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PULMONARY CHANGES AND BRONCHIAL HYPERRESPONSIVENESS IN RATS SUBJECTED TO ACUTE ACROLEIN EXPOSURE

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ABSTRACT

Acrolein is a toxic substance resulting from cigarette smoke and partial combustion of animal fats and vegetable oils, and mainly used in industry for the production of acrylic acid and plastics. The main forms of human exposure to this substance is through cigarette smoke and car exhaust. Its high toxicity is responsible for many adverse health effects, especially changes in the respiratory system. This study aimed to analyze the changes in lung function and hyperresponsiveness of the smooth muscle of the airways of rats, resulting from acute exposure to acrolein. Exposure was by inhalation of acrolein vapor generated by bubbling nitrogen gas (N_2) in a 1.5 mL container of liquid acrolein. Albino rats, 200±20 g were divided into control group (C), which was exposed to inhalation of saline vapor for 1 h, acrolein group (A), exposed to acrolein inhalation of vapor generated by bubbling N_2. Our results demonstrated changes in all variables, Newtonian resistance (R_N), tissue resistance (G), tissue elastance (H), hysteresis (η) and the estimate of inspiratory capacity (IC), and there was greater hyperresponsiveness in smooth muscle of the airways (R_N, G and H). Our findings reinforce the harmful nature of exposure to acrolein to health.

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INTRODUCTION

Acrolein is a pollutant originating from various sources, such as cigarette smoke, exhaust gases of gasoline and diesel engines, the partial combustion of animal fats and vegetable oils, and particularly of glycerol combustion (IARC, 1995; US EPA, 2003). It is important for acrylic acid production industry and plastics and is used directly as a biocide in the control of algae, aquatic weeds and molluscs in the water recirculation process (Stevens *et al.*, 2008; U.S. EPA, 2003).

Acrolein in cigarette smoke is a major source of exposure of humans to this substance. The factor responsible for its presence in smoke, is the addition of glycerol (1 to 5% by weight) and sugars (20% by weight) in its composition (Stevens *et al.*, 2008). In places where smoking is high, such as bars and restaurants, its concentration varies from 1.0 x 10^{-3}-119.5 x 10^{-3} ppm (IARC, 1995). The main factors that influence the level of exposure to acrolein by inhalation are the location (rural or urban), concentration and time of exposure to smoking, vehicular exhaust, occupational exposure and

distance of residence from some source of acrolein (ATSDR, 2007). Occupational exposure to acrolein occurs through inhalation of or contact with this compound at workplaces where it is produced or used, while the exposure of the general population occurs primarily through contact with air (HSDB, 2003; US EPA, 2003). A study in Canada suggests that the population is exposed to an average concentration of acrolein of 1.3 μg/m^3 (0.5 x 10^{-3} ppm),from which one can estimate that an adult is exposed to inhalation of 26 μgacrolein per day (Environment Canada, 2000; ATSDR, 2007). The toxicity of acrolein directly affects the upper airways and lung periphery, causing irritation, cell hyperplasia, andchanges in respiratory rate, among others (Parent *et al.*, 1992; Kutzman *et al*., 1985;Costa *et al*., 1986). The increase in cases of chronic respiratory diseases such as childhood asthma, chronic bronchitis and chronic obstructive pulmonary disease (COPD) in recent years have called attention to the quality control of indoor air (CDC, 2007). The concentration of acrolein in these environments can exceed up to 20 times the concentration outdoors, which could mean a health risk, since the public spends most of the time in homes, offices, shopping centers, restaurants, etc. (Environment Canada, 2000). Heated animal or vegetable oils, wood stoves, cigarette smoke, incense and candles contribute to its concentration in indoor environments (Seaman *et al*, 2007). A concentration of 2.02 μg/m^{3}acrolein was measured in a closed environment at a public university in Fortaleza, Brazil (Cavalcante *et al*., 2006).

