	PNM (cell $\times 10^{-3}/\mu\text{m}$)	MN (cell $\times 10^{-3}/\mu\text{m}$)		C (%)	N (%)	
SAL-SAL	3.7 $\pm$ 0.3	6.4 $\pm$ 0.3	0.07 $\pm$ 0.02	9.8 $\pm$ 1.2	90.4 $\pm$ 1.2	1.00 $\pm$ 0.11
OVA-SAL	5.6 $\pm$ 0.4*	6.2 $\pm$ 0.3	0.24 $\pm$ 0.02*	60.8 $\pm$ 1.5*	38.5 $\pm$ 1.3*	1.22 $\pm$ 0.08*
SAL-ROFA	5.4 $\pm$ 0.3*	6.0 $\pm$ 0.4	0.14 $\pm$ 0.01	62 $\pm$ 1.5*	37.4 $\pm$ 1.4*	1.07 $\pm$ 0.13
OVA-ROFA	7.0 $\pm$ 0.2*	4.9 $\pm$ 0.3*	0.44 $\pm$ 0.05* [§]	73.8 $\pm$ 2.3* [#]	24.9 $\pm$ 2.2*	1.27 $\pm$ 0.06* [§]

PMN and MN, fraction areas of polymorpho- and mononuclear cells, respectively; C and N, percent of collapsed and normal areas; BCI, bronchoconstriction index; SAL-SAL, OVA-SAL, SAL-ROFA and OVA-ROFA, groups that received saline solution twice, ovalbumin and saline, saline and residual oil fly ash, and ovalbumin and residual oil fly ash, respectively. Data were gathered from 10 to 15 random, non-coincident fields per mouse.

* Different from SAL-SAL, $p < 0.05$.

Different from OVA-SAL, $p < 0.05$.

§ Different from SAL-ROFA, $p < 0.05$.

