



Dexamethasone reduces bronchial wall remodeling during pulmonary migration of *Strongyloides venezuelensis* larvae in rats

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ABSTRACT

Strongyloidiasis is an intestinal parasitosis with an obligatory pulmonary cycle. A Th2-type immune response is induced and amplifies the cellular response through the secretion of inflammatory mediators. Although this response has been described as being similar to asthma, airway remodeling during pulmonary migration of larvae has not yet been established. The aim of this study was to identify the occurrence of airway remodeling during *Strongyloides venezuelensis* (S. v.) infection and to determine the ability of dexamethasone treatment to interfere with the mechanisms involved in this process. Rats were inoculated with 9,000 S. v. larvae, treated with dexamethasone (2 mg/kg) and killed at 1, 3, 5, 7, 14 and 21 days. Morphological and morphometric analyzes with routine stains and immunohistochemistry were conducted, and some inflammatory mediators were evaluated using ELISA. Goblet cell hyperplasia and increased bronchiolar thickness, characterized by edema, neovascularization, inflammatory infiltrate, collagen deposition and enlargement of the smooth muscle cell layer were observed. VEGF, IL1- β and IL-4 levels were elevated throughout the course of the infection. The morphological findings and the immunomodulatory response to the infection were drastically reduced in dexamethasone-treated rats. The pulmonary migration of S. venezuelensis larvae produced a transitory, but significant amount of airway remodeling with a slight residual bronchiolar fibrosis. The exact mechanisms involved in this process require further study.

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1. Introduction

Strongyloidiasis is an intestinal parasitosis caused by the nematode *Strongyloides* spp. found in tropical areas [1]. Usually, the filariform larvae pass through the lungs to complete their cycle life [2,3]. This passage has been known to produce symptoms such as cough, shortness of breath, wheezing and transient pulmonary infiltrates [4]. The immune response is predominantly a Th2-type response with great leukocyte recruitment and overproduction of cytokines [5–7] and has been described as being similar to that observed in atopic diseases, such as allergy and asthma [8].

Airway remodeling is an important feature of asthma and induces structural and inflammatory cells to undergo Th2-type changes in the airway walls, including subepithelial fibrosis, mucous gland hypertrophy and airway smooth muscle (ASM) layer hypertrophy/hyperplasia [9,10]. During helminth infections, the Th2 response is more commonly associated with host protection, but the exact mechanisms involved have not been fully elucidated [11]. Currently, there is a lot of information about airway remodeling in asthma, but almost no information about remodeling during the passage of *Strongyloides* larvae in the airways.

Larval migration in the lung has been analyzed in many species such as *Ascarididae*, *Ancylostomatoidea*, *Nippostrongylus* and so on. Pathological changes by migrating larvae of nematodes are fundamental and important issue for understanding of these infections. Although, there are others rodents which are definitive host of *Strongyloides venezuelensis* (S.v.), in this study, we utilized an experimental model in rats, a natural host of S. v. larvae, to demonstrate the occurrence of airway remodeling during the pulmonary cycle of these nematodes, and we showed that dexamethasone, a known efficient immunomodulator of many inflammatory responses, can effectively inhibit with this process.

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2. Materials and methods

2.1. Animals and parasites

The rats (Specific Pathogen-Free) were obtained by the Department of Parasitology at the State University of Campinas (UNICAMP) and maintained at the School of Medicine of Ribeirão Preto (University of São Paulo, Brazil). In this study, the male Wistar rats (100–120 g weight) were fed laboratory chow and given tap water to drink *ad libitum*. *S. v.* were isolated from rodents (*Bolomys lasiurus*) and were maintained at the Department of Parasitology (UNICAMP) through serial passages in Wistar rats. The infective larvae were prepared for inoculation according to our previously published protocol [12]. For each infection, 9000 *S. v.* L3 larvae in 300 µl of PBS were inoculated subcutaneously (s.c) in the abdominal region of the rat. The experimental procedures were approved and complied with the guidelines established by the animal experiments ethics committee.

2.2. Experimental protocol

The rats were randomly divided into four groups: Control; Control + Dexamethasone (Dexa); Infected; and Infected + Dexa. The larvae were inoculated in both Infected and Infected + Dexa groups. The control groups received 300 µl of PBS. Dexamethasone disodium phosphate (Decadron®, Aché Laboratories, Campinas, Brazil) was daily administered (2 mg/kg), starting 1 h before inoculation in the treatment groups. The experimental period was 21 days. Rats were euthanized on days 1, 3, 5, 7, 14 and 21, after intraperitoneal anesthesia with ketamine and xylazine. The chest cavity was opened and the trachea was exposed. A blunt cannula was inserted and tied to the trachea. The lungs were inflated according to previously described procedures [12].

2.3. Infective larvae counts

The lungs were harvested, tweezed and incubated for 2 h at 37 °C. After centrifugation, the pellets were collected and *S.v.* larvae were counted under light microscopy at 50× magnification.

2.4. Histological analysis

The lungs were inflated with 10% buffered formalin. After 48 h of fixation, the tissue was processed through conventional paraffin embedding. Five-micrometer-thick sections were cut and assessed with the following stains: HE, to analyze global morphologic alterations in the airways; periodic acid-Schiff (PAS), to demonstrate goblet cells in the bronchiolar epithelial layer and picosirius red, to demonstrate collagen in the bronchiolar chorion. Immunohistochemical staining was applied to analyze the ASM layer using primary antibody α -smooth muscle actin (clone 1A4, 1/800; RDI, Flanders, NJ, USA).

2.5. Morphometric analysis

A researcher unaware of the protocol design (CTS) utilized the Leica QWin software (Leica Microsystems Image Solutions, Cambridge, UK) in conjunction with a Leica DMR microscope (Leica, Microsystems GmbH, Wetzlar Germany), video camera (Leica Microsystems Ltd, Heeburg, Switzerland) and an online computer to perform the morphometric analyzes. Goblet cells were identified as PAS positive cells in the bronchiolar epithelium and counted manually in ten randomly chosen bronchioles. Counts were performed at 200× magnification and were expressed as cells per millimeter length of airway wall [13]. Bronchiolar wall thicknesses were measured in ten randomly chosen, non-coincident fields at a magnification of 400× across a total area of 0.6 mm. ASM thicknesses brown layer were measured in ten non-coincident fields of randomly chosen bronchioles, at a magnification of 400× across a total area of 0.6 mm. Blood vessels in the bronchiolar

chorion were counted manually in tissue sections stained with HE in ten randomly chosen, non-coincident fields at magnification of 400× across a total area of 0.6 mm². For evaluation of the presence of collagen in the bronchiolar chorion, we determined the surface density of collagen fibers by optical density in the image analysis (Leica QWin software; Leica Microsystems Image Solutions, Cambridge, UK). The thresholds for collagen fibers were established for each slide after enhancing the contrast to a point at which the fibers were easily identified as red bands. Ten randomly chosen, non-coincident fields were measured for each group, at 400× across a total area of 0.6 mm².

2.6. Cytokine assays

Enzyme-linked immunosorbent assays (ELISA) were used to determine the levels of vascular endothelial growth factor (VEGF), interleukin 1-beta (IL1- β) and IL-4. Tissue samples (0.1 g) were homogenized (Ultra-Turrax T8, IKA-Werke, Staufen, Germany) in 1.5 ml of RPMI 1640 (Sigma, St. Louis, MO). After centrifugation, the supernatants were stored at –70 °C until the assays were analyzed. The cytokine levels were determined according to the manufacturer's instructions (BD Pharmingen). Sensitivities were > 10 pg/ml.

2.7. Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (SD). Differences between groups were tested by one-way analysis of variance (ANOVA) and the Newman-Keuls post-hoc test. For differences between two groups, the Student's *t* test was used. A 5% level of significance was chosen to denote significant differences between the means. Results were analyzed using the GraphPad Prism 4.0 statistic program (GraphPad Software Inc., San Diego, California, USA).

3. Results

3.1. Number of *S. v.* larvae recovered from the lungs during the course of the infection

Lung maceration showed infective larvae on the 1st day post infection, and increasing dramatically to reach a peak on the 3rd day. Infections were almost resolved by day 7 and reached zero by the end of the experimental period. However, rat lungs that were treated with dexamethasone showed a high number of larvae throughout the experimental period, starting on the 1st day, to reach a peak on day 3. After day 5, the larvae numbers reduced, but still remained increased ($P < 0.05$); (Fig. 1). No larvae were recovered from the control groups.

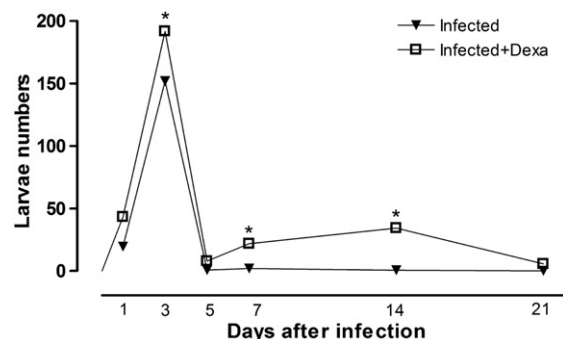


Fig. 1. Number of larvae recovered after infection with *S. venezuelensis* and treatment with dexamethasone (Dexa). The lungs were tweezed in saline and maintained at 27 °C for 2 h. Lungs were centrifuged, and the pellet was examined under light microscopy at 50× magnification for detection of larvae. Data are expressed as mean $\pm$ SD. * $P < 0.05$. Infected vs. Infected + Dexa.

3.2. Dexamethasone treatment reduced the airway wall remodeling induced by *S. v.* larvae migration through the lungs

S. v. larvae migration throughout the lungs induced a significant amount of airway wall remodeling and epithelial goblet cell hyperplasia and increased bronchiolar wall thickness. This was characterized by edema, congestion, a hypertrophied ASM layer and collagen deposition. Dexamethasone reduced most of these findings in infected-treated rats.

Bronchiolar goblet cells were hyperplastic, showing disruption and shedding of cells with marked mucus production in infected, untreated rats. This process started on day 3, peaked on day 5, remained increased until on day 14. However, treated-rats showed goblet cell hyperplasia values similar to the infected, untreated rats until the 3rd day, followed by an important reduction on the 5th day and ultimately maintained values less than 1% until the end of the experiment. Values were significant on all days, except for days 3 and 21, when compared with infected, untreated rats ($P<0.001$); (Fig. 2). Bronchiolar wall thicknesses were increased in those rats inoculated with *S. v.* larvae, starting on day 3, reaching a peak on day 5 and reducing gradually to control values after day 14. Treated-rats showed increased bronchiolar wall thickness on days 3 and 5, although reduced in relation to infected, untreated rats. After day 7, no increased bronchiolar wall thickness was observed in these rats. Significance values were determined in comparison to the infected, untreated mean values ($P<0.001$); (Fig. 3). New blood vessels were observed in the bronchiolar chorion during *S. v.* infection. They were augmented through days 3 to 7 of the experiment and after that, returned to control values. Dexamethasone treatment significantly reduced these findings during infection, especially on days 3 and 5 of the experiment. Significance values were determined in comparison to the infected, untreated mean values ($P<0.05$); (Fig. 4). The

