Abstract

Second-hand smokers, in various settings such as recreational areas, schools, workplaces, homes and other enclosed public spaces also experience the detrimental effects of exposure to cigarette smoke (CS). Out of the 8 million deaths attributed to smoking, 1.2 million are a result of non-smokers being exposed to second-hand smoke. As a preventive strategy to these damages, Eucalyptol has been shown to be effective in the treatment of lung lesions caused by smoking. This study aimed to assess the effects of indoor second-hand cigarette smoke on respiratory mechanics and lung tissue, in addition to investigating the potential benefit of Eucalyptol against lung damage caused by this type of aggression. The experiments were conducted for 14 days with 40 BALB/c mice, divided into 5 groups: a control group, two groups nebulized with saline solution, divided into active and passive (second-hand) exposure to CS, two groups pre-treated with eucalyptol (10 mg/mL by nebulization) divided into active and passive exposure to CS. Lung mechanics data were collected on a flexiVent® small animal mechanical ventilator. In addition, ex vivo analysis of lung tissue micromechanics, morphometric analyzes and qualitative analyzes of lung tissue were performed. The results showed that 14 days of second-hand exposure generated significant tissue damage, causing infiltration of inflammatory cells and altering respiratory mechanics. However, pre-treatment with eucalyptol was able to prevent all changes caused by CS in both exposure protocols. We conclude that indoor second-hand smoke is capable of causing damage similar to active tobacco consumption and that Eucalyptol can be used as a preventive or nutritional resource against the changes caused by the inhalation of cigarette smoke.

Keywords: Second-hand smoke. Indoor pollution. Tobacco Smoke Pollution. Respiratory mechanics. Eucalyptol.
1 Introduction

According to the World Health Organization (WHO), tobacco consumption is one of the greatest threats to public health, being described as an epidemic, contributing to the death of more than 8 million people, in addition to being the leading cause of preventable death in the world (WHO, 2020).

In addition to the numerous health risks associated with cigarette smoke (CS), it is important to recognize that smokers are not the sole group affected. Individuals called second-hand smokers, in various settings such as homes, workplaces, recreational areas, schools, and other enclosed public spaces also experience the detrimental effects of exposure to CS (WUNSCH FILHO et al., 2010).

Second-hand smoking refers to the inhalation of tobacco products, specifically the smoke emitted from the burning end of a cigarette (sidestream smoke) as well as the smoke exhaled by active smokers (mainstream smoke). It is estimated that approximately one-third of the global population consists of passive (second-hand) smokers who are consistently exposed to cigarette smoke. Out of the 8 million deaths attributed to smoking, 1.2 million are a result of non-smokers being exposed to second-hand smoke (WHO, 2020).

In order to contain the advancement of the health repercussions of active or passive smokers, several pharmacological approaches such as antioxidants, anti-inflammatories, anticholinergics, β2-agonists, phosphodiesterase inhibitors, glucocorticoids, or the combination of several drugs have been developed (BARNES et al., 2013), among these, some found in medicinal plants.

Eucalyptol, also known as 1,8-cineole, is a natural saturated monoterpen found in Eucalyptus globulus Labill essential oil. Literature reports have already highlighted its antioxidant and anti-inflammatory properties, demonstrated in experimental studies for the treatment of endothelial injury and hypertension (CHEN et al., 2023), hemorrhage-induced brain injury (XU; GUO; SUUN, 2021), lipopolysaccharide-induced lung injury (ZHAO et al., 2014), and protection against influenza-virus-induced pneumonia (LI et al., 2016).

Although tobacco consumption has decreased in recent years, the rate of reduction does not appear to be fast enough, even with goals to reduce tobacco use by 30% among adults by 2025 (CLAIRE et al., 2020). Therefore, the use of natural products such as Eucalyptol stands out as an adjunct pharmacological strategy in the prevention and treatment of lung injuries caused by active or passive exposure to CS.

Furthermore, there is a need for a better understanding of the alterations caused in individuals passively exposed to CS.

Based on the aforementioned, this study aims to investigate the alterations in respiratory mechanics and the action of eucalyptol as an alternative for the prevention and treatment of lung alterations caused by indoor second-hand cigarette smoke. To achieve this, we employed analyses of respiratory system mechanics, pulmonary tissue mechanics, histopathology, and lung parenchyma morphometry.

2 Material and Methods

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. 8523, revised 1985); and the National Council for Controlling Animal Experimentation of the 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 No. 01261850/2021). Invasive procedures were performed under anesthesia (see below) and every effort was made to minimize suffering.

Forty females BALB/c mice, (7–8 weeks of age), 25 ± 5 g BW, had water and feed ad libitum, were used in this study. Mice were housed in plastic cages under controlled environmental conditions, in which a temperature of 20-22 °C, housed in a room with a 12 h light/12 h dark cycle.

Experimental Groups

The animals were divided into five groups (n = 8), the animals in the Saline Ambient Air (SAA) group were exposed to ambient air and nebulized with saline solution; the Saline Active Smoking (SAS) group was nebulized with saline solution before the first exposure of the day being actively exposed to cigarette smoke; the Saline Passive (second-hand) Smoking (SPS) group was nebulized with Eucalyptol before the first exposure of the day being actively exposed to cigarette smoke; the Eucalyptol Active Smoking (EAS) group nebulized with Eucalyptol before the first exposure of the day being actively exposed to cigarette smoke, and the Eucalyptol Passive (second-hand) Smoking (EPS) group that was nebulized with Eucalyptol before the first exposure of the day being passively exposed to cigarette smoke.
Pre-treatment with Eucalyptol

Nebulization was performed at the concentration of 10 mg/mL of Eucalyptol according to Lee et al (2016); Kennedy-Feitosa et al (2019) for the period of 15 minutes per day. The compound was inhaled for 14 days, always 1 hour before the first exposure to cigarette smoke.

The animals were placed in the nebulizer chamber (A) (29 cm long, 16 cm wide, and 19 cm high), and received the compound through a connection between the suction cup (B) of the nebulizer (C) and the chamber, as shown in the image below (Figure 1).

Figure 1. Schematic diagram of eucalyptol nebulization. Prepared by the author. A: nebulization chamber; B: inhalation cup; C: nebulizer.

Indoor second-hand smoke and active cigarette smoke exposure

The protocol used for indoor second-hand cigarette smoke exposure is based on the protocol adapted from the induction model (YPSILANTIS et al., 2012). The animals were placed in the exposure chamber (A) (40 cm long, 30 cm wide, and 25 cm high), housed in an exhaust; the cigarette (B) was placed inside a Kitassato (C), using a positive pressure created by the airflow inside a controlled air pump, generating a flow rate of 0.9 L/min (D), keeping the cigarette lit, and directing, by means of drag, the smoke from inside the Kitassato toward the exposure chamber (Figure 2).

Briefly, the animals were placed in the exposure chamber (E) (40 cm long, 30 cm wide, and 25 cm high), housed in an exhaust. Cigarettes were coupled (F) directly through the filter into a pump with controlled suction, generating a flow rate of 0.9 L/min (G) and the cigarette smoke was sucked up and sent directly into the inhalation chamber (Figure 2). Both exposures were performed 6 times a day, lasting 40 minutes, with an interval of 1 h between exposures. 5 cigarettes were used for each exposure, with consumption of 8 minutes per cigarette, totaling 30 cigarettes per day.

