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Effects of Eucalyptol in respiratory system mechanics on acute lung injury after exposure to short-term cigarette smoke

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ABSTRACT

Eucalyptol is a compound that has demonstrated antioxidant, anti-inflammatory and bronchodilator effects, but there are no investigations about the effects of this constituent on the respiratory system mechanics in relation to acute lung injury caused by short-term cigarette smoke (CS) exposure. In view of the above, this work investigated the effects of Eucalyptol on the mechanics of the respiratory system of mice in short-term CS exposure. For this, we used data from respiratory mechanics *in vivo*, and histopathology and lung parenchymal morphometry analysis *in vitro*. The experiments were performed on C57black/6 mice divided into 5 groups. One group exposed to ambient air (AA + T), and another to cigarette smoke (CS + T) for 5 consecutive days and treated with 1% Tween 80 solution. The other groups were exposed to cigarette smoke for 5 consecutive days, and treated with Eucalyptol at doses of 30 mg/kg (CS + E30), 100 mg/kg (CS + E100), 300 mg/kg (CS + E300). Our results demonstrated significant changes in all variables of respiratory mechanics and lung parenchyma morphometry analyzed for the AA + T group compared to the CS + T group, confirming the establishment of the lesion induced by exposure to cigarette smoke. We also observed that mice treated with Eucalyptol orally at a dose of 300 mg/kg (CS + E300) showed improvement in all variables compared to the group exposed to cigarette smoke and treated with 1% Tween 80 (CS + T) demonstrating the effectiveness of Eucalyptol in preventing lung injury induced by exposure to CS. In conclusion, our results demonstrated that the Eucalyptol was able to prevent the acute lung injury in mice submitted to short-term cigarette smoke exposure.

1. Introduction

Tobacco smoking remains the overwhelming risk factor for COPD in the world. Although acute lung inflammation caused by short-term exposure to cigarette smoke (CS) does not represent a model that holds all the characteristics of COPD, both have common basic pathophysiological mechanisms: hypoxemia, pulmonary edema, and oxidative stress (Suda et al., 2011; Bezerra et al., 2006; Lanzetti et al., 2008a; Valença et al., 2009). A greater understanding of the inflammatory mechanisms involved in this pathology achieved in the last decades has resulted in the identification of several processes and goals for the development of new anti-inflammatory therapies (Barnes, 2013).

Pharmacological treatment through the use of bronchodilators and corticosteroids is widely used in many lung diseases. However, these have high costs and several associated side effects, evidencing the need to seek alternative therapies that minimize these difficulties. Corresponding to this need, the study of pharmacological attributes of products of plant origin used for medicinal purposes should be seen as an important economic and scientific strategy in the investigation of therapeutic alternatives (Greiner et al., 2013).

An example of biologically active plant compounds, Eucalyptol (also known as 1,8-Cineol), a major constituent of leaf oil of eucalyptus species such as *Eucalyptus globulus* and *Eucalyptus tereticornis*, is a terpenoid free of side effects seen on steroids. Thus, systemic Eucalyptol therapy seems to be favorable because of its lipophilicity related to the terpene group, and its excretion predominant by exhalation (Juergens et al., 2004a; Aparicio et al., 2007; Liu and Chang, 2011).

Studies have shown that the biological activities of Eucalyptol include various mechanisms and important molecules in the development of inflammation, acute and chronic, respiratory system, such as attenuation of tracheobronchial resistance (Nascimento et al., 2009), decrease of molecules involved in the production of mucus (Zhou et al., 2007), proinflammatory cytokines (Juergens et al., 2004b; Bastos et al., 2011; Sadlon and Lamson, 2010; Zhao et al., 2014; Li et al., 2016) and proteases (Kim et al., 2015).

Despite these promising effects and the need for alternative

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therapies in COPD and acute affections that affect the respiratory system, the scientific literature lacks studies investigating the effects of Eucalyptol in respiratory system mechanics in the face of lung inflammation induced by short-term cigarette smoke exposure. Consequently, in the present study we investigated the effects of Eucalyptol in respiratory mechanics *in vivo*, histopathology and lung parenchymal morphometry analysis *in vitro*, of animals short-term cigarette smoke exposure.