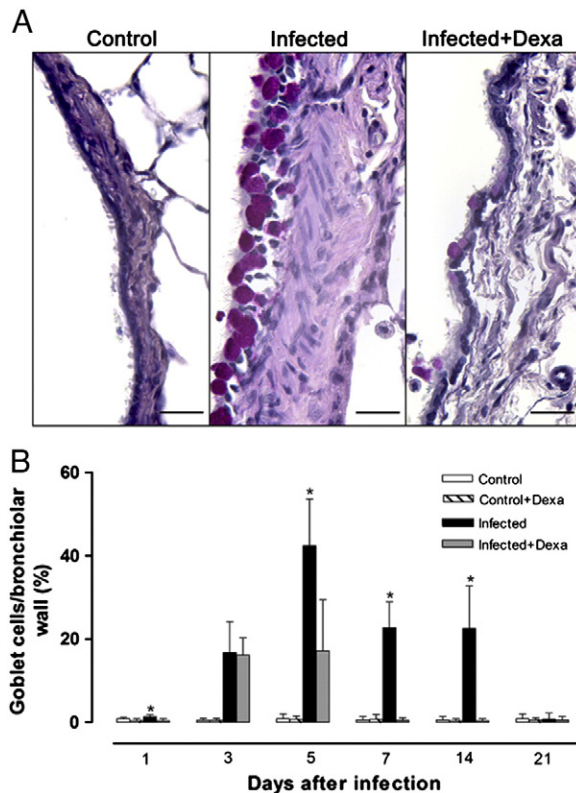


Fig. 2. A) Goblet cells in bronchiolar epithelium after infection with *S. venezuelensis* and treatment with dexamethasone (Dexa). Representative PAS-stained 5- μ m sections of Control, Infected and Infected + Dexa groups on day 5. Magnification of panels is 400 $\times$. B) Percentage of goblet cells with mucus production in bronchiolar epithelium. Data are expressed as mean $\pm$ SD. $P^* < 0.001$, $^{\#}P < 0.05$, $^{**}P < 0.01$. Infected vs. Infected + Dexa.

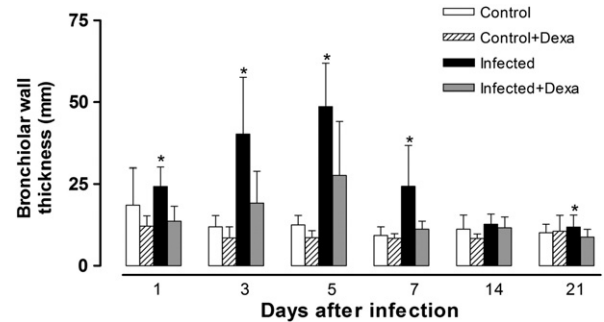


Fig. 3. Bar graphs showing the bronchiolar wall thickness after infection with *S. venezuelensis* and treatment with dexamethasone (Dexa). Data are expressed as mean $\pm$ SD. $P^* < 0.001$, $^{\#}P < 0.05$, $^{**}P < 0.01$. Infected vs. Infected + Dexa.

hypertrophic bronchiolar ASM layer was an important finding during *S. v.* infection, especially on day 5. After day 7, the values had returned to basal levels. Dexamethasone treatment inhibited the hypertrophy of the ASM layer observed on day 5 in the infected, untreated rats. Significance values were determined in comparison to the infected, untreated mean values ($P<0.05$); (Fig. 5). Collagen fibers showed a significant increase in infected rats after day 3 and reached their peak on day 5. Although exhibiting gradually smaller values, collagen fibers were slightly elevated in relation to the other groups until the end of the experiment. On the contrary, no augmentation of collagen fibers was observed in treated-infected rats. Significance values were determined in comparison to the mean values of the infected, untreated rats ($P<0.05$); (Fig. 6).

3.3. Dexamethasone treatment reduced cytokine levels during *S. v.* larvae migration through the lungs

VEGF increased during the course of the experimental period, starting on day 1, and reached a peak on day 5, remained increased until on day 21. Our results showed an important inhibition of VEGF levels starting on the 5th day in treated-rats, drastically reduced on the last experimental days. Significance values were determined in comparison to the mean values of the infected, untreated rats ($P<0.05$); (Fig. 7A). Pulmonary levels of IL-4 production was augmented at all intervals analyzed, starting on day 3, progressively increasing and reached a peak at day 14. On the contrary, dexamethasone treatment showed a significant inhibition of the IL-4 levels. Significance values were determined in comparison to the mean values of the infected, untreated rats ($P<0.05$); (Fig. 7B). Levels of IL1- β showed a significant increase throughout days 3 and 5. After day 7 the levels gradually decreased until reaching basal values on day 21. Dexamethasone treatment significantly reduced the levels of IL1- β , although they still showed a small peak at day 3. Significance

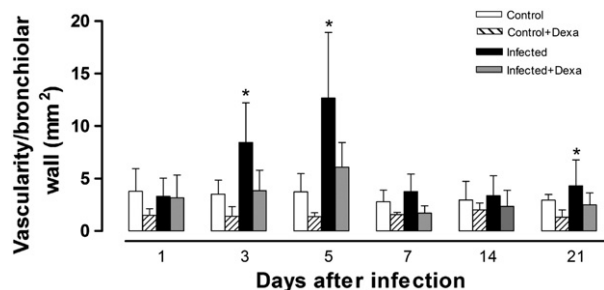


Fig. 4. Bar graphs showing vascularity in the bronchiolar wall after infection with *S. venezuelensis* and treatment with dexamethasone (Dexa). The blood vessels in the peribronchiolar were counted. Data are expressed as mean $\pm$ SD. $P^* < 0.001$, $^{\#}P < 0.05$, $^{**}P < 0.01$. Infected vs. Infected + Dexa.

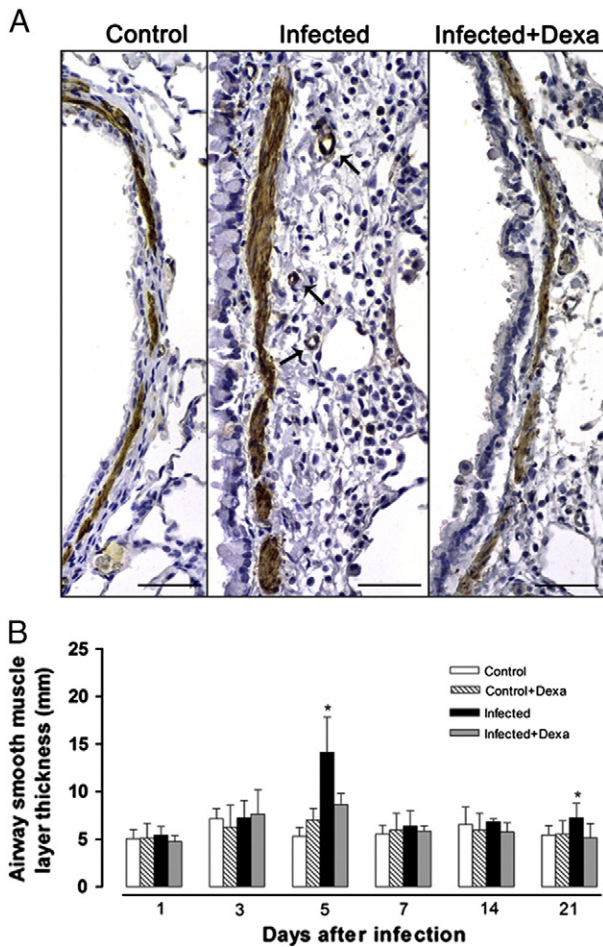


Fig. 5. A) Smooth muscle layer in the bronchiolar wall after infection with *S. venezuelensis* and treatment with dexamethasone (Dexa). Representative immunohistochemistry to alpha-actin (5- μ m sections) of Control, Infected and Infected + Dexa groups on day 5. Note the presence of vessels (arrows). Magnification of panels is 200 $\times$. B) Smooth muscle layer thickness in the bronchiolar wall. Data are expressed as mean $\pm$ SD. * P <0.001, # P <0.05, ** P <0.01. Infected vs. Infected + Dexa.