Respiratory system mechanics

After the exposure period, the animals were sedated with diazepam 5 mg/kg intraperitoneally (ip) and anesthetized with 10% Ketamine hydrochloride (100 mg/kg) and Xylazine hydrochloride (10 mg/kg), intraperitoneally, the doses were sufficient to keep them in the anesthetic plane (suppression of the corneal-palpebral reflex), after tracheostomy the animals were positioned in dorsal decubitus and fixed on a heated table (37°C) and stabilized on their limbs with adhesive tape. An 14 gauge cannula (Eastern medikit LTD) was placed in the trachea during the tracheostomy procedure and then connected to a computer-controlled ventilator for small animals (Scireq®-flexiVent®, Montreal, QC, Canada).
The animals were ventilated with baseline settings: respiratory frequency of 120 breaths/min, tidal volume of 10 mL/kg, limiting pressure of 30 cmH₂O, and Positive End-Expiratory Pressure (PEEP) of 3 cmH₂O. Mice were then paralyzed (pancuronium bromide - 0.5 mL/kg, i.p., Cristália, Itapira, Brazil).

Along with Mechanical Ventilation (MV), an integrated testing platform in lung mechanics was used. This platform mainly consists of a flexiVent® mechanical respirator for small animals (SCIREQ, Montreal, Canada), allowing the application of arbitrary waveforms to the injected volumes or pressures that are applied to the lung, with the simultaneous acquisition of all relevant variables for determining organ mechanics (SERRA et al., 2017).

Soon after the animal was connected to the ventilator, it was paralyzed with panoarium bromide as described above. Then, after five minutes to accommodate the animal, it was checked for possible leaks, obstructions, correction of the animal's position in relation to the ventilator, and confirmation that the animal was not performing spontaneous inspirations in order not to compromise the analysis, only after the protocol.

During the data collection protocol, a preconditioning phase was initially added in order to standardize the lung history of the analyzed animals, i.e., a recruitment maneuver was performed, which consists of offering an equal airflow to all mice (GRASSO et al., 2007). Twelve quick-prime perturbations were used for data collection, where they were used to determine the parameters of the constant phase model: Newtonian resistance ($R_n$), tissue elastance ($H$) and resistance ($G$) (SERRA et al., 2017).

Thereafter, starting at the functional residual capacity defined by the PEEP, the flexiVent delivered seven inspiratory pressure steps for a total pressure of 30 cmH₂O, followed by seven expiratory steps, pausing at each step for 1 sec. At each step, plateau pressure (P) was recorded and related to the total volume (V), which allowed the drawing of a Pressure-Volume (PV) curve using a ramp-style pressure-driven maneuver (PVR-P). Static compliance ($C_{st}$) was calculated as the slope of the curve (SALAZAR; KNOWLES, 1964).

**Lung tissue micromechanics**

Lung micromechanics was analyzed in an organ bath by 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 were gathered by a dedicated computer.

One of the ends of the lung parenchyma strip (approximately 2 × 2 × 6 mm) 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 Krebs-Henseleit solution, aerated with carbogen gas mixture (95% O₂ and 5% CO₂), and the bath temperature controlled at 37 °C. The length of the sample was then slowly adjusted until baseline force reached 1 gram-force.

Since the mechanical behavior of lung tissue depends on its tension history, preconditioning was performed to minimize any tension history and return the tissue to a standard and stable point. Each strip was preconditioned for 10 minutes under sinusoidal oscillations with an amplitude amounting to 10% of the strip resting length ($L_0$) and frequency of 1 Hz, so that a stable stress-strain loop was observed. Soon after, uniaxial mechanical measurements consisting of dynamic or quasistatic stress-strain curves were generated 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 ($L_0$) of the tissue strip was set to increased in 15% ($L_i$) 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 $L_0$.

**Histological analysis**

The left lung was embedded in paraffin for later obtaining histological sections 4 μm thick. The slides were stained with Hematoxylin-eosin and analyzed under optical microscopy (Axioplan, Zeiss, Oberkochen, Germany), according to their qualitative and quantitative aspects. Each animal was represented by one slide. The slides were stained with Hematoxylin-eosin and analyzed by optical microscopy.

They were performed by the conventional point-couting technique, using an eyepiece containing a reference system of 100 points and 50 lines.
The qualitative analysis was performed by means of photomicrographs of the areas of atelectasia, alveolar wall thickening, presence of cellular infiltrate and alveolar collapse. Quantitative analysis was done by morphometry to verify the percentage of normal and collapsed alveoli; mean alveolar diameter Lm for the length of the distal air spaces and inflammatory cell count (Gondim; Serra; Cavalcante, 2019).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.00 (GraphPad, San Diego, CA, USA). Results are presented as mean ± SD, where n represents the number of samples. For comparison among groups, we used one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. A difference was considered significant if \( p < 0.05 \).

3 Results

Our results demonstrated that indoor second-hand exposure to cigarette smoke caused significant changes in the respiratory system, similar to active exposure. In addition, it was possible to verify that pre-treatment with Eucalyptol prevented pulmonary alterations caused by cigarette smoke.

In order to analyze the lung function of mice exposed actively and passively to cigarette smoke and the effect of Eucalyptol action on the respiratory system of these animals, the mechanics of the respiratory system was analyzed, making it possible to obtain data on the Newtonian resistance \( (R_N) \), tissue resistance \( (G) \), tissue elastance \( (H) \), static complacency \( (Cst) \), inspiratory capacity \( (IC) \) and hysteresis. On the other hand, these variables were considered significant if \( p < 0.05 \).

Table 1. Alveolar collapse, amount of PMN and mean alveolar diameter and collapsed alveoli. Morphometric parameters. Values are mean ± SD of groups SAA, SAS, EAS, SPS, and EPS. Data were collected from ten matched fields per animal.

*Represents a statistically significant difference between the SAS and SPS groups compared to the SAA group (\( p<0.05 \)).

Table 4 Discussion

The adaptations made in the two CS exposure protocols were performed with the objective of having an Active Exposure Protocol (AEP) and a exposed to indoor Passive (second-hand) Smoke Protocol (PEP) with the same duration, amount of cigarettes, and intervals between the two types of exposure.
Figure 3. Correlation of data related to Newtonian resistance ($R_N$), tissue resistance ($G$), tissue elastance ($H$), static complacency ($C_{st}$), inspiratory capacity (IC) and PV area. Values are mean ± SD of SAA, SAS, EAS, SPS and EPS. * Represents a statistically significant difference between the SAS and SPS groups compared to the SAA group (p<0.05). # Represents a statistically significant difference between the SPS group compared to the SAS group (p<0.05).

Figure 4. Correlation of mechanical properties of lung tissue strips: elastance (E), resistance (R) and hysteresis (H). Values are mean ± SD of SAA, SAS, EAS, SPS and EPS. * Represents a statistically significant difference between the SAS and SPS groups compared to the SAA group (p<0.05).
ASSESSMENT OF INDOOR SECOND-HAND CIGARETTE SMOKE ON THE RESPIRATORY MECHANICS AND PREVENTIVE BENEFITS OF EUCALYPTOL: A MURINE MODEL

As for the proposed model of AEP to CS, our results corroborate previous models present in the literature used by Hizume (2010), Castardeli et al (2005). Thus, suggesting that this model can be used in studies with AEP to CS proving to be effective for studies that seek to evaluate repercussions to exposure to active smoking.

Furthermore, our results also showed that second-hand smoke exposure was sufficient to promote tissue and functional changes in the animals’ respiratory system, proven by statistically significant differences in all evaluated parameters (figure 3, figure 4 and table 1).

According to the results of lung mechanics, the proposed model of PEP to CS, was able to develop injury in the proposed exposure period. The presence of changes in lung mechanics was observed through analysis of airway resistance ($R_N$), tissue elastance ($H$) and tissue resistance ($G$), as well as static complacency ($C_{st}$), inspiratory capacity ($IC$) and area of the curve $PV$ (Figure 3).

The $R_N$ represents the resistance to air passage in the airways, and a statistically significant increase was observed between the groups SPS and SAA (Figure 3). These changes in $R_N$ may be related to the accumulation of secretion in the airways resulting from inflammatory processes that hinder the passage of air (OLIVEIRA et al., 2015).

The presence of oxidation intermediates in CS may induce reactions in the body that favor epithelial remodeling, increase of calliciform cells and hypertrophy of mucous cells, where these provide considerable increase in mucus production, causing impairment in mucociliary transport, resulting in accumulation of secretion and inflammatory processes of the bronchial mucosa (TACAO et al., 2015).