2. Materials and methods

2.1. Animals

All animals received humane care and the experiments complied with the following guidelines: ARRIVE, the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978), and National Council for Controlling Animal Experimentation, Ministry of Science, Technology and Innovation (CONCEA/MCTI), Brazil. This study was approved by the Ethics Committee on the Use of Animals of the State University of Ceará (Protocol 06808872018). The experimental study was carried on in the Laboratory of Biophysics of Breathing.

C57black/6 mice with body mass of 25 ± 5 g and access to water and food *ad libitum*, were used in this study. The animals from groups exposed to cigarette smoke were exposed for 5 days. Mice were exposed to 12 commercial cigarettes per day for 5 days using an inhalation chamber (40 cm long, 30 cm wide, and 25 cm high). The animals were placed in the inhalation chamber, housed inside an exhaust hood. The cigarettes were coupled to a 60 mL plastic syringe, and the cigarette smoke was sucked into the syringe and then immediately expelled into the inhalation chamber. The animals were kept in this condition, with presence of cigarette smoke in this environment, for 6 min. Then the inhalation chamber cap was removed, and the exhaust fan connected to evacuate the smoke for 1 min. Exposure to cigarette smoke was repeated four times (4 × 6 min) with a 1 min escape interval after each exposure. This procedure was repeated three times a day (8 h am, 12 h am and 4 h pm) (Valença et al., 2009).

We used 40 animals randomly divided into five groups. In the first group (n = 8), the animals were exposed to Ambient Air for 5 days and received a daily treatment of vehicle (Tween-80 [1%] solution) (AA + T Group). In the second group (n = 8), the animals were exposed to smoke from 12 cigarettes per day for 5 days and received a daily treatment of vehicle (Tween-80 [1%] solution) (CS + T group). In the third group (n = 8), animals were exposed to smoke from 12 cigarettes per day for 5 days and received a daily treatment of 30 mg/kg Eucalyptol (CS + E30 group). In the fourth group (n = 8), animals were exposed to smoke from 12 cigarettes per day for 5 days and received a daily treatment of 100 mg/kg Eucalyptol (CS + E100 group). In the fifth group (n = 8), animals were exposed to smoke from 12 cigarettes per day for 5 days and received a daily treatment of 300 mg/kg Eucalyptol (CS + E300 group).

Eucalyptol (Sigma Chemical Co., St. Louis, MO, USA) was diluted with saline (30, 100, 300 mg/kg) and administered intragastrically once daily before first exposure to cigarette smoke.

2.2. Respiratory system mechanics

24 h after the end of the exposure period, the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p., Hypnol® 3%, Syntect, Brazil) and tracheotomized. The animals were intubated with a 18-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. Mices were then paralyzed with pancuronium bromide (0.5 mL/kg, i.p., Cristália, Brazil) administered intraperitoneally.

Initially we standardized the mechanical history of the respiratory system with two deep inflations (DI, 6-s long, peak pressure: 30 cmH_2O). Followed by 5 min of ventilation at baseline. Soon after, the impedance of the respiratory system (Z_{rs}) was measured with the forced oscillation technique (Hantos et al., 1992), 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 (Hirai et al., 1999):

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

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

where R_N is the Newtonian resistance, which represents the central airways 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) defined by the PEEP, 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. Another forced oscillation technique ensued to determine respiratory system mechanics.

2.3. Methacholine challenge

Immediately after measurements of respiratory system mechanics, two DIs were done, followed by 5 min of ventilation with baseline settings. Airway smooth muscle hyperresponsiveness was evaluated by inhalation of methacholine (MCh) (Sigma-Aldrich) 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.

The nebulization was carried out during 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), followed by two DIs and another forced oscillation data gathering.

2.4. Histological study

Immediately after the determination of respiratory system mechanics, the rib cage was opened and Heparin (1000 IU) was injected in right ventricle of the heart. The trachea was clamped at end-expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly euthanized the animals. The lungs were perfused with saline and, then, removed in bloc. The right lung was isolated, frozen in liquid nitrogen, and stored for biochemistry analysis; the left lung was kept at functional residual capacity and fixed in Millonig's formaldehyde (100 mL HCHO, 900 mL H_2O, 18.6 g NaH_2PO_4, 4.2 g NaOH). Slides containing left lung sections were stained with hematoxylin and eosin (HE) and examined by optical 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 or normal pulmonary areas, and the amount of polymorphonuclear (PMN) cells, as well as pulmonary tissue were determined by the pointcounting technique (Weibel, 1990). 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., 2009).