The use of animal models is a good alternative to exposure of humans in the quantification of pulmonary effects in response to exposure to pollutants and toxic compounds (Fich *et al*., 2002). Several studies using animal models for the study of the effects of exposure of acrolein to health, such as exposure of rats for 1 and 4 hto varying concentrations of acrolein of 14-81 ppm and 4.8 to 12.1 ppm, respectively, showing congestion and intra-alveolar hemorrhage, fibrin deposition in the peripheral airways and necrosis of the bronchiolar epithelium (Ballantyne *et al*., 1989).

Also demonstrated were changes in lung function such as increased airway resistance and tidal volume and decreased respiratory rate. All these findings are consistent with several studies on acute exposure to acrolein (Murphy *et al*, 1963; Davis *et al*, 1967). In a study of rats exposed subchronically to acrolein (6 h/day, 5 days/week for 62 days) at doses ≤ 4.0 ppm, changes were observed in the lung structure and function, suggesting airway obstruction, andthere was an increase in the amount of elastin and collagen, edema and necrosis of the bronchiolar epithelium. Mortality in the 4.0 ppm group was elevated and associated with severe bronchopneumonia (Kutzman *et al*., 1985; Costa *et al*., 1986). In the present study, we determined the changes in lung function and hyperresponsiveness of the smooth muscle of the airways of rats, resulting from acute exposure to acrolein. Exposure was performed by inhalation of acrolein vapor generated by bubbling nitrogen gas (N_2) (10 mL/min) in a 1.5-mLcontainer of liquid acrolein.

MATERIALS AND METHODS

Animals and exposure chamber

All animals were housed in a conventional animal facility and placed in sterile plastic cages, where the rats had access to food and water *ad libitum*.

All experimental procedures were in accordance with the basic principles of research involving the use of animals recommended by the Ethics Commission for the Use of Animals (CEUA) of the State University of Ceará. Twenty-four male Wistar albino rats (*Rattus norvegicus*), weighing 200 ± 20 g, were divided into two controls. The control group (C), n = 12, was exposed to inhalation of saline vapor for 1 h, generated by bubbling N_2 (10 mL/min) in a 1.5-mLvolume of saline. The acrolein group (A), n = 12, was exposed to inhalation of acrolein vapor generated by bubbling N_2 (10 mL/min) in a1.5mL volume of liquid acrolein (Figure 1) as proposed by Leikauf et al. (1989). At the end of exposure, the amount of inhaled acrolein for the animals was 0.3973g.

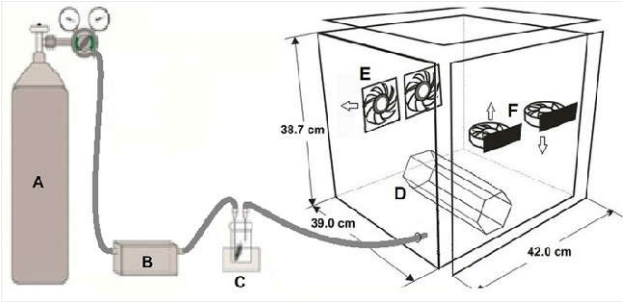

Figure 1. Acrolein/saline vapor generation system. A - nitrogen (N_2) cylinder, B - flowmeter. C - container with acrolein/saline, D - exposure chamber

The animals were placed in groups of three in a exposure chamber measuring 387 mm high, 390 mm wide and 420 mm deep (Figure 1) in containment boxes in the shape of hexagonal prisms, each box housing a single animal . The containment boxes and the chamber were constructed of polymethylmethacrylate plates. The front and rear boxes and containment are closed with EVA (ethyl vinyl acetate) rubber with central holes to allow the animal's exposure to the chamber atmosphere. There were fans for homogenization of the pollutant in the chamber and for exhaustion to avoid saturation of the pollutant, CO_2 (carbon dioxide), CO (carbon monoxide) and water vapor released in the animal's breath. Analysis of pulmonary function At 24 hours after the end of the exposure period, the animals were anesthetized with intraperitoneal sodium pentobarbital (30 mg/kg, Hypnol® 3%, Syntect, Brazil), and subjected to tracheotomy for the introduction of a 14G cannula (Eastern Medikit LTD) which was fixed to the trachea. The cannula was then connected to a ventilator for small animals (Scirec$^{©}$-*flexVent* $^{®}$) controlled by a computer. The animals were ventilated at baseline settings at a frequency of 90 breaths/min, a tidal volume of 10 mL/kg, with maximum pressure of 30 cmH_2O, and a positive end-expiratory pressure (PEEP) of 3 cmH_2O. It was necessary that the animal was sufficiently sedated and paralyzed, to haveno interference from the activity of the respiratory muscles for the lung function variables to be reliably determined. Accordingly, after initiation of ventilation, the animals were paralyzed with intraperitoneal pancuronium bromide (0.5 mL/kg,Pancuron$^{®}$, Cristália, Brazil).