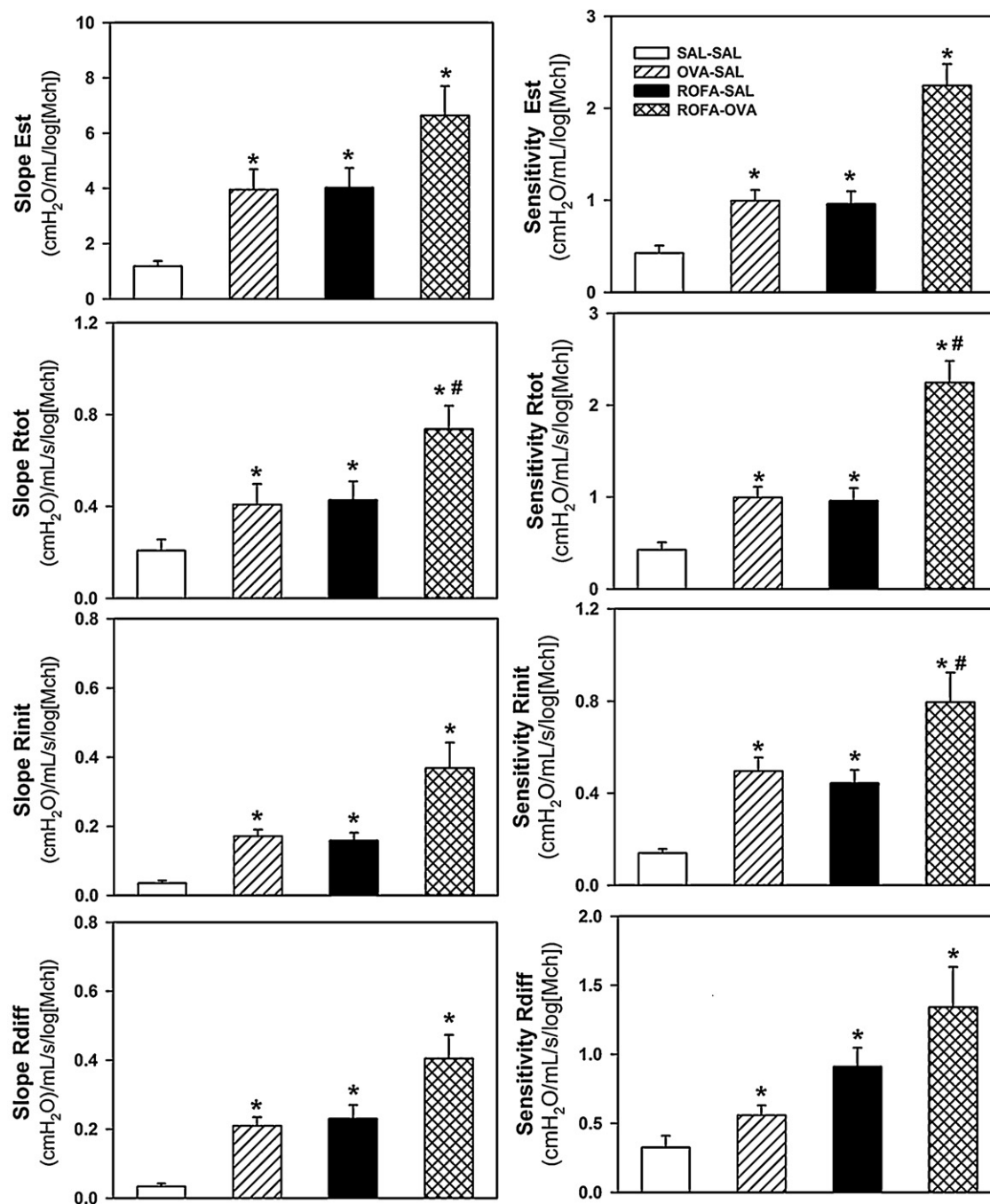


Fig. 2. Slope and sensitivity of pulmonary mechanics after a dose–response curve with increasing doses of methacholine. Lung static elastance (Est), initial (Rinit), difference (Rdiff) and total (Rtot) resistances 24 h after acute exposure to residual oil fly ash (ROFA) or saline (SAL) in animals that had been exposed to saline (SAL) or ovalbumin (OVA). Columns represent the average of 10 mice, 3 determinations per animal in each dose. Bars represent SEM. *Significantly different from SAL-SAL and #significantly different from OVA-SAL and SAL-ROFA. In all instances $p < 0.05$.

larger particulate matter, as measured during granulometry, unfasted themselves during preparation of the intranasally instilled solution and penetrated deeper into the airways, damaging the lung parenchyma (Donaldson and Stone, 2003). Last but not least our ROFA also contained smaller particles that could induce lung lesions.

Our study was done considering the same time lag after exposure, as previously reported in the literature (Laks et al., 2008; Mazzoli-Rocha et al., 2008; Rhoden et al., 2004; Wegesser et al., 2009). The dose of ROFA utilized in this study was about 2.5 times smaller than the average daily exposure to PM in many cities such as São Paulo, where our ROFA was collected. In spite of this, after a

single exposure to ROFA, we observed a pronounced infiltration of PMN cells with an increased fraction of collapsed air spaces (Table 1). These alterations in cellularity and morphometry were associated with an impairment of lung mechanics similar to that observed after exposure to other particulate matter (Laks et al., 2008; Mazzoli-Rocha et al., 2008; Riedel et al., 2006).

Decays in respiratory function and histology similar to those produced by ROFA were observed in the chronic allergic inflammation model induced by ovalbumin (Fig. 1 and Table 1). It is known that ovalbumin sensitization followed by an ovalbumin challenge can induce an experimental condition that mimics asthma in many aspects, but not all (Kucharewicz et al., 2008). We found that