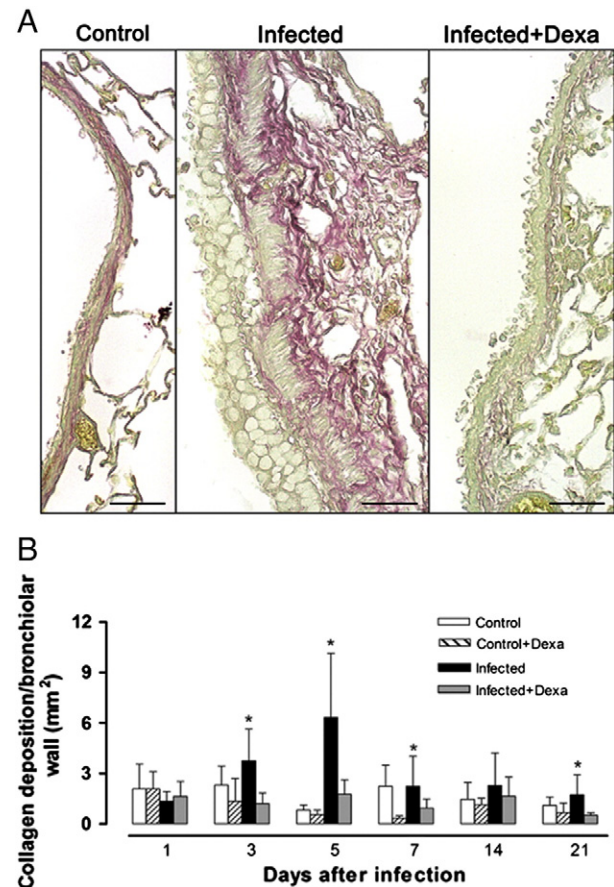


Fig. 6. A) Collagen fibers in the bronchiolar wall after infection with *S. venezuelensis* and treatment with dexamethasone (Dexa). Representative picro-sirius red stain (5- μ m sections) of Control, Infected and Infected + Dexa groups on day 5. Magnification of panels is 200 $\times$. B) Collagen fiber densitometry (%) in the bronchiolar wall. Data are expressed as mean $\pm$ SD. * P <0.001, # P <0.05, ** P <0.01. Infected vs. Infected + Dexa.

values were determined in comparison to the mean values of the infected, untreated rats (P <0.05); (Fig. 7C).

4. Discussion

Our data showed an airway remodeling in bronchiolar wall during *S. venezuelensis* larvae migration. Herein, we demonstrated that this passage can promote a significant amount of airway remodeling characterized by goblet cell hyperplasia and increased thickness of the bronchiolar wall with edema, congestion, neovascularization, collagen deposition and an enlarged ASM layer. These characteristics are the same as those described for asthmatic patients [14,15].

There are a number of similarities between the immune response against helminths and asthma. In asthma, the bronchial inflammation is orchestrated by Th2 inflammatory profile, with elevated levels of IgE and increase of eosinophil numbers, which results in airway remodeling with increase of vascularity, muscular hypertrophy and airway wall thickening [16,17]. Studies have suggested that a high prevalence of helminthic infection would predispose to an allergic reaction. Helminths can stimulate the production of IgE antibody against their own antigens, which sensitizes the mast cells, increasing the allergic process [18,19]. The inverse association has been shown, suggesting that helminthic infection have a protective role in the development of asthma. The excess of IgE stimulated by helminthic infection can suppress the allergic response by producing mast-cell saturation and

inhibition of IgE synthesis [20]. An explanation of these divergences is that the relation of asthma with helminthic infection could vary according to kind of parasites [21]. In this study, we speculate that during the passage of parasite by lung, the larvae break into alveoli promoting the direct inflammatory response, and it is possible that it could play an important role in the development of asthma; however, asthma is characterized by repeated episodes of allergic inflammation of the airway walls, whereas, in our model, just one infective cycle of *S. v.* was sufficient to promote airway remodeling.

In our study, bronchiolar remodeling was transitory and was initiated by the larvae migration throughout the lungs. Inflammatory cells, cytokines and growth factor mediators may be implicated in the mechanisms [22,23]. VEGF, expressed by many cell types including epithelial cells, inflammatory cells and smooth muscle cells during inflammatory processes [24,25], was greatly increased, and its peak coincided with the highest bronchiolar wall thickness. It has primarily been implicated in vascular alterations, especially submucosal edema and congestion of neovessels. In fact, VEGF is a potent permeability factor with angiogenic proprieties [26–28]. Interestingly, VEGF levels and vascularity in the bronchial wall did not follow the same pattern in our analyses. We think that this discrepancy helps to distinguish that the increased bronchial wall vascularity coincided with most of larvae passage through the airways, wherever higher VEGF levels could represent also the inflammatory aspects observed into the lungs at the 7th day of the pulmonary cycle of the infection [12].