Cellularity was assessed at 1000× magnification across 10–15 random non-coincident microscopic fields in each animal. Morphometric analysis and determination of bronchoconstriction index were done at 400× magnification. 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).

2.5. Statistical analysis

Results are presented as mean ± SD, where n represents the number of samples. Data normal distribution and homogeneities of variances were tested with Kolmogorov-Smirnov (with Lilliefors's correction) and Levene median tests, respectively. If both conditions were satisfied, Student's t-test was used. If any condition was refused, Mann-Whitney non-parametric test was used instead. A difference was considered significant if $p < 0.05$.

3. Results

Fig. 1 shows respiratory system mechanical data of AA + T (R_N = 0.179 ± 0.037, G = 2.77 ± 0.54, H = 11.77 ± 1.99, C_{ST} = 0.097 ± 0.011, CI = 0.98 ± 0.07, PV Loop Area = 2.92 ± 0.43), where the animals were exposed to ambient air and treated with 1% Tween 80 for 5 consecutive days. In the CS + T (R_N = 0.316 ± 0.051, G = 7.53 ± 0.97, H = 25.51 ± 3.63, C_{ST} = 0.073 ± 0.013, CI = 0.70 ± 0.13, PV Loop Area = 5.02 ± 0.78), CS + E30 (R_N = 0.293 ± 0.040, G = 7.34 ± 0.88, H = 26.53 ± 3.31, C_{ST} = 0.073 ± 0.011, CI = 0.73 ± 0.17, PV Loop Area = 5.02 ± 0.61), CS + E100 (R_N = 0.277 ± 0.019, G = 6.41 ± 1.19, H = 24.65 ± 2.54, C_{ST} = 0.075 ± 0.014, CI = 0.74 ± 0.11, PV Loop Area = 4.13 ± 0.82), CS + E300 (R_N = 0.180 ± 0.025, G = 2.88 ± 0.61, H = 13.12 ± 2.80, C_{ST} = 0.075 ± 0.014, CI = 0.74 ± 0.11, PV Loop Area = 4.13 ± 0.82) groups, where the animals were exposed to cigarette smoke and treated for 5 consecutive days with 1% Tween 80 and 30 mg/kg, 100 mg/kg and 300 mg/kg Eucalyptol, respectively.

Our results demonstrated significant changes in all respiratory mechanics variables analyzed for the AA + T group compared to the CS + T group, confirming the establishment of the lesion induced by short-term cigarette smoke exposure. As well as, between the AA + T group, compared to the CS + E30 and CS + E100 groups, demonstrating that treatment with Eucalyptol at doses of 30 mg/kg and 100 mg/kg could not avoid injury induced by short-term cigarette smoke exposure.

We also observed that mice treated with oral Eucalyptol at the dose of 300 mg/kg (CS + E300) showed improvement in all variables of respiratory mechanics compared to the group exposed to cigarette smoke and treated with 1% Tween 80 (CS + T), demonstrating the effectiveness of Eucalyptol in avoiding lung injury induced by short-term cigarette smoke exposure.

Fig. 2 shows the variation in ΔR_N after nebulization of MCh (30 mg/mL) in all groups. We observed an increase (ΔR_N) of the groups CS + T, CS + E30 and CS + E100 in comparison to the AA + T group, evidencing an airway hyperresponsiveness (AHR). This behavior was not observed in the CS + E300 group when compared to the CS + T group.

Fig. 3 depicts representative lung histological images of the AA + T, CS + T, CS + E30, CS + E100 and CS + E300 groups. There was the presence of alveolar collapse, thickened septa and cellular infiltration in the photomicrographs of the pulmonary parenchyma of the CS + T, CS + E30 and CS + E100 groups.

Table 1 displays the alveolar collapse, amount of polymorphonuclear cells, mean alveolar diameter and bronchoconstriction index. We observed an increase in alveolar collapse, amount of polymorphonuclear cells, mean alveolar diameter and bronchoconstriction index of the CS + T, CS + E30 and CS + E100 groups when compared to the AA + T group. Altogether, these findings may suggest pulmonary inflammation and bronchoconstriction.

