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Histopathologic evaluation, anesthetic protocol, and physiological parameters for an experimental Balb/c mouse model of asthma

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Abstract

Asthma is known to induce airway remodeling, which subsequently promotes pulmonary dysfunction. The aim of this study was to present data on the management, physiological parameters, and anesthetic procedure for using a Balb/c mice asthma model in research. The study was approved by the Ethics Committee on Animal Use of Pontifícia Universidade Católica do Paraná. The animals (n = 32) were randomly divided into four groups: Control-A, ovalbumin (OVA)-A, Control-B, and OVA-B. The OVA groups were sensitized with an intraperitoneal injection of a suspension containing 10 µg of OVA on days 0, 2, 4, 7, 9 and 10, and airways were challenged by intratracheal instillation of a 20 µg OVA suspension on days 15, 18, and 21. On these same days, the control groups received saline. On day 22 (Control-A and OVA-A groups) and on day 29 (Control-B and OVA-B groups), the animals were euthanized in order to investigate the efficiency of the protocol using histopathological examination. Detailed information on the anesthetic protocol and the monitoring of physiological parameters are presented. Results demonstrated that the OVA-A and OVA-B groups exhibited increased inflammatory infiltrate, muscle and epithelial thickening, epithelial desquamation, goblet cell metaplasia, and collagen deposition in comparison with their respective control groups ($p < 0.001$). In conclusion, the studied experimental Balb/c mouse model of asthma proved to be effective as it caused the lung remodeling observed in the asthma inflammatory process. Moreover, anesthetic and physiological parameters, scarcely described, are demonstrated here.

Introduction

Asthma is a chronic inflammatory disease of the airways characterized by recurrent and variable episodes of airflow obstruction and bronchial hyperresponsiveness [1]. The disease affects the quality of life of 300 million individuals of all ages and is considered a problem worldwide [2].

The inflammatory process within the airways of patients with asthma induces significant structural changes in the respiratory architecture, which subsequently promotes pulmonary dysfunction [3,4]. Therefore, it is important to investigate the pathophysiologic events responsible for the development of asthma. To this end, the use of pre-clinical models in research is important.

Different animal species have been used in the study of the inflammatory processes in the airways such as mice [5], rats [6], guinea pigs [7], rabbits [8], and horses [9]. In particular, the Balb/c mice used in our study are isogenic animals that have been used frequently. These mice have the ability to develop a strong TH2 type immune response, with specific IgE levels, after allergic sensitization and challenge [10] to ovalbumin (OVA), which is the current gold standard in small animal models for inducing an immune reaction [1].

In this study, we demonstrate a protocol of an experimental model of asthma using adjuvant-free OVA in Balb/c mice, with the purpose of investigating the airway remodeling that occurs in the asthma process. Moreover, it is our aim to present detailed data regarding the management, physiological parameters, and anesthetic procedure for using Balb/c mice as a model for asthma in research, which are not often described in research papers.

Materials and methods

Animals

Thirty-two male Balb/c mice, aged 6 to 8 weeks, were obtained from the central animal facility at Pontifícia Universidade Católica do Paraná (PUCPR) and evaluated in the present study. They were maintained in collective cages (up to four animals per cage) with dimