



Repeated stress reduces mucociliary clearance in animals with chronic allergic airway inflammation[☆]

Rafael Almeida-Reis^a, Alessandra C. Toledo^a, Fabiana G. Reis^a, Ricardo H. Marques^{a,c}, Carla M. Prado^d, Marisa Dolhnikoff^b, Milton A. Martins^a, Edna A. Leick-Maldonado^a, Iolanda F.L.C. Tibério^{a,*}

^a Department of Clinical Medicine, School of Medicine, University of São Paulo, São Paulo, Brazil

^b Department of Pathology, School of Medicine, University of São Paulo, São Paulo, Brazil

^c Department of Physiology, Lusiada Faculty of Medical Sciences, Santos, Brazil

^d Department of Biological Sciences, Federal University of São Paulo, Diadema, Brazil

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ABSTRACT

We evaluated if repeated stress modulates mucociliary clearance and inflammatory responses in airways of guinea pigs (GP) with chronic inflammation. The GP received seven exposures of ovalbumin or saline 0.9%. After 4th inhalation, animals were submitted to repeated forced swim stressor protocol (5×/week/2 weeks). After 7th inhalation, GP were anesthetized. We measured transepithelial potential difference, ciliary beat frequency, mucociliary transport, contact angle, cough transportability and serum cortisol levels. Lungs and adrenals were removed, weighed and analyzed by morphometry. Ovalbumin-exposed animals submitted to repeated stress had a reduction in mucociliary transport, and an increase on serum cortisol, adrenals weight, mucus wettability and adhesivity, positive acid mucus area and IL-4 positive cells in airway compared to non-stressed ovalbumin-exposed animals ($p < 0.05$). There were no effects on eosinophilic recruitment and IL-13 positive cells. Repeated stress reduces mucociliary clearance due to mucus rheological-property alterations, increasing acid mucus and its wettability and adhesivity. These effects seem to be associated with IL-4 activation.

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1. Introduction

Evidence suggests a deleterious effect of chronic stress on pulmonary allergic disorders in humans and animal models (Ritz et al., 2000; Portela et al., 2002, 2007; Forsythe et al., 2004). Miller et al. (2002) proposed a glucocorticoid-resistance model assuming that chronic stress reduces the immune system's sensitivity to the glucocorticoid hormones derived from the hypothalamic–pituitary–adrenocortical (HPA) and sympathetic adrenal medullary axes. In this regard, stress increases eosinophilic inflammation due to an increase in IL-5 production in mild asthmatics (Fritz and Overholser, 1989; Gutstadt et al., 1989; Liu et al., 2002).

Considering the link between stress and allergic asthma, Elenkov and Chrousos (1999) have proposed that stress could also increase the Th1/Th2 shift. The main inflammatory cell populations involved in asthma pathophysiology were represented by eosinophils and Th2 lymphocytes, which produce several cytokines (IL-4, IL-5 and IL-13), which are potentially responsible for many of the structural and functional lung alterations observed (Zimmermann et al., 2003).

Another aspect of asthma pathophysiology that has been scarcely analyzed is mucociliary clearance (Knowles and Boucher, 2002). Some studies showed a reduced number of ciliated cells, goblet cells metaplasia and large amounts of hyperviscous mucus in asthmatic patients (Pavia et al., 1985; Houtmeyers et al., 1999; Del Donno et al., 2000). Mucociliary clearance represents one of the most important respiratory defenses. However, the influence of stress on mucociliary clearance of airways with chronic Th2 inflammation needs to be fully investigated (Pavia et al., 1985; Houtmeyers et al., 1999).

We tested the hypothesis that repeated stress modifies mucus clearance and its rheological properties in an animal model of chronic allergic airway inflammation. For stress induction we used the forced swim stressor model, as a model of behav-

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* Corresponding author at: Departamento de Clínica Médica, Faculdade de Medicina da Universidade de São Paulo, Av. Dr. Arnaldo, 455 - Sala 1210, 01246-903 São Paulo, SP, Brazil, Tel.: +55 11 3061 7317; fax: +55 11 3085 0992.

E-mail addresses: ioalvo@uol.com.br, dosreisrafael@usp.br (I.F.L.C. Tibério).

ioral despair and depression (Porsolt, 1977; Živković et al., 2005). In order to characterize the mechanisms involved, we quantified the Th2-inflammatory response, by measuring the number of eosinophils and IL-4 and IL-13 positive cells in airway walls.

2. Materials and methods

2.1. Animals

Male guinea pigs, weighing 300–400 g, were maintained in an animal facility with a 12-h light/dark cycle and fed water and chow ad libitum. The animals received humane care in compliance with the “Guide for Care and Use of Laboratory Animals” (National Institutes of Health, publication 86-23, revised 1985) and approved by the Ethical Committee of the University of São Paulo.

2.2. Sensitization protocol

The animals were placed in an acrylic box (30 cm × 15 cm × 20 cm) connected to an ultrasonic nebulizer (Soniclear, São Paulo, Brazil). An aerosol of ovalbumin solution (Salto's, São Paulo, Brazil) diluted in NaCl 0.9% (saline) was generated for 15 min or until respiratory distress occurred (onset of sneezing, coryza, cough, and/or the indrawing of the thoracic wall). This protocol was repeated twice a week for 4 weeks, with increasing concentrations of ovalbumin from 1 to 5 mg/mL (Tibério et al., 1997). Control animals were submitted to the same protocol with only saline solution (NaCl 0.9%) (Fig. 1A).

2.3. Forced swim stressor

Twenty-four hours after the fourth inhalation animals were submitted to the forced swim-stress protocol, 5 days a week for 2 weeks to induce the behavioral stress (Fig. 1). The stressing condition was interrupted for 2 days to avoid lactic acid buildup, pain, fatigue and intense stress in the animals. Each guinea pig was placed alone into a transparent box (50 cm × 40 cm × 30 cm), containing clean potable water at 25 °C. Although guinea pigs are sensitive to changes in water temperature we previously had shown that at this temperature the animals exhibited stable, forced swim-stressed behavior (Capellozzi et al., 2007). In addition, it is important to emphasize that the functional pulmonary evaluations were performed 24 h after the last exposure to the forced swim-stress protocol to exclude any effects of the water temperature on pulmonary mechanical measurements. The performance of each animal was observed and monitored. The animals swam up to 10 min or until they presented signs of fatigue or danger of drowning. It is important to remember that guinea pigs only breathe through their snouts minimizing aspiration of water.

2.4. Experimental design

Thirty-two guinea pigs were divided into four groups: (a) animals that received aerosolized saline 0.9% (SAL, $n=8$), (b) animals that received aerosolized ovalbumin (OVA, $n=8$), (c) animals submitted to repeated, forced swim-stress that received aerosolized saline 0.9% (SAL-S, $n=8$) and (d) animals submitted to repeated, forced swim-stress that received aerosolized ovalbumin (OVA-S, $n=8$).

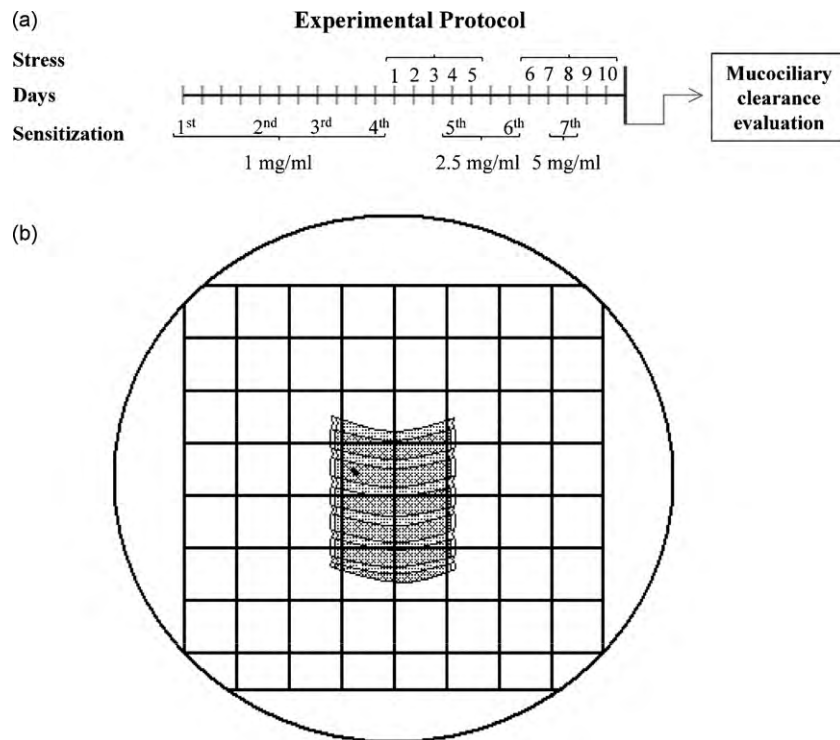


Fig. 1. (A) Timeline of the experimental protocol. The guinea pigs were submitted to 7 inhalations (2 per week—with 2- to 3-day intervals, during 4 weeks) with aerosols of saline or ovalbumin solution with increasing doses of antigen. From the first to the fourth inhalations, the dose used was 1 mg/mL of ovalbumin (2 weeks). In the fifth and sixth inhalation sessions (third week), animals were inhaled with 2.5 mg/mL of ovalbumin and in the seventh inhalation session (beginning in the fourth week) the dose of 5 mg/mL of antigen was used. The forced swim-stress starts 24 h after the fourth inhalation during 5 days a week for 2 weeks. The solution of ovalbumin or saline was continuously aerosolized for 15 min or until respiratory distress occurred (sneezing, coryza, cough or retraction of the thoracic wall). Seventy-two hours after the seventh inhalation (24 h after the last stress session), all guinea pigs were anesthetized and exsanguinated. Their lungs were removed and submitted to the experimental protocol of evaluation of the mucociliary clearance. (B) Schematic representation of ex vivo mucociliary tracheal transport evaluation. Tracheal tissue under matted lens. The progression of the leading edge of the nankeen drop along a longitudinal axis toward the oropharynx was timed and expressed as a velocity.

Seventy-two hours after the last inhalation (24 h after the last stress session), guinea pigs were anesthetized with pentobarbital sodium (50 mg/kg ip), tracheostomized and mechanically ventilated at 60 breaths/min with a tidal volume of 8 mL/kg using a Harvard 683 ventilator (Harvard Apparatus, Holliston, USA). The transepithelial potential difference was measured in the trachea in vivo. Immediately after this measurement, two pieces of the trachea were removed to measure their mucociliary transport and ciliary beat frequency. Afterwards, the animals were laparotomized and their adrenal glands removed and weighed. Lungs were washed with heparinized saline, removed, fixed with 4% buffered formaldehyde and embedded in paraffin. Histological sections were stained with LUNA, a combination of Schiff periodic acid and Alcian blue (PAS-AB), or submitted to immunohistochemistry analysis for IL-4 and IL-13 expression (Santa Cruz Biotechnology, Santa Cruz, USA) as previously described (Hocke et al., 2006; Arantes-Costa et al., 2008).

2.5. Transepithelial electric potential difference

The transepithelial electric potential difference was performed as previously described (Nakagawa et al., 2004). Briefly, the measurements of transepithelial electric potential (PD) were performed using two flexible microelectrodes saturated with KCl, connected to two calomel half-cells: one for the reference and another for the test. Both calomel half-cells were connected to the high input of a grounded electrometer (Fisher Accumet 950; Fisher Scientific, Pittsburgh, USA). The reference electrode was guided in with a polyethylene catheter and placed near the trachea in the subcutaneous tissue of the animal. The test electrode was carefully placed in contact with the tracheal epithelium surface. Whenever the electrode pairs differed by 1 mV of PD, the bridges were discarded. We performed three measurements per guinea pig and results were presented in mV.

2.6. Tracheal mucociliary transport (MCT)

Based on previously described MCT analysis in rat bronchus (Rivero et al., 2001), we performed the evaluation of ex vivo tracheal mucociliary transport, as shown in Fig. 1B. After dissection, the surface of the trachea received a stream of nebulized saline to provide humidification. Afterwards, the surface of trachea was placed under the optical microscope (10× objective, 10× ocular), equipped with a reticulated eyepiece. Ex vivo mucociliary transport was monitored by direct observation of the displacement of a China ink (Acrilex, São Paulo, Brazil) drop (50 µL) placed on the surface of the trachea. The progression of the leading edge of the nankeen drop along a longitudinal axis toward the oropharynx was timed and expressed as a velocity. MCT was calculated by dividing the distance traveled (50 µm) by the elapsed time (s). We performed three measurements for each animal (Fig. 1B).

2.7. Ciliary beat frequency (CBF)

CBF was measured by a modification on the videoscopic technique previously described by Pazetti et al. (2008). Briefly, this technique consists of focusing on a group of cilia through an optical microscope connected to a video camera – Model 3CCD Iris (Sony, Park Ridge, USA), with the resulting image being sent to a monitor OE141 (Olympus, Tokyo, Japan). A stroboscopic light Model 5000 (Machine Vision Strobe, Salem, USA) was placed in front of the ciliary epithelium emitting flashes (0–30 Hz). The incident light illuminating the ciliated epithelium was reflected from the cilia packed together and the thin layer of mucus covering the cilia. We performed three measurements for each animal. The results were expressed in Hz.

2.8. Mucus properties analysis

The contact angle and in vitro cough clearance were measured according to previous studies (Teixeira et al., 2006). The contact angle evaluates mucus wettability, the tendency of a biological fluid to spread when deposited on a solid plane surface measuring the interaction between the surface and the mucus molecules. Cough clearance or adhesivity was quantified by determining the displacement of mucus in millimeters (King et al., 1985).

2.9. Serum cortisol analysis

Cortisol Kits B060-101 (AutoDELFIA, Turku, Finland) were used for the detection of cortisol in serum using a solid-phase, time-resolved fluoroimmunoassays. These are based on the competitive reaction between the Eu-labeled cortisol and the serum cortisol sample for the limited number of binding sites on cortisol-specific, biotinylated monoclonal antibodies.

2.10. Morphometric studies

Histological sections were stained with a combination of Schiff Periodic Acid and Alcian Blue (PAS-AB) at a pH of 2.5. With this technique, neutral and acidic glycoproteins are stained in red and blue, respectively. The quantification of neutral and acidic mucus substances and the total airway epithelial-wall area of non-cartilaginous airways used an optical microscope, provided with an integrating eyepiece containing a known area ($10^4 \mu\text{m}^2$ at a magnification of 1000×) of 50 lines and 100 points. Neutral and acidic mucus areas as well as airway epithelial area were determined by point counting technique. The percentage area of neutral and acidic mucus was obtained by dividing the number of the points hitting each type of positive mucus area by the number of points hitting the total airway epithelial area and expressed as a percentage (Arantes-Costa et al., 2008).

The density of eosinophils and the number of IL-4 and IL-13 positive cells in the airway walls were determined. Using a 100-point grid with a known area ($10^4 \mu\text{m}^2$ at a 1000× magnification) attached to the ocular of the microscope, we counted the number of points hitting the airway wall, from basement membrane through smooth muscle and adventitia excluding the epithelial area. The number of eosinophils, IL-4 and IL-13 positive cells within this airway wall was counted. The densities were calculated as the number of these cells in each field divided by the number of points in the airway wall. Measurements are expressed as cells/ $10^4 \mu\text{m}^2$. Five airways were evaluated per animal (Leick-Maldonado et al., 2004).

2.11. Data analysis

Statistical analysis was performed using SigmaStat software, version 3.5 (Systat Inc., Chicago, USA). All data passed on normality test and values were expressed as mean and standard error (SE). For the comparison of the four experimental groups, data were evaluated by two-way analysis of variance (factors: ovalbumin and stress exposures). Multiple comparisons were made using Holm–Sidak method. A *p* value of less than 0.05 was considered significant.

3. Results

3.1. Mucociliary transport

Fig. 2 shows the values of mucociliary transport in all experimental groups. There were no significant differences between the saline-exposed groups (SAL and SAL-S groups). Ovalbumin-exposed guinea pigs (OVA and OVA-S groups) had lower values than

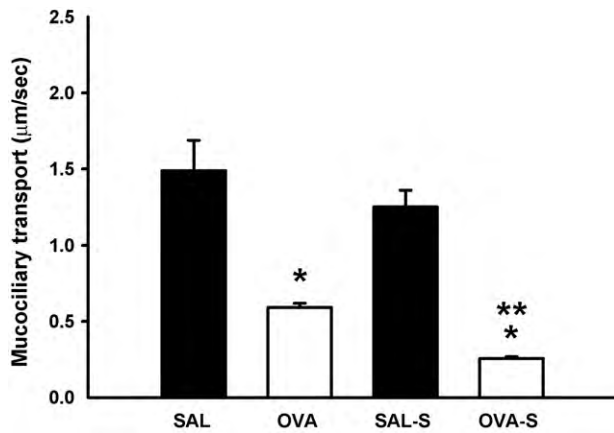


Fig. 2. Mean and SE of the values of the mucociliary clearance transport ($\mu\text{m/s}$) of the four experimental groups. The OVA and OVA-S groups had lower values than the controls ($^*p < 0.001$). There were significant differences between ovalbumin-exposed groups ($^{**}p < 0.05$).

controls ($p < 0.001$). Guinea pigs subjected to the repeated, forced swim-stress (OVA-S) had lower values than OVA animals ($p < 0.05$).

3.2. Contact angle

Fig. 3 shows the values of contact angle of mucus in all experimental groups. There were no significant differences between the saline-exposed groups (SAL and SAL-S groups). Ovalbumin-exposed guinea pigs (OVA and OVA-S groups) had greater values than the controls ($p < 0.001$). Guinea pigs subjected to the repeated, forced swim-stress (OVA-S) had greater values than that observed in OVA animals ($p < 0.05$).

3.3. Ciliary beat frequency

There were no differences among the values of ciliary beat frequency in trachea obtained in all experimental groups. The tracheal transepithelial potential values in ovalbumin-exposed groups (OVA and OVA-S groups) were lower values than those observed in control groups (SAL and SAL-S groups, $p < 0.05$).

3.4. Airway epithelial area

There were no significant differences between the normal saline-exposed groups, in the airway epithelial area. Ovalbumin-

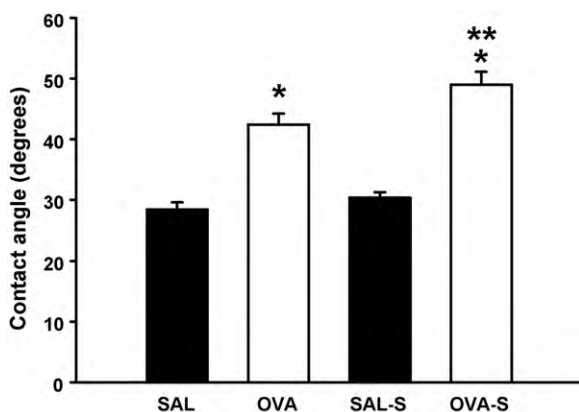


Fig. 3. Mean and SE of values of the contact angle of mucus of the four experimental groups. The ovalbumin-exposed guinea pigs (OVA and OVA-S groups) had bigger values than the controls ($^*p < 0.001$). OVA-S group had bigger values than those observed in OVA animals ($^{**}p < 0.05$).

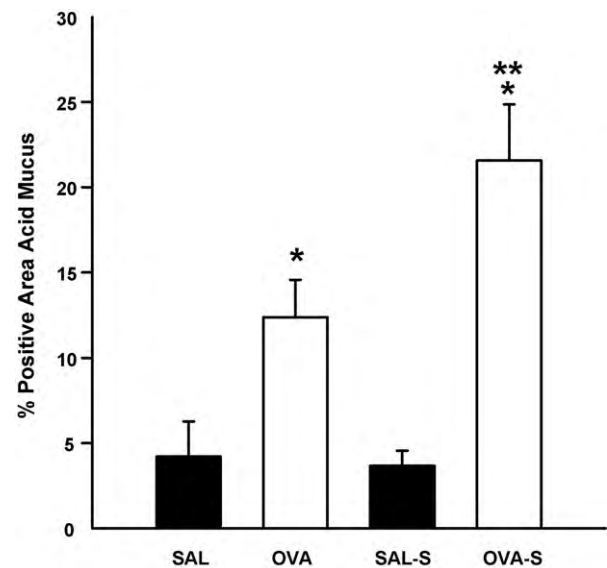


Fig. 4. Mean and SE of the percentage of acid mucus positive area in airway epithelia of the four experimental groups. The ovalbumin-exposed animals had greater values of positive acid mucus area than those observed in the saline-exposed animals ($^*p < 0.01$). The OVA-S group had greater values of positive acid mucus area than the OVA group ($^{**}p < 0.05$).

exposed guinea pigs (OVA and OVA-S) had greater values than the controls ($p < 0.05$).

3.5. Area of acid mucus

Fig. 4 shows the percentage of acid mucus positive area in the airway epithelia in all experimental groups. Ovalbumin-exposed groups (OVA and OVA-S) had greater values of acid mucus positive area when compared to the control groups ($p < 0.05$). Furthermore, ovalbumin-exposed guinea pigs subjected to repeated, forced swim-stress (OVA-S) had greater values of fractional positive area of acid mucus than that observed in OVA animals ($p < 0.05$). There were no differences among the values of neutral mucus obtained in all experimental groups.

3.6. Airway eosinophilic inflammation

Ovalbumin-exposed animals (OVA and OVA-S groups) had an increase in the number of eosinophils in airway walls compared to saline-exposed animals ($p < 0.001$). There was no difference between OVA and OVA-S groups.

3.7. Adrenal weight and serum cortisol

Animals subjected to the repeated, forced swim-stress (SAL-S and OVA-S groups) had greater values of serum cortisol than OVA group ($p < 0.05$). The adrenal weight in animals subjected to the repeated, forced swim-stress (SAL-S and OVA-S groups) was greater than that in non-stressed animals (SAL and OVA groups, $p < 0.05$).

3.8. IL-4 and IL-13 positive cells

Ovalbumin-exposed animals (OVA and OVA-S groups) had an increase in the number of IL-13 positive cells in airway walls compared to saline-exposed animals ($p < 0.001$). However, there were no differences in these values between OVA and OVA-S groups (**Fig. 5A**).

The values of IL-4 positive cells in airway walls in all experimental groups were described in **Fig. 5B**. There were no significant

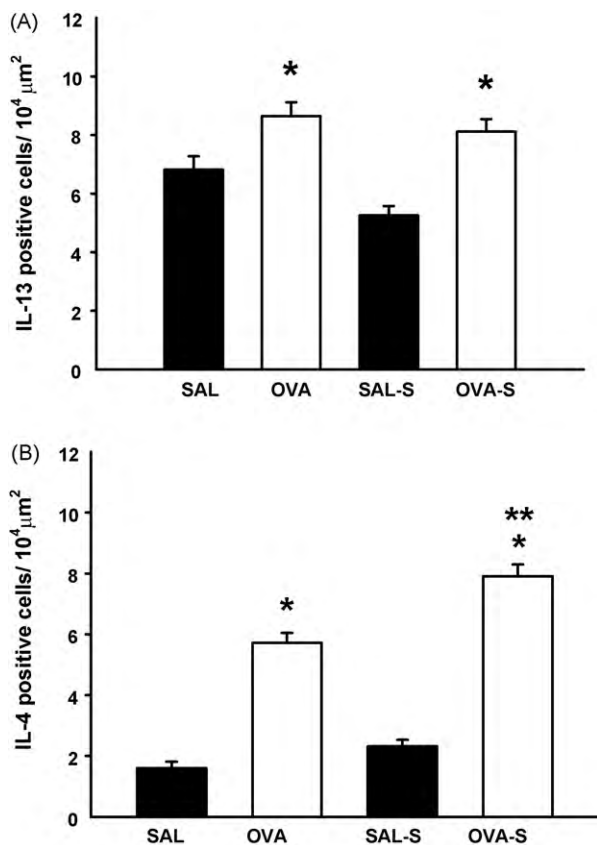


Fig. 5. (A) Mean and SE of IL-13 positive inflammatory cells in airway walls in all the experimental groups. There were no significant differences between the saline-exposed groups (SAL and SAL-S groups). The ovalbumin-exposed guinea pigs (OVA and OVA-S groups) had bigger values than the controls ($p < 0.001$). There were no differences between the OVA and OVA-S groups. (B) Mean and SE of IL-4 positive inflammatory cells in airway walls in all the experimental groups. There were no significant differences between the saline-exposed groups (SAL and SAL-S groups). The ovalbumin-exposed guinea pigs (OVA and OVA-S groups) had bigger values than the controls ($p < 0.001$). Guinea pigs submitted to the forced swim-stress (OVA-S) had bigger values than those observed in OVA animals ($p < 0.001$).

differences between the values of saline-exposed groups (SAL and SAL-S groups). Ovalbumin-exposed guinea pigs (OVA and OVA-S groups) had greater values than the controls ($p < 0.001$). Ovalbumin-sensitized guinea pigs animals subjected to repeated, forced swim-stress (OVA-S) had greater values of IL-4 positive cells in airway walls than that observed in OVA animals ($p < 0.001$).

Fig. 6 shows representative photomicrographs of airway walls stained with PAS-Alcian Blue (A, B, C and D panels) and LUNA (E, F, G and H panels) and immunohistochemistry to detect IL-4 (I, J, K and L panels) and IL-13 (M, N, O and P) expression on airway epithelial cells. Guinea pigs exposed to saline 0.9% (A, E, I and M panels) and those repeatedly exposed to ovalbumin (B, F, J and N panels) are represented. The guinea pigs subjected to repeated, forced swim-stress and exposed to saline 0.9% (C, G, K and O panels) or ovalbumin (D, H, L and P panels) were also represented. The ovalbumin-exposed guinea pigs (OVA and OVA-S) had an increase in the percentage of acid mucus positive area, eosinophils and IL-13 positive cells in airway walls compared to the controls (SAL and SAL-S). The repeated stress exposures in sensitized animals (OVA-S) increased IL-4 positive cells in airway walls and the percentage of acid mucus positive area, compared to the OVA group. No differences were observed between the OVA and OVA-S groups, concerning the eosinophils number and IL-13 positive bronchial epithelial and inflammatory cells.

4. Discussion

Although stress can worsen asthma, the data corroborating this assumption are scarce (Vig et al., 2006). Few studies have been conducted to evaluate the effects of stress on asthma pathophysiology, particularly those analyzing the modulation of the mucociliary clearance (Busse et al., 1995).

In this animal model of chronic allergic airway inflammation, repeated stress reduced the mucociliary clearance. This result was associated with an increase in the percentage of positive epithelial acid mucus area (Fig. 4), its wettability (Fig. 3) and adhesivity and the number of IL-4 positive cells (Fig. 5B) in airway walls. The presence of stress-induced alterations was reinforced by the fact that there was a significant increase in the adrenal weight and cortisol levels in the animals submitted to repeated stress. However, it is noteworthy to consider that in this protocol of stress induction there are both psychological and physical stressors, and it is not possible to separate the role of each component in the functional and inflammatory responses observed.

In order to assess the importance of stress-induced functional alterations of the mucociliary clearance in this animal model of chronic airway inflammation, we used an ex vivo model of mucociliary transport, based on previous studies (Rivero et al., 2001; Teixeira et al., 2006; Pazetti et al., 2008). We showed for the first time that repeated stress reduces the mucociliary clearance transport (Fig. 2) due to alterations on mucus properties, particularly increasing its wettability and adhesivity as well as the percentage of positive epithelial acid mucus area. It is important to clarify that we evaluated the mucus wettability and adhesivity but not the viscosity, which is an internal property of a fluid that offers resistance to flow.

We did not observe that repeated stress influenced changes in the transepithelial potential difference and ciliary beat frequency. Many factors were involved in the regulation of ciliary beat frequency such as Ca^{2+} and cAMP, intracellular concentration. Ca^{2+} is involved in rapid, but short, stimulation of ciliary beat frequency. On the other hand, cAMP is involved in long-lasting enhancement of ciliary beat. In a recent study, it was showed that PDE4 inhibitors such as roflumilast and romipal improved ciliary beat frequency in large and small airways (Wohlsen et al., 2010).

One hypothesis to explain the effects of repeated stress on mucus clearance was related to mucus properties, since we observed an increase in the percentage of positive epithelial acid mucus area. Other authors had shown that significant irritants to airway epithelium, such as air pollutants, were able to cause a shift toward an increase in the proportion of cells with production of acidic mucus (Saldiva et al., 1992; Pires-Neto et al., 2006; Arantes-Costa et al., 2008). Acidifying mucus may lead to intra- and intercellular edema, as well as contribute to altered mucociliary clearance, affecting various lung functions (Holma and Hegg, 1989).

We showed that repeated stress induces an increase in IL-4 positive cells (Fig. 5B) in airway walls of sensitized animals. The mechanisms involved in the stress effects on mucus properties and clearance can be related, at least in part, to the increase in IL-4, but not IL-13 positive cells in airway walls (Fig. 5A). Cohn et al. (1997) showed that IL-4R α receptor activation led to mucus production in airway cells. Dabbagh et al. (1999) showed that airway epithelial cells expressed IL-4R and IL-4 constitutively, and that IL-4-induced MUC2 gene expression and mucus glycoconjugate production contributed to the differentiation of epithelium into goblet cells.

The eosinophilic response observed was increased by repeated ovalbumin exposures but unaffected in animals also subjected to repeated stress (OVA vs OVA-S, $p > 0.05$). It is well known that the modulators of stress responses, such as adrenal steroids, catecholamines and neuropeptides may have different effects

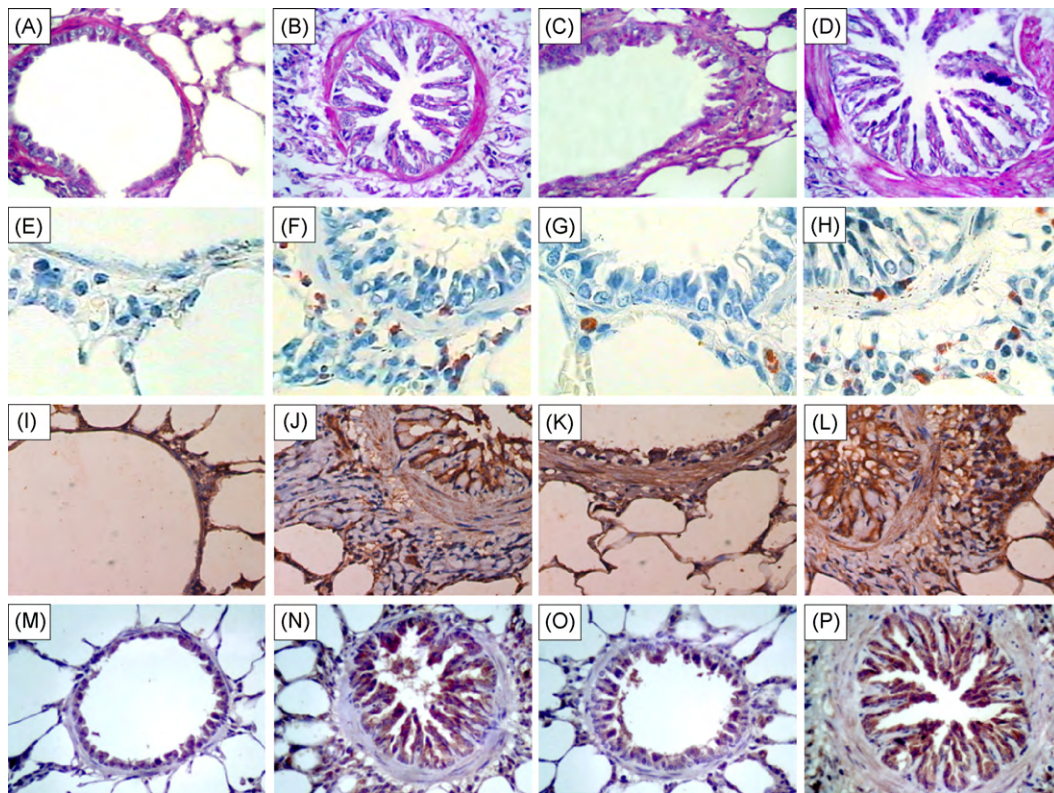


Fig. 6. Representative photomicrographs of airway walls stained with PAS-Alcian Blue (A, B, C and D panels) ($\times 400$), LUNA (E, F, G and H panels) ($\times 1000$), immunohistochemistry technique to detect IL-4 (I, J, K, L panels) and IL-13 (M, N, O and P panels) ($\times 400$) from guinea pigs exposed to saline (A, E, I and M panels) or those repeatedly exposed to ovalbumin (B, F, J and N panels). The guinea pigs submitted to repeated exposures to forced swim-stress and exposed to saline (C, G, K and O panels) or ovalbumin (D, H, L and P panels) are also represented. The OVA-S groups had an increase in IL-4 positive cells in airway walls and the percentage of acid mucus positive area compared to the OVA group. There were no differences between the OVA and OVA-S groups concerning the eosinophils number and IL-13 positive cells in airway walls.

on eosinophil recruitment and apoptosis (Takeaki et al., 1998; McEwen, 2000).

Portela et al. (2002) observed that sensitized and stressed rats had an increase in the formation of the airway edema and in the lymphocytic infiltration, but not in the number of eosinophils. In addition, the catecholamine levels were unaffected in the present stress model (data not shown). Concerning the effects of neuropeptides on eosinophil recruitment, Tibério et al. (2003) showed that both substance P and neurokinin A infusion contributed to eosinophil lung recruitment in distal airways and in the alveolar wall. Although neuropeptides depletion reduced CD4⁺ lymphocytes in animals with chronic allergic airway inflammation, the absence of these mediators did not influence airway eosinophilic recruitment (Tibério et al., 1997). Taken together, it seems that the stress determinants of the eosinophilic recruitment depend on complex interactions and may be influenced by the type (acute or chronic) and/or intensity of stress and inflammatory stimuli.

The present study has some limitations. We only evaluated IL-4 and IL-13 positive cells in airway walls. Although these are important mediators of mucociliary clearance the importance of other cytokines, in this response, could not be excluded. The inclusion of other methods to evaluate the expression of the receptors involved in these responses could also amplify the comprehension of the mechanisms involved, since both IL-13 and IL-4 are thought to signal via the IL4-R α receptor. Another limitation of the present study was that, due to ethical issues, the serum for cortisol analysis was collected in animals of all groups under anesthesia, which may have reduced its levels.

In conclusion, repeated exposures to stress in guinea pigs with chronic airway inflammation leads to secretory changes in the res-

piratory tract, marked by the increase in the epithelial acidic mucus substances with consequent impairment of the mucociliary clearance, which is an important defense mechanism of the respiratory tract. These effects seem to be related to an increase in IL-4 expression airway wall cells.

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References

- Arantes-Costa, F.M., Lopes, F.D.T.Q.S., Toledo, A.C., Magliarelli-Filho, P.A., Moriya, H.T., Carvalho-Oliveira, R., Mauad, T., Saldiva, P.H.N., Martins, M.A., 2008. Effects of residual oil fly ash (ROFA) in mice with chronic allergic pulmonary inflammation. *Toxicol. Pathol.* 36, 680–686.
- Busse, W.W., Kiecolt-Glaser, J.K., Coe, C., Martin, R.J., Weiss, S.T., Parker, S.R., 1995. Stress and asthma. *Am. J. Respir. Crit. Care Med.* 151, 249–252.
- Capelozzi, M.A., Leick-Maldonado, E.A., Parra, E.R., Martins, M.A., Tiberio, I.F.L.C., Capelozzi, V.L., 2007. Morphological and functional determinants of fluoxetine-induced pulmonary disease in an experimental model. *Respir. Physiol. Neurobiol.* 156, 171–178.
- Cohn, L., Homer, R.J., Marinov, A., Rankin, J., Bottomly, K., 1997. Induction of airway mucus production by t helper 2 (th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J. Exp. Med.* 186, 1737–1747.
- Dabbagh, K., Takeyama, K., Lee, H.M., Ueki, I.F., Lausier, J.A., Nadel, J.A., 1999. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *J. Immunol.* 162, 6233–6237.