We waited five minutes for acclimation of the animal, checked for possible leaks, obstructions, animal positioning corrections in relation to the ventilator and confirmed that the animal was not performing spontaneous inspirations by lung function analyses. Initially, we proceeded with the standardization of the mechanical conditions of the respiratory system with the

application of two deep inflations (DIs) with maximum pressure of 30 cmH_2O and 3 s long. To reduce the bronchoprotective effect caused by DIs (Kapsali *et al*., 2000) the animal was ventilated for 20 min at baseline settings. With the completion of the DI, the inspiratory capacity (IC) was determined as the volume of air displaced from the PEEP until the pressure reached 30 cmH_2O. Soon after, the impedance of the respiratory system (Z_{rs}) was measured by the forced oscillation technique sequentially in 30-s intervals for 6 min. Z_{rs} was determined by measuring the displacement volume and pressure ventilator cylinder piston while 3-s perturbations of oscillatory volume were delivered to the airways.

These perturbations were done using 13 superimposed sinusoidal waves with variations in amplitude and frequency (1-20.5 Hz). The frequencies were set at mutually conditioned values to reduce the harmonic distortion that can occur in nonlinear systems (Hantos *et al.,*1992). Before starting the protocol, we obtained dynamic calibration signals required to correct the physical characteristics of the mechanical ventilator in subsequent measurements of Z_{rs}. Z_{rs} was determined by Fourier transform of the volume signals of the ventilator piston and cylinder pressure, as described previously (Hirai, *et al*., 1999). $Z_{rs}$was calculated according to the model in whichR_N is the Newtonian resistance representing the resistance of the central airways, i is $\sqrt{1}$, f is frequency (Hz), I is the inertance of the airways, and G and Hrespectively characterizethe dissipative and elastic properties of the lung tissue (Hantos *et al.*, 1992). Another important characteristic of the constant phase model is the ratio G/H, known as hysteresivity (η).

Hyperresponsiveness of airway smooth muscle Soon after the collection of the initial data two DIswere again performed followed by 20 min of ventilation at baseline settings. The hyperresponsiveness of airway smooth muscle was assessed by inhalation of acetyl choline (ACh) (Sigma-Aldrich) carried by aerosol produced by an ultrasonic nebulizer (Inalasonic®, NS) connected to the inspiratory line of the mechanical ventilator. For this procedure, 4 mL of 30 mg/mL ACh were added to the nebulizer container. Nebulization was carried out during 30 s of mechanical ventilation (Xue et al., 2008) and the mean amount delivered to the animal was 1.2 mg/kg MCh. Immediately after nebulization, the focused oscillation technique was again performed sequentially at 30-s intervals for 6 min. At the end of data collection, two DIs were applied, and 30s later, forced oscillation was repeated to check the return of parameters to levels prior to nebulization with ACh. Data for airway hyperresponsiveness collected after nebulization with ACh are shown in R_N, G and H, where is the variation of the parameters in relation to pre-nebulization data.

Lung histology

Slides of lung sections were stained with hematoxylin and eosin (HE) and examined by light microscopy according to qualitative aspects. For descriptive analysis, the entire slide surface was observed with all pulmonary structures represented, at 100x and 400x.

Statistical analysis

The pulmonary function data are presented as the mean ± standard deviation. The differences between the values of the groups were evaluated using the Student t-test.

All statistical analyses were performed using Sigma Plot (Systat Software, Inc.). p<0.05 was considered statistically significant.

RESULTS

The results of the pulmonary function of the animals are shown in Table 1.

Table 1. Experimental measurements of the variables related to the calculation of the respiratory system impedance (Z_{rs}) and inspiratory capacity (IC). Values are given as mean ± standard deviation. * p<0.05 is considered statistically significant

Measure	Group	Value	p value (*t student)*
Newtonian Resistence (R_N) ($cmH_2O.s/mL$)	C	0.0523±0.0143	$p = 0.0111^*$
	A	0.0733±0.0219	
Tissue Resistence (G) (cmH_2O/mL)	C	0.545±0.101	$p = 0.0053^*$
	A	0.745±0.200	
Tissue Elastance (H) (cmH_2O/mL)	C	2.783±1.168	$p = 0.0395^*$
	A	4.022±1.574	
Hysteresivity (η)	C	0.176±0.028	$p = 0.0061^*$
	A	0.224±0.046	
Inspiratory Capacity (IC) (mL)	C	10.699±1.095	$p = 0.0013^*$
	A	8.611±1.627	

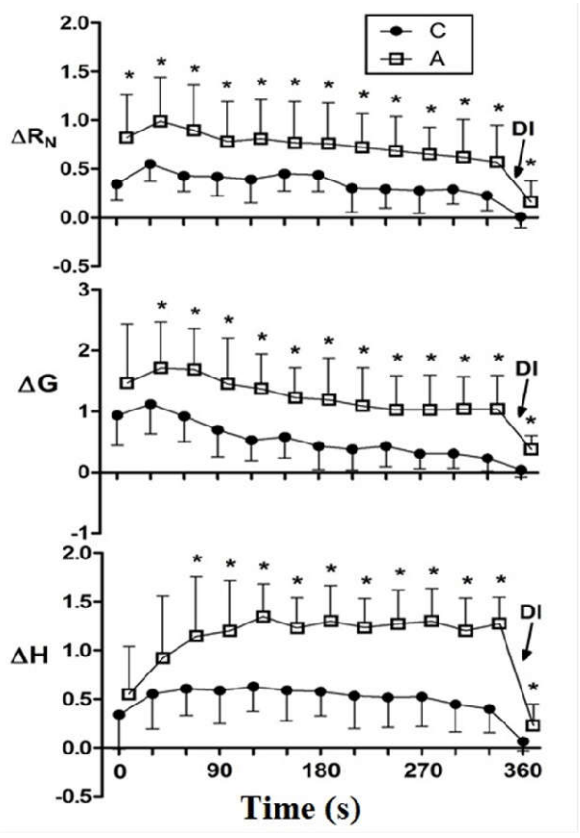

Figure 2- Experimental measurements of groups C (closed circles) and A (open squares) with time, of parameters obtained by calculating the respiratory system impedance (Z_{rs}) after nebulization with Ach (30 mg/mL) for 30 s. Two deep inflations (DIs) were soon given after 6 min from start of collections. * p<0.05 was considered statistically significant

All values of group A studied showed statistically significant differences when compared to group C (p<0.05, Student t-test). Data from airway hyper responsiveness, referring to the R_N, G and H are shown in Figure 2. We observed several points with statistically significant differences between the groups for all the parameters examined.

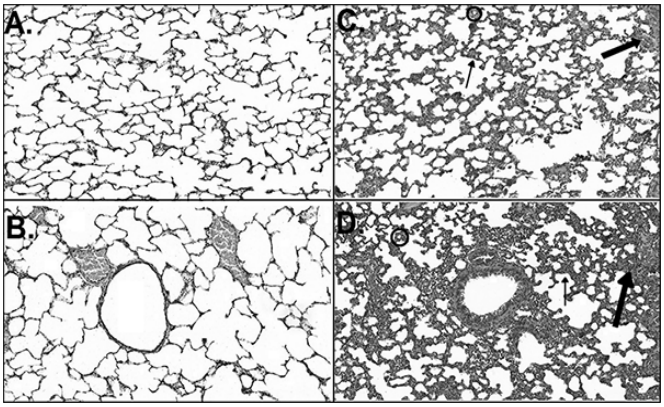

Figure 3. Representative photomicrographs of lung parenchyma (A) and airway (B) of animals exposed to saline vaporand lung parenchyma (C) and airway (D) of animals exposed to acrolein vapor generated by bubbling N_2.Fine arrows: thickened septa; thick arrows: cellular infiltrate and circles: alveolar colapse

Histological analysis (Figure 3) revealed a thickening of alveolar septa, areas of atelectasis, cellular infiltration and constriction of the airways in the lungs of group A.

DISCUSSION

Acrolein is a pollutant with highly irritability to airways, causing damage to the epithelium and bronchoconstriction (Beauchamp *et al*., 1985; Lyon *et al*., 1970; Costa *et al*., 1986). Due to its high water solubility, when inhaled, deposition occurs predominantly in the proximal airways (Costa *et al*., 1986; Lyon *et al*., 1970), but it also reaches more distal airways (Beauchamp *et al*., 1985), causing dysfunction of the alveolar macrophages and increased susceptibility to infections (Astry *et al.*, 1983). In rats chronically exposed to 4.0 ppm acrolein, there was a decrease in lung function, characterized by increased pulmonary resistance, lung volumes and capacities and the quasi-static compliance. Besides functional changes, alveolitis, hemorrhage, edema, type II pneumocyte hyperplasia and slight increase in collagen fibers were observed (Costa *et al*., 1986). One study conducted the exposure of wistar rats to the exhaust gases from the glycerol combustion for 13 weeks (Serra *et al,* 2017) and the major pollutants generated by the combustion process of glycerol is acrolein. The authors found alterations in the group exposed to exhaust gases from the combustion of glycerol in all variables of the respiratory system analyzed, emphasizing its toxic pulmonary effect.

Other studies ofacrolein exposure demonstrated that after inhalation of 0.4-1.0 ppm acrolein for two hours, there was an increase in total lung resistance and decreased respiratory rate (Murphy *et al.*, 1963). Animals that inhaled acrolein for two hours (15 mL/min bubbled with N_2) after intravenous administration of ACh showed an increase in pulmonary resistance for five minutes and bronchial responsiveness for more than four hours (Leikauf *et al*., 1989). Isolated strips of tracheal smooth muscle showed hyperresponsiveness to ACh after *in vivo* inhalation of acrolein (Ben-Jebria *et al*., 1995) and exposure to acrolein (1-2 ppm) for four hours resulted in an imbalance in lung antioxidant defenses, favoring an oxidative stress condition and severe lung injury (Arumugan *et al*., 1999). In our study, we observed changes in all variables related to $Z_{rs}(R_N,\ G,\ H,\eta)$ and the estimate of inspiratory capacity (IC) of the animals of group A compared to the animals of group C.

Newtonian resistance (R_N) has been used as a good estimate of the total resistance of the central airways (Bates, 2009). We can assume that significantly higher R_N values in group A compared to group C (Figure 1) may represent greater narrowing or increased stiffness of smooth muscle of the airways in group A, which is evidenced in the histological photomicrographs (Figure 3, C and D). Acute exposure to acrolein appears to cause bronchoconstriction as a bronchoprotective reflex response, and studies have shown a dose-dependent relationship between exposure to acrolein and increased airway resistance in animals (Leikauf *et al*., 1989). Tissue resistance (G) and tissue elastance (H) (Figure 1) are related to the intrinsic properties of the tissue, and the analysis of these parameters is not as simple as that of Newtonian resistanceR_N. There are several hypotheses to explain their changes, and one of them points to changes in the rheological properties of the tissue (Bates, 2009). Another way would be through the influence of narrowing of the airways on these parameters, where narrowing could result in the distortion of the lung parenchyma with closure of small airways, effectively providing less lung with proportionally higher tissue elastance (H) (Wagers *et al*., 2004).

Tissue resistance (G) reflects the dissipation of viscous energy in the lung tissue, a parameter that is also changed due to the distortion of the lung parenchyma, which occurs when airways constricted (Wagers *et al*., 2004). This may explain the significant increases in the G and H, values, since there was an increase in this parameter related to airway resistance(R_N). Another possibility for this increase is the presence of mucus in small caliber airways, which could lead to their occlusion, as withthe production of areas with atelectasis. Histological analysis (Figure 3b) demonstrated both the narrowing of airways and thickening of alveolar septa in group A, which may result in the increase in G and H. Regarding hysteresivity (η), it is known that its value rises as the lung becomes mechanically heterogeneous (Bates, 2009), and thus the histeresivity can be used as an indicator of these ventilation heterogeneities. We can then assume that the increase in η values is due to the presence of ventilation inhomogeneities resulting from increased R_N.Decreased inspiratory capacity (IC) is consistent with the effective stiffening of lung tissue indicated by increased H.

With regard to hyperresponsiveness of airway smooth muscle, it is known that bronchoconstrictors have been widely used in studies of animals with pulmonary inflammation. This is due to the knowledge that the primary problem in asthma is excessive shortening of the smooth muscle of the airway, either by better response of smooth muscle itself (Fredberg *et al.*, 1997) or by an increased stiffness and thickness of the airway wall (Moreno *et al.*, 1996; Wagers *et al.,* 2004). Exposure to acrolein can induce airway hyperresponsiveness, like with other spasmogenic stimuli. Studies have reported that animals previously exposed to acrolein show higher response to the bronchoconstrictor effects of ACh (Leikauf *et al*., 1989). The greater responsiveness of smooth muscle of the airways of the animals of group A in response to the ACh nebulization was evident (Figure 3). We observed significant differences between the means of the groups at various points throughout the study, corroborating the findings of previous studies. The peak ofΔR_Nrepresents the point at which the greatest airway narrowing occurs, after ACh nebulization. However, due to the removal of ACh by the circulation and its enzymatic degradation (Lauzon *et al.*, 2000), the peak of ΔR_N is not

maintained. Despite this removal, ΔR_Ncontinued with a high plateau and did not return to baseline. A possible explanation for this finding was that since ACh was removed, there was a complete relaxation of the airway smooth muscle, which can be achieved only after it is physically stretched (Wagers *et al*., 2004). Another explanation is that bronchoconstriction caused by ACh results in the complete closure of small airways (Evans *et al.,* 2003), and these cannot be opened only with the relaxation of their smooth muscle but can be after administering a DI (recruitment maneuver) (Naureckas *et al*., 1994; Perun *et al*, 1995), which can be observed in the parameters ΔR_N, ΔG and ΔH..

The behavior of ΔH during challenge with ACh, may also be explained by the closure of small airways, resulting in effectively less lung with proportionally greater elastance (Wagers *et al.,* 2004). In addition, the increase in ΔR_N due to narrowing of the airways may cause a distortion of the lung parenchyma, resulting in increased intrinsic stiffness of the tissue, causing an increase in elastance (Bates *et al.*, 1994) and development of ventilation inhomogeneities throughout the lung, which causes an increase in tissue stiffness (Bates *et al.*, 1994). It was said earlier that Gsupposedly reflects the viscous energy dissipation in lung tissue and that this is not independent of$H$, due to both reflecting tissue characteristics. It is expected then that changes such as distortion of the lung parenchyma caused by the contraction of the airways result in an increase in ΔH, as stated earlier, and consequentlyΔG. We noted that the behavior of parameter ΔG, resembled that of parameter ΔR_N, indicating that this is far more influenced by changes that occur in the smooth muscle of the airways.

Conclusion

In conclusion, acute exposure to acrolein caused changes in all parameters studied related to lung mechanics, and there was a greater hyper responsiveness in the smooth muscle of the airways and histological changes. These findings reinforce the harmful nature of exposure to acrolein to health.

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