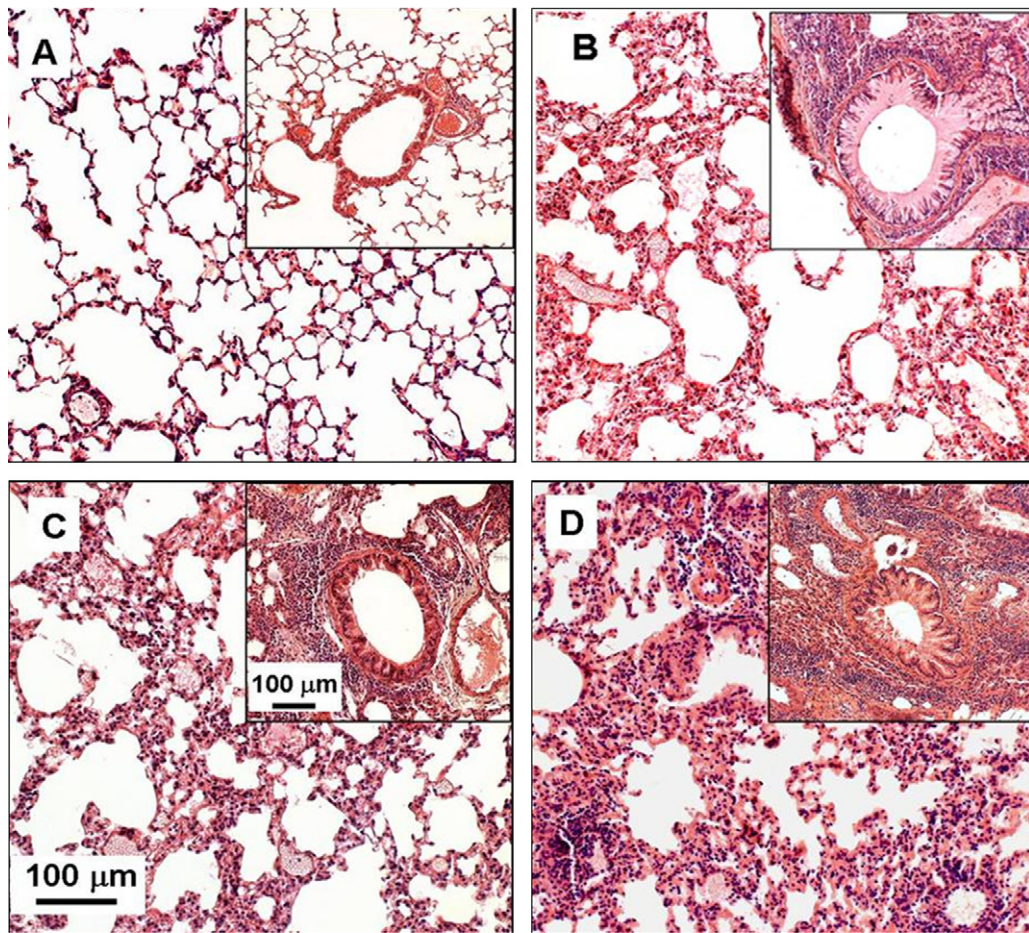


Fig. 3. Photomicrographs of pulmonary parenchyma stained with hematoxylin/eosin (200 $\times$) depicting representative lungs of animals that received saline and saline (panel A), ovalbumin and saline (panel B), saline and residual oil fly ash (panel C), and ovalbumin and residual oil fly ash (panel D). Inserts show representative airways in each group; bar = 100 μ m. Intact parenchyma and alveolar shape were observed in panel (A); however, in panels (B–D) parenchyma thickening, alveolar destruction, high cellular infiltration, and airway constriction were detected.

ovalbumin increased pulmonary resistances, as expressed by Rinit (central), Rdiff (peripheral) and Rtot (central and peripheral), and elastance (Fig. 1), as previously reported (Xisto et al., 2005). Other authors also found increased total pulmonary resistance using different methods (Hessel et al., 1995; Wagers et al., 2002). It is accepted that both central and peripheral airways are inflamed, as well as lung tissue (Bousquet et al., 2000).

The inflammatory process results from a complex interaction between inflammatory mediators and cells (Kay, 2005). In this study, the animals sensitized and challenged with ovalbumin presented an increased number of PNM cells (Table 1). Additionally, mast cells potentially modulate the levels of airway inflammation and remodeling (Broide, 2008). Studies on airway remodeling in mast cell-deficient mice chronically challenged with allergen reveal that mast cells mediate chronic airway inflammation as well as remodeling features (Yu et al., 2006). We observed an increased proliferation of mast cell in animals with chronic allergic inflammation (Table 1) as well as an increased bronchoconstriction (Fig. 3B, insert) index (Table 1). This bronchoconstriction most probably responds for the increased pulmonary resistance, expressed in this study as Rinit (central airways) and Rtot (central and peripheral resistances) (Fig. 1).

In summary, these findings suggest that acute ROFA exposure or chronic OVA can independently impair pulmonary mechanical properties and yield lung inflammation.

4.2. Lung mechanics and histological alterations induced by the association of chronic OVA and acute ROFA exposure

It has been recently reported that acute exposure to ROFA is able to further increase respiratory responsiveness in a murine model of chronic allergic inflammation, without exacerbating inflammation (Arantes-Costa et al., 2008). However, the extent of lung mechanical impairment in animals treated with ROFA and OVA has not been assessed yet. In the present study chronic ovalbumin administration or acute ROFA exposure similarly degraded lung mechanics and the association of these two factors did not result in a synergic effect (Fig. 1). On the other hand, after MCh challenge, OVA-ROFA animals presented an even higher pulmonary hyperresponsiveness, with increased reactivity and sensitivity of Rtot and Rinit (Fig. 2), bronchoconstriction index, and the amount of mast cells (Table 1 and Fig. 3D, insert). Interestingly, the amount of PMN in OVA-ROFA did not differ from those in OVA-SAL and SAL-ROFA (Table 1).