Regarding parameters $G$ and tissue elastance $H$, statistically significant increases were found between the SAS and SAA groups, as well as between the SPS and SAA groups (figure 3). These values suggest changes in the rheological properties of lung tissue (BATES, 2009).
G and H values were observed to be markedly elevated in the SAS and SPS groups when compared to the SAA animals. These findings can be ascribed to changes in the tissue, such as the thickening and collapse of alveolar septa, along with the existence of cellular infiltrates in the pulmonary parenchyma of animals belonging to the SAS and SPS groups (figure 5).

These data corroborate the ex vivo analyses performed by micromechanics using strips of the lung parenchyma, where there were significant changes in the parameters of resistance ($R$), the elastance ($E$) and hysteresivity in the SAS and SPS groups when compared to the SAA group at all frequencies obtained (0.1, 0, 3, 1, 3, 10 Hz).

The changes in the parameters of $E$, $R$, and hysteresis ex vivo may be related to heterogeneities in the lung tissue parenchyma due to the presence of alveolar infiltrate (presence of polymorphonuclear cells), remodeling and inflammatory processes of the extracellular matrix from active and passive exposure to CS. The increase in hysteresis may refer to tissue damage, such as collagen fiber degradation caused by oxidative stress and inflammatory reactions induced by exposure to agents present in CS (AMARAL et al., 2020).

Regarding the parameters of the Pressure-Volume curve ($PV$), there is a statistically significant reduction in the variables of $Cst$ and $IC$ between the SAS and SAA groups, and between the SPS and SAA groups. The reduction in $Cst$ can occur due to increased $H$, in view of the fact that antagonistic findings are expected in these variables. According to Serra et al (2017), the reduced $IC$ is related to a stiffness in the lung tissue, which can be corroborated with the increased parameters in $G$ and $H$. As a result of the factors already presented, the mechanics of the respiratory system suffers functional losses, such as difficulties in the inspiration process resulting in a smaller expiratory volume and consequently a smaller $IC$ (BALTAR et al., 2010; STEIDL et al., 2013).

The reduction in $IC$ is a consequence of damage to collagen and elastin fibers in the lung tissue caused by the release of arachidonic acid metabolites and pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF, resulting from exposure to CS (WONG; MAGUN; WOOD, 2016). Since elastin is the main protein component of the extracellular matrix in the lungs, its degradation leads to direct pulmonary mechanical damage, causing deficits in its functionality (LAURELL; ERIKSSON, 2013).

The area of the pressure-volume curve is taken as a representation of areas of atelectasis in the lung tissue, where a statistically significant increase is observed between the SAS and SAA groups, and between the SPS and SAA groups.

Areas of atelectasis result from an ineffective distribution of surfactant on the alveolar surface. The presence of this substance in the alveolar region is extremely important, since it provides a decrease in the surface tension of the alveoli, thus preventing them from collapsing and generating areas of atelectasis.

Continuous inflammatory processes can contribute to the increase of atelectasis due to changes in the metabolism of surfactant molecules, making the alveoli more susceptible to atelectasis (TAM et al., 2011). This justifies the presence of collapsed alveoli in the SAS and SPS groups, as shown in Table 1.

The changes caused by passive and active exposure can also be visualized via histological analysis, characterized by the thickening of the alveolar septa, an augmentation in the proportion of collapsed alveoli, a reduction in the average alveolar diameter, and an escalation in the presence of Polymorphonuclear cells (PMN cells) in the lung parenchyma of the SAS and SPS groups (Table 1 and figure 6).

Constituents present in gaseous and particulate phases of CS, when accumulating on the surface of epithelial cells, promote damage caused by oxidative stress and inflammatory reactions, such as the release of cytokines, lipid mediators and enzymes that can promote edema and tissue damage (CANTIN; RICHTER, 2012). Despite the lack of statistical significance in most of the evaluated parameters between the two SAS and SPS groups, the percentage difference indicates a tendency towards increased damage in the group exposed to second-hand smoke, with these alterations observed at the level of the airways and lung parenchyma.