4. Discussion

Products of plant origin, such as Eucalyptol, have been investigated as possible therapeutic tools for respiratory system diseases caused by cigarette smoke and other pollutants capable of inducing acute injuries or aggravating chronic diseases (Lanzetti et al., 2008b; Pires et al., 2011; Zin et al., 2011; de Moura et al., 2012). Thus, considering the scarcity of reports on the effects of these compounds on pulmonary function, this research sought to expand knowledge about the therapeutic use of Eucalyptol using daily doses of 30, 100 or 300 mg/kg in animals submitted to short-term CS exposure, in the analysis of the mechanics of the respiratory system. The concentrations used in the present work are assured by a previously study (Caldas et al., 2016), where, from hematological and biochemical analyzes, no signs of toxicity or deaths were recorded during the 50 consecutive days of treatment oral administration with at doses of 100, 500 or 1000 mg/kg Eucalyptol.

In relation to our results, we assessed respiratory mechanics by the forced oscillation technique using a constant phase model and quasi-static PV curve (Fig. 1). In the constant phase model, the Newtonian resistance (R_N) represents a good estimate of the total central airway resistance (Salazar and Knowles, 1964), and tissue resistance (G) and tissue elastance (H) are related to the intrinsic properties of the tissue (Fredberg and Stamenovic, 1989). In the quasi-static PV curve, the static compliance (C_{ST}) indirectly measures the degree of lung tissue distensibility, and estimate of inspiratory capacity (IC) quantifies the volume of air received by the lungs up to a pressure of 30 cmH_2O. PV loop area provides an estimate of the amount of atelectasis (airspace closure) that existed before the PV loop manoeuvre (Fredberg and Stamenovic, 1989). To estimate IC and C_{ST}, the pressure and volume data were measured in the upper flatter portion of the expiratory limb of the PV curve.

Therefore, we can assume that the significantly higher values of R_N in groups CS + T, CS + E30 and CS + E100 compared to the group exposed to ambient air (AA + T) (Fig. 1), have indicated greater narrowing of the airway lumen or increased stiffness of smooth muscle of the airways. In fact, this hypothesis was supported by morphometric data (Table 1, BCI) both confirming narrower airways. In addition, such phenomenon can be explained by the fact that the inhalation of irritants, such as environmental pollutants and cigarette smoke, promote activation of pattern recognition receptors, such as Toll-like Receptors 4 (TLR4) in the epithelial cells of the pathways aeration, culminating in hypersecretion of mucus (Nadigel et al., 2011; Freeman et al., 2013; Vassallo et al., 2010). On the other hand, we observed that the CS + E300 group did not present an increase in the resistance of the airways, avoiding the installation of the lesion in the smooth muscles of the airways. This result corroborates previous research, in which Eucalyptol reduced the expression of TLR4 in mice submitted to pulmonary injury (Zhao et al., 2014; Lee et al., 2016).

Tissue resistance (G) and tissue elastance (H) are related to the intrinsic properties of the tissue. There are several hypotheses to explain their changes. One of them may be the change in tissue rheological properties (Bates et al., 2009). Our histological analyzes demonstrate the thickening of the alveolar septa, alveolar collapse and cellular