On the other hand, IL1- β and IL-4 seemed to contribute to goblet cell hyperplasia and hypersecretion of mucus though synthesis

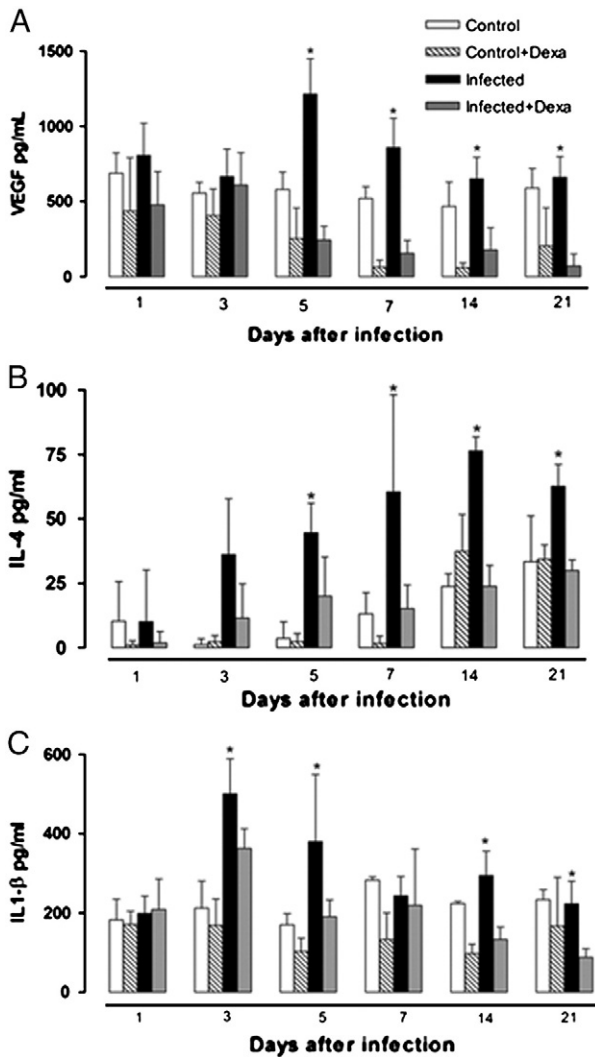


Fig. 7. Bar graphs showing cytokines in the lung after infection with *S. venezuelensis* and treatment with dexamethasone (Dexa). A) Levels of Vascular Endothelial Growth Factor (VEGF). B) Levels of interleukin 4 (IL-4). C) Levels of IL-1 β . Data are expressed as mean $\pm$ SD. * $P < 0.001$, # $P < 0.05$, ** $P < 0.01$. Infected vs. Infected + Dexa.

augmentation, a release of mucus glycoprotein and MUC5AC gene secretion in bronchial epithelial cells [22,29,30]. IL-1 β peaked during the highest rates of larvae migration, suggesting this passage may be directly implicated in its induction. In contrast, IL-4 showed progressive augmentation until day 14. Another interesting finding was the augmented ASM layer, observed exclusively on day 5, which showed a complete reversion during the remainder of the experimental period. ASM remodeling is associated with an enlarged smooth muscle mass involving hyperplasia and hypertrophy and changes in the phenotype of the muscle that facilitates proliferative, synthetic and contractile functions. Numerous factors, such as inflammatory mediators, cytokines, growth factors, and ECM proteins could be involved [24,31]. It is likely associated with the *S.v.* infection airway hyperresponsiveness described previously in rats [19]. The exact mechanisms underlying these alterations deserve further investigation, but some studies have suggested that IL-4 could play an important role through induction of a mast cell and airway smooth muscle interaction [32]. Surprisingly, IL-4 levels in control and control + dexa groups increase gradually in the course of experiment. This observation needs further investigation. Finally, the Th2 lymphocytes, mast cells and eosinophils, as well as structural cells, have been linked to the airway fibrotic process through the production of profibrotic cytokines [33,34]. Among them, TGF- β is the most potent profibrotic