In relation to the parameters of the constant phase model, we noted significant increases in the SAS group compared to the control group (SAA). The values of $R_N$, $G$ and $H$ showed respective percentage increases of 63.61%, 149.91%, and 57.01% in the SAS group. Similarly, the SPS group exhibited substantial increases of 117.82%, 186.19%, and 92.45% in $R_N$, $G$ and $H$, respectively, compared to the SAA group. Conversely, reductions in percentage values were observed in the SAS group compared to the SAA group. There was a decrease of 36.47%, 27.02%, and 88.79% in $Cst$, $IC$ and curved area $PV$, respectively. The SPS group also demonstrated reductions of 42.21%, 39.43%, and 95.33% in $Cst$, $IC$ and curved area $PV$.

The difference between percentage values in lung mechanics parameters observed in the SPS group compared to the SAS group also suggests that the filter present in the cigarette, in the active exposure model of the SAS group, may retain certain particles from tobacco combustion, contributing to an attenuation of damage to the respiratory system.
The reports concerning the importance of the filter in containing particulate matter present in CS are not recent, works such as those by Selke (1978) and Browne; Keith; Allen (1980), have already shown that the use of the filter can decrease the constituents in CS by up to 70%.

The filters perform a reduction in the emission of particulate matter due to their size, circumference, number of fiber filaments and use of additives in the fibers. These combined cellulose acetate and activated charcoal filters are based on the premise that these devices have the capacity to selectively remove the toxic components of CS (ADAM et al, 2010). Although the filter suggests a certain protection to the constituents present in CS as observed in the aforementioned studies, according to our results, it did not prevent the involvement of lesions in the airways and lung parenchyma in all parameters analyzed.

In an attempt to analyze whether inhaled Eucalyptol at a dose of 10 mg/kg would be effective in preventing the appearance of lung damage induced mainly by second-hand smoke exposure, we compared the findings of the EAS and EPS groups with the control group (SAA). Eucalyptol administered by nebulization to animals exposed to CS was able to avoid lung injury, where no significant difference between all the variables of respiratory mechanics (Figure 3), lung tissue mechanics (Figure 4) and histology (table 1 and figure 5).

Analysis of lung mechanics have shown that Eucalyptol was effective in preventing injuries induced by active exposure to CS (KENNEDY-FEITOSA et al., 2019; GONDIM; SERRA; CAVALCANTE, 2019) and acute lung injury induced by lipopolysaccharide (ZHAO et al., 2014).

Antioxidant substances, such as Eucalyptol, can exhibit different protective properties and act at different stages of the oxidative process, functioning through different mechanisms: inhibiting or slowing down oxidation by inactivating free radicals through hydrogen or electron donation, or modulating gene expression through interaction with transcription factor proteins, such as nuclear factor kappa B (NF-κB) and nuclear erythroid 2-related factor 2 (Nrf2), as observed by Kennedy-Feitosa et al (2019). It is suggested that the interaction with transcription factor proteins is the main antioxidant mechanism of action of eucalyptol, as the molecule itself lacks hydroxyl groups, double bonds, and aromatic rings capable of directly stabilizing radical species.

5 Conclusions

In conclusion, the present study showed that indoor second-hand cigarette smoke, and not just active exposure, is capable of causing changes in lung tissue and respiratory mechanics in a murine model. As for the use of Eucalyptol as a protective agent, it proved to be effective in preventing lung injuries and dysfunctions in respiratory mechanics caused by active exposure and second-hand smoke, proving to be a potential adjuvant in the protection of the respiratory system caused by inhalation of cigarette smoke.

CREDIT AUTHORSHIP

CONTRIBUTION STATEMENT


DECLARATION OF INTEREST

The authors disclose that they have no known competing financial interests or personal relationships that could have appeared to influence the study reported in this manuscript.

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