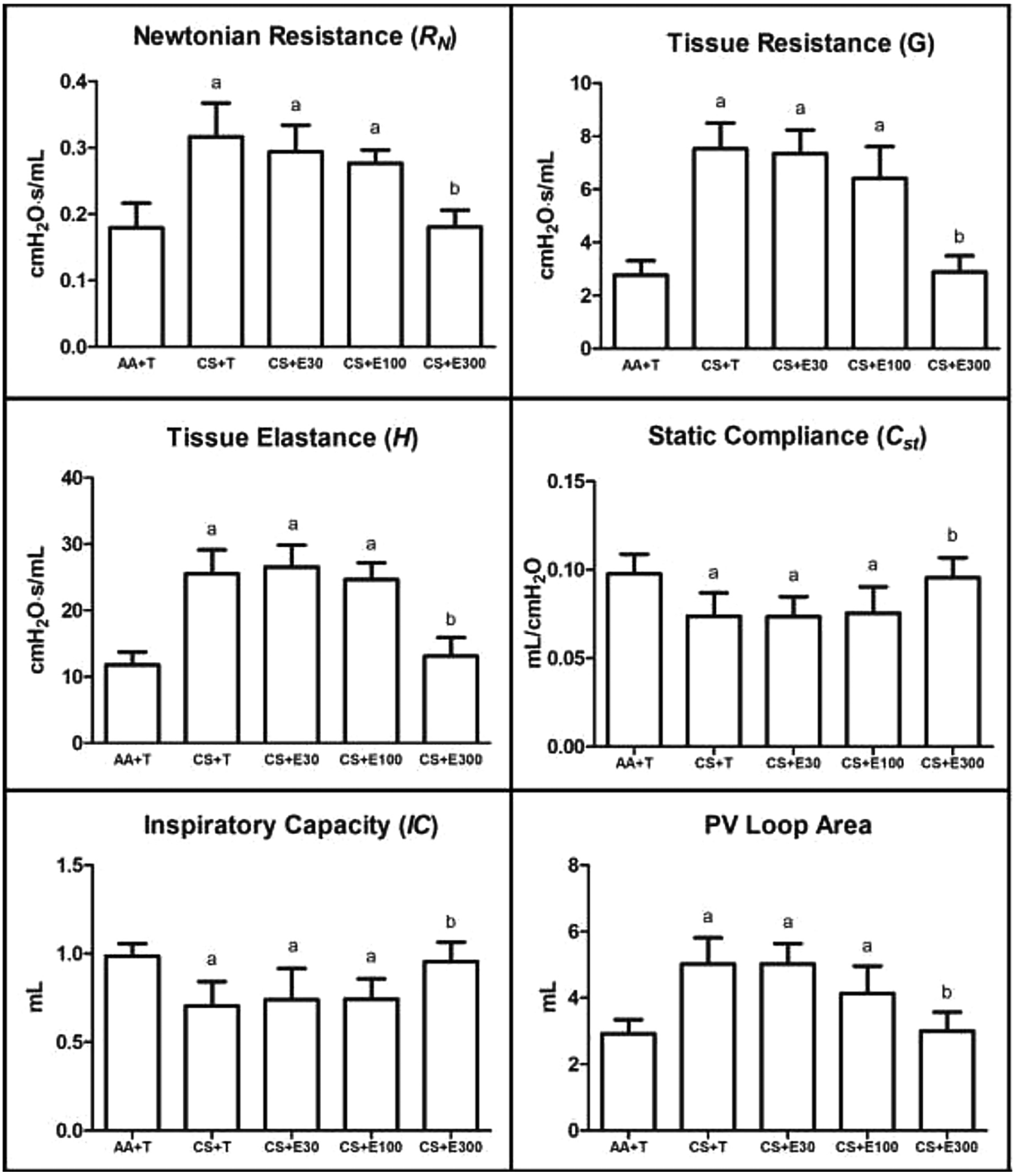

Fig. 1. Pulmonary mechanical. Data obtained by performing the forced oscillation technique (R_N, G and H) and PV curve (C_{ST}, IC and PV loop area) in animals exposed to Ambient Air for 5 days and received a daily treatment of vehicle (Tween-80 [1%] solution) (AA + T Group), or exposed to cigarette smoke for 5 days, and received a daily treatment of vehicle (Tween-80 [1%] solution) (CS + T group), 30 mg/kg Eucalyptol (CS + E30 group), 100 mg/kg Eucalyptol (CS + E100 group), or 300 mg/kg Eucalyptol (CS + E300 group). 8 animals per group. Values are mean ± SD. One-way ANOVA followed by Student–Newman–Keuls test was peformed. [a] Different from AA + T group ($p < 0.05$). [b] Different from CS + T group ($p < 0.05$).

infiltration in the pulmonary parenchyma in the CS + T, CS + E30 and CS + E100 groups (Fig. 3). In addition, the increase in the number of polymorphonuclear cells (PMN cells) and in the percentage of collapsed alveoli, and the decrease in mean alveolar diameter (Table 1), may indicate the release of inflammatory cytokines, lipid mediators and enzymes capable of promoting edema and tissue injury (Holz et al., 2008). These findings may explain the increase of G and H (Fig. 1) in the pulmonary mechanics of the CS + T, CS + E30 and CS + E100 groups when compared to the AA + T group.