cytokine and has been shown to play an important role in airway remodeling in asthmatic patients [35,36]. Although not evaluated herein, we speculate that it might be involved in bronchiolar remodeling post *S.v.* infection through increased submucosal collagen deposition via inflammatory cells. Interestingly, collagen deposition was the unique factor that remained altered at the end of the experiment, indicating that although in small proportions, modifications to the bronchiolar walls can remain after *S.v.* infection.

Corticosteroids have been used as a potent anti-inflammatory drug, especially in asthma [37,38], through reduction of most of the inflammatory features of the Th2 response [39]. Previous studies in our laboratory have shown that *S.v.* infection treated with dexamethasone caused an increase in the alveolar hemorrhage, impeded granuloma formation in the lungs, and reduced blood leukocytosis and inflammatory cell migration to target organs (lungs and intestines) [12,40]. In this study, we administered the dexamethasone just to observe if its effects into the airway remodeling could be similar to that described in asthma, since dexamethasone is commonly used in the treatment of asthmatic patients. However, is not easy to identify punctually the mechanism of dexamethasone action during the parasitic infection cycle. Recently, we observed (data not published yet) that probably, dexamethasone could have a role in increasing larvae numbers through promoting parasite moulting by the production of ecdysteroid-like molecules, hormones that control moulting in female helminths. Unfortunately, these data could not be proved yet, but these studies have shown that it could be one of the mechanisms which dexamethasone treatment could promote parasitic dissemination and hyperinfection. In fact, others studies have implicated dexamethasone treatment in the parasitic dissemination to others organs [41,42], but the exact mechanisms remain to be clarified.

Our data suggest that dexamethasone could interfere in the process of airway remodeling through inhibition of inflammatory mediators involved. Some studies have shown that corticosteroids can suppress VEGF production in airway smooth muscle cells, inhibiting the increased microvascular permeability, formation of edema and angiogenesis [43,44]. It also reduced IL-1 β and IL-4 levels, which inhibited goblet cell hyperplasia. However, effective inhibition of goblet cell hyperplasia occurred only after day 5, which suggests that other mechanisms can be involved in this process. Corticosteroids have been related to the increase the expression of the IL-1 receptor antagonist in the airway epithelial cells [45,46] and could act directly on submucosal gland cells by reducing the expression of mucin genes, such as MUC5 [47,48]. Moreover, corticosteroids have been described to reduce smooth muscle cell proliferation, ASM actin expression and contractile function through attenuation of mRNA translation and enhancement of protein degradation [49–51]. In this model, collagen deposition was reduced significantly. A possible explanation is that the dexamethasone action on eosinophil and TGF- β 1 expression. Glucocorticoids reduce circulating eosinophils through suppression of eosinophil maturation by inhibiting the production and release of IL-5 from cells within the bone marrow [52].

In conclusion, the present study showed, for the first time to our knowledge, that *S. v.* larvae passage in the lungs promotes a substantial amount of airway remodeling, although it is almost completely reversible. It could be compatible with a defense mechanism, directly related to the presence of the parasites in the lower airways. Moreover, bronchial wall remodeling during the cycle life of helminths can be relevant in endemic areas where they can occur simultaneously with asthma, maybe adding deleterious effects in the airways, especially in children. Dexamethasone is known to inhibit inflammation and immune response and can effectively inhibit this process. Future studies to assess the effects of dexamethasone on airway remodeling would be of great interest to enhance the understanding of the mechanisms behind the structural alteration occurring in the airway walls during *S.v.* infection in rats and the possible relationship of other helminths with pulmonary cycle and allergic diseases, such as asthma.

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