- Del Donno, M., Bittesnich, D., Chetta, A., Olivieri, D., Lopez-Vidriero, M.T., 2000. The effect of inflammation on mucociliary clearance in asthma. *Chest* 118, 1142–1149.
- Elenkov, I.J., Chrousos, G.P., 1999. Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. *Trends Endocrinol. Metab.* 10, 359–368.
- Forsythe, P., Ebeling, C., Gordon, J.R., Befus, A.D., Vliagoftis, H., 2004. Opposing effects of short- and long-term stress on airway inflammation. *Am. J. Respir. Crit. Care Med.* 169, 220–226.
- Fritz, G.K., Overholser, J.C., 1989. Patterns of response to childhood asthma. *Psychosom. Med.* 51, 347–355.
- Gutstadt, L.B., Gillette, J.W., Mrazek, D.A., Fukuhara, J.T., LaBrecque, J.F., Strunk, R.C., 1989. Determinants of school performance in children with chronic asthma. *Am. J. Dis. Child.* 143, 471–475.
- Hocke, A.C., Ermert, M., Althoff, A., Brell, B., N'Guessan, P.D., Suttrop, N., Ermert, L., 2006. Regulation of interleukin IL-4, IL-13, IL-10, and their downstream components in lipopolysaccharide-exposed rat lungs. Comparison of the constitutive expression between rats and humans. *Cytokine* 33, 199–211.
- Holma, B., Hegg, P.O., 1989. pH and protein dependent buffer capacity and viscosity of respiratory mucus. Their interrelationships and influence on health. *Sci. Total Environ.* 84, 71–82.
- Houtmeyers, E., Gosselink, R., Gayan-Ramirez, G., Decramer, M., 1999. Regulation of mucociliary clearance in health and disease. *Eur. Respir. J.* 13, 1177–1188.
- King, M., Brock, G., Lundell, C., 1985. Clearance of mucus by simulated cough. *J. Appl. Physiol.* 58, 1776–1782.
- Knowles, M.R., Boucher, R.C., 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J. Clin. Invest.* 109, 571–577.
- Leick-Maldonado, E.A., Kay, F.U., Leonhardt, M.C., Kasahara, D.I., Prado, C.M., Fernandes, F.T., Martins, M.A., Tibério, I.F.L.C., 2004. Comparison of glucocorticoid and cysteinyl leukotriene receptor antagonist treatments in an experimental model of chronic airway inflammation in guinea-pigs. *Clin. Exp. Allergy* 34, 145–152.
- Liu, L.Y., Coe, C.L., Swenson, C.A., Kelly, E.A., Kita, H., Busse, W.W., 2002. School examinations enhance airway inflammation to antigen challenge. *Am. J. Respir. Crit. Care Med.* 165, 1062–1067.
- McEwen, B.S., 2000. The neurobiology of stress: from serendipity to clinical relevance. *Brain Res.* 886, 172–189.
- Miller, G.E., Cohen, S., Ritchey, A.K., 2002. Chronic psychological stress and the regulation of pro-inflammatory cytokines: a glucocorticoid-resistance model. *Health Psychol.* 21, 531–541.
- Nakagawa, N.K., Donato-Junior, F., Kondo, C.S., King, M., Auler-Junior, J.O.C., Saldiva, P.H.N., Lorenzi-Filho, G., 2004. Effects of acute hypovolaemia by furosemide on tracheal transepithelial potential difference and mucus in dogs. *Eur. Respir. J.* 24, 805–810.
- Pavia, D., Bateman, J.R.M., Shehan, N.F., Agnew, J.E., Clarke, S.W., 1985. Tracheo-bronchial mucociliary transport in asthma: impairment during remission. *Thorax* 40, 171–175.
- Pazetti, R., Pêgo-Fernandes, P.M., Lorenzi-Filho, G., Saldiva, P.H.N., Moreira, L.F.P., Jatene, F.B., 2008. Effects of cyclosporine A and bronchial transection on mucociliary transport in rats. *Ann. Thorac. Surg.* 85, 1925–1929.
- Pires-Neto, R.C., Lichtenfels, A.J., Soares, S.R., Macchione, M., Saldiva, P.H.N., Dolhnikoff, M., 2006. Effects of São Paulo air pollution on the upper airways of mice. *Environ. Res.* 101, 356–361.
- Porsolt, R.D., 1977. Depression: a new animal model sensitive to antidepressant treatments. *Nature* 266 (5604), 730–732.
- Portela, C.P., Leick-Maldonado, E.A., Kasahara, D.I., Prado, C.M., Tibério, I.F.L.C., Martins, M.A., Palermo-Neto, J., 2007. Effects of stress and neuropeptides on airway responses in ovalbumin-sensitized rats. *Neuroimmunomodulation* 14, 105–111.
- Portela, C.P., Tibério, I.F.L.C., Leick-Maldonado, E.A., Martins, M.A., Palermo-Neto, J., 2002. Effects of diazepam and stress on lung inflammatory response in OVA-sensitized rats. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 282, L1289–L1295.
- Ritz, T., Steptoe, A., DeWilde, S., Costa, M., 2000. Emotions and stress increase respiratory resistance in asthma. *Psychosom. Med.* 62, 401–412.
- Rivero, D.H.R.F., Lorenzi-Filho, G., Pazetti, R., Jatene, F.B., Saldiva, P.H.N., 2001. Effects of bronchial transection and reanastomosis on mucociliary system. *Chest* 119, 1510–1515.
- Saldiva, P.H.N., King, M., Delmonte, V.L., Macchione, M., Parada, M.A., Daliberto, M.L., Sakae, R.S., Criado, P.M., Silveira, P.L., Zin, W.A., 1992. Respiratory alterations due to urban air pollution: an experimental study in rats. *Environ. Res.* 57, 19–33.
- Takeaki, N., Fujimori, H., Kozumi, Y., Ishihara, K., Mue, S., Ohuchi, K., 1998. Effects of glucocorticoids on apoptosis of infiltrated eosinophils and neutrophil in rats. *Eur. J. Pharmacol.* 354, 73–81.
- Teixeira, M.A., Chaguri, L.C.A.G., Carissimi, A.S., Souza, N.L., Mori, C.M.C., Saldiva, P.H.N., Lemos, M., Macchione, M., Guimarães, E.T., King, M., Merusse, J.L.B., 2006. Effects of an individually ventilated cage system on the airways integrity of rats (*Rattus norvegicus*) in a laboratory in Brazil. *Lab. Anim.* 40, 419–431.
- Tibério, I.F., Leick-Maldonado, E.A., Miyahara, L., Kasahara, D.I., Spilborghs, G.M., Martins, M.A., Saldiva, P.H., 2003. Effects of neuropeptides on airway and alveolar eosinophil recruitment. *Exp. Lung Res.* 29, 165–177.
- Tibério, I.F.L.C., Turco, G.M.G., Leick-Maldonado, E.A., Sakae, R.S., Paiva, P.S.O., Warth, M.P.T.N., Lapa e Silva, J.R., Saldiva, P.H.N., Martins, M.A., 1997. Effects of neurokinin depletion on airway inflammation induced by chronic antigen exposure. *Am. J. Respir. Crit. Care Med.* 155, 1739–1747.
- Wohlsen, A., Hirtle, A., Tenor, H., Marx, D., Beume, R., 2010. Effect of cyclic AMP-elevating agents on airway ciliary beat frequency in central and lateral airways in rat precision-cut lung slices. *Eur. J. Pharmacol.* 635, 177–183.
- Vig, R.S., Forsythe, P., Vliagoftis, H., 2006. The role of stress in asthma: insight from studies on the effect of acute and chronic stressors in models of airway inflammation. *Ann. N. Y. Acad. Sci.* 1088, 65–77.
- Zimmermann, N., Hershey, G.K., Foster, P.S., Rothenberg, M.E., 2003. Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J. Allergy Clin. Immunol.* 111, 227–242.
- Živković, I.P., Rakin, A.K., Petrović-Djergović, D.M., Kosec, D.J., Mičić, M.V., 2005. Exposure to forced swim stress alters morphofunctional characteristics of the rat thymus. *J. Neuroimmunol.* 160, 77–86.