In the PV curve, static compliance (C_{ST}) indirectly measures the degree of lung tissue distensibility, the inspiratory capacity (IC) estimate quantifies the volume of air received by the lungs up to a pressure of 30 cmH_2O, while the PV Loop area provides an estimate of the amount of atelectasis (occlusion of air spaces) that existed prior to the PV curve maneuver (Fredberg and Stamenovic, 1989). In this way, the reduction of C_{ST} and IC, corroborates with the stiffening of lung tissue indicated by the increase of G and H, in the groups CS + T, CS + E30 and CS + E100 groups, in relation to the AA + T group (Fig. 1). In this

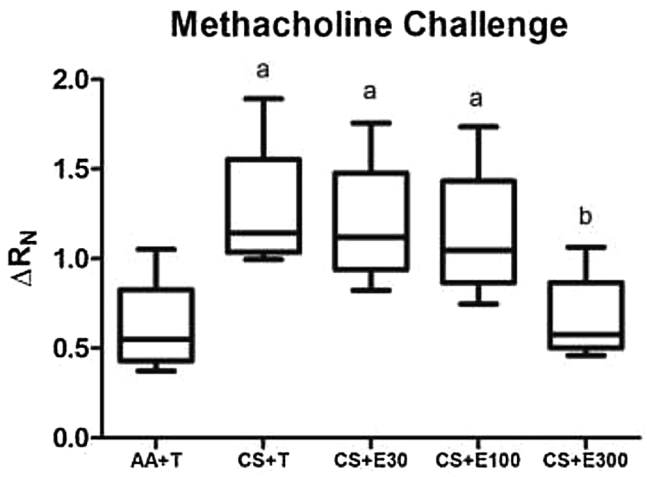

Fig. 2. Methacoline challenge. Methacoline challenge as a function of number of measurements after MCh nebulization (30 mg/mL for 30 s) in animals exposed to Ambient Air for 5 days and received a daily treatment of vehicle (Tween-80 [1%] solution) (AA + T Group), or exposed to cigarette smoke for 5 days, and received a daily treatment of vehicle (Tween-80 [1%] solution) (CS + T group), 30 mg/kg Eucalyptol (CS + E30 group), 100 mg/kg Eucalyptol (CS + E100 group), or 300 mg/kg Eucalyptol (CS + E300 group). 8 animals per group. [a] Different from AA + T group ($p < 0.05$). [b] Different from CS + T group ($p < 0.05$).

sense, the larger PV Loop area (Fig. 1) can also be attributed to tissue changes, such as: edema, alveolar collapse and greater presence of PMN cells, and possibly a mechanism associated with alveolar surfactant (Muller et al., 1998; Wagers et al., 2001).

Airway hyperresponsiveness (AHR) was mensured by pulmonary response to MCh challenge assessed by ΔR_N (Fig. 3). After challenge with MCh, we observed significant alterations in the CS + T, CS + E30 and CS + E100 groups when compared to the AA + T group, evidencing an AHR of the animals of these groups. This result was expected because AHR is a characteristic present in lung injury. AHR may be present due to the increase of PMN cells, such as eosinophils. Eosinophils may contribute to remodeling of the airways and to the development of AHR (Southam et al., 2007). Greater presence of PMN cells and increase of R_N in the CS + T, CS + E30 and CS + E100 groups compared to the CS + T group, may explain this finding.

Zhao and collaborators (Zhao et al., 2014) demonstrated that the oral treatment of 30 mg/kg and 100 mg/kg Eucalyptol inhibited acute lung inflammation induced by lipopolysaccharides (LPS). However, despite the evidence that such doses are effective against LPS-induced injury, our data show that oral administration of 30 mg/kg (CS + E30 group) and 100 mg/kg (CS + E100 group) of Eucalyptol in animals short-term CS exposure was not enough to avoid the installation of the pulmonary lesion and, consequently, the changes in the mechanics of the respiratory system (Fig. 1) and morphometry of the pulmonary parenchyma (Table 1). Studies evaluating lung function with the use of essential oils as a form of treatment are common. Similar results to ours were founded in a study using 300 mg/kg EOCZ in mice of OVA-induced lung injury (Serra et al., 2018).

Similarly, it was observed that Eucalyptol when administered orally in daily doses of 300 mg/kg in animals short-term cigarette smoke exposure (CS + E300), was able to avoid the installation of lung injury in these animals. This can be verified by the statistical difference observed in all variables of the CS + E300 group, when compared to the CS + T group (Fig. 1). Previous studies correlate the beneficial effects of Eucalyptol through its performance on several pathophysiological mediators, also identified in affections of the human respiratory system (Juergens et al., 2004b; Zhao et al., 2014; Li et al., 2016; Kim et al., 2015), but especially by the intracellular mechanism in attenuating the activation of the p65 subunit of NF-kappa B after exposure to CS, causing reduction of transcription of cytokines and activity of pro-inflammatory cells (Kennedy-Feitosa et al., 2016).