Increased lung responsiveness associated with pollutant exposure in chronic allergic inflammation models was also reported in other studies (Gavett et al., 1999; Hamada et al., 1999; Wang et al., 2008). Wang et al. (2008) found that urban PM exposure in ovalbumin-challenged A/J mice resulted in a significant increase in lung hyperresponsiveness 4 days after exposure, and Gavett et al. (1999) using BALB/c mice observed pulmonary hyperresponsive-

ness with increased respiratory system resistance and decrease in respiratory system compliance only 8 days after ROFA exposure. Interestingly, we found an increased lung hyperresponsiveness 1 day after pollutant exposure. These discrepancies may be due to different methodological issues. Wang et al. (2008) used a less reactive mouse strain and pollutant; indeed, BALB/c was shown to be more sensitive to PM inhalation (Vancza et al., 2009). On the other hand, Gavett et al. (1999) used the same strain and pollutant but our protocol of sensitization and challenge lasted longer than theirs.

PMN cell infiltration did not increase when ROFA exposure was associated with chronic allergic inflammation (Table 1), as previously reported (Arantes-Costa et al., 2008; Gavett et al., 1999; Goldsmith et al., 1999). On the other hand, a significant increase in the amount of eosinophils and neutrophils in asthmatic animals exposed to pollutants was also described (Hamada et al., 1999; Poynter et al., 2006). The discrepancies could be explained by different methodological approaches, since the ovalbumin challenge of Hamada et al. (1999) consisted of six nebulizations of ovalbumin, against our three intratracheal instillations; in the study by Poynter et al. (2006) the pollutant exposure was repeated during 5 consecutive days, versus our single exposure.

Additionally, our results showed increased lung collapsed areas and bronchoconstriction indexes in OVA-ROFA mice, which may be responsible for the higher reactivity and sensitivity in MCh dose-response curve for Rtot and Rinit. Indeed, MCh produces an inhomogeneous patchy pattern of ventilation distribution (Bates et al., 1997) that could explain these findings in the central airways.

Ovalbumin sensitization and challenge causes an inflammatory response in the airways. It is known that Th1 and Th2 responses are present in models of allergic inflammation (Kucharewicz et al., 2008). The Th2 response typically involves an increase in interleukins IL-4, IL-5, IL-10 and IL-13 (Lambrecht, 2001). In allergic inflammation the involvement of Th1 cytokines (IL-2, TNF- α , INF γ among others) may explain IgE-independent mechanisms (Wilder et al., 1999). On the other hand, PM-induced inflammation starts through macrophage activation that is antagonized by various mechanisms involving mediators and cytokines especially those of the Th2 family (Mills et al., 2000; Scapellato and Lotti, 2007) and BALB/c mice respond more importantly to antigens with a Th2 profile (Mills et al., 2000). The proinflammatory pathway of nuclear factor kappa B (NF- κ B) is also involved, but NF- κ B activation is suppressed by several agents, including Th2 cytokines and interferons among others (Ahn and Aggarwal, 2005). These findings are in line with our results, since we demonstrated that either OVA or ROFA could trigger inflammation, but their association did not result in a synergistic effect.

Interestingly, the mechanical response as evaluated by MCh dose-response curves did not follow the pattern of inflammation. Both OVA and ROFA triggered higher and similar sensitivities and reactivities for Est, Rtot, Rinit and Rdiff. However, the association of OVA and ROFA produced a further increase in hyperresponsiveness after methacholine challenge. Under similar conditions to ours, smooth-muscle-specific actin content was increased in OVA-treated mice, which resulted in stronger airway contraction (Xisto et al., 2005). ROFA binds to the cell surface, activating transient receptor potential vanilloid 1 (TRPV1), thus increasing the intracellular concentration of Ca²⁺ (Agopyan et al., 2004), which could potentiate smooth muscle contraction. Hence, by two different mechanisms the OVA-ROFA association resulted in increased pulmonary resistance in the face of methacholine stimulation.

In conclusion, our study suggests that acute exposure to ROFA or chronic allergic inflammation induced by ovalbumin similarly altered lung mechanics, histology and pulmonary responsiveness to injected MCh. Although together they did not worsen pulmonary mechanics and the influx of PMN, they led to a more pronounced pulmonary responsiveness, bronchoconstriction, and amount of

mast cells, suggesting that ROFA exposure can be deleterious to hyperresponsive lungs.

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