5. Conclusion

In conclusion, our results demonstrated that the Eucalyptol was able to prevent the acute lung injury in mice submitted to short-term cigarette smoke exposure, and supposedly can be used as a preventive or nutritional resource against acute affections of the respiratory system, since the administration of this compound was always performed prior to exposure to the offending agent.

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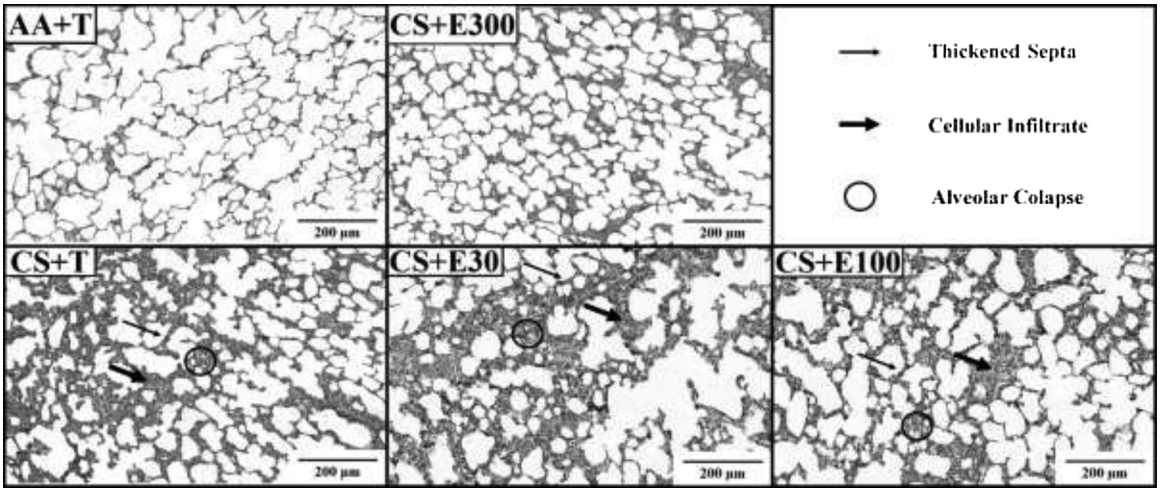

Fig. 3. Histological study. Photomicrographs of pulmonary parenchyma in animals exposed to Ambient Air for 5 days and received a daily treatment of vehicle (Tween-80 [1%] solution) (AA + T Group), or exposed to cigarette smoke for 5 days, and received a daily treatment of vehicle (Tween-80 [1%] solution) (CS + T group), 30 mg/kg Eucalyptol (CS + E30 group), 100 mg/kg Eucalyptol (CS + E100 group), or 300 mg/kg Eucalyptol (CS + E300 group). Photomicrographs of lung parenchyma stained with hematoxylin–eosin.

Table 1
Morphometric parameters. Values are mean ± SD of AA + T, CS + T, CS + E30, CS + E100 and CS + E300 groups. The data were collected in ten matched fields per mice. [a] Different from AA + T group ($p < 0.05$). [b] Different from CS + T group ($p < 0.05$). By one-way ANOVA followed by the multiple comparisons corrected with the Bonferroni's test. PMN, polymorphonuclear; BCI, bronchoconstriction index.

Groups	Alveolar Collapse (%)	PMN Cells ($x10^{-3}/\mu m^2$)	Mean alveolar diameter (μm)	BCI
AA + T	7,69 ± 1,27	15,91 ± 4,18	46,76 ± 3,79	2,03 ± 0,18
CS + T	26,67 ± 4,54 [a]	29,33 ± 5,73 [a]	60,33 ± 6,23 [a]	2,68 ± 0,24 [a]
CS + E30	23,68 ± 3,57 [a]	28,06 ± 5,51 [a]	58,41 ± 6,87 [a]	2,65 ± 0,29 [a]
CS + E100	19,95 ± 2,33 [a,b]	26,46 ± 5,96 [a]	56,25 ± 6,75 [a]	2,51 ± 0,26 [a]
CS + E300	9,45 ± 3,88 [b]	18,19 ± 5,76 [b]	47,72 ± 5,07 [b]	2,05 ± 0,23 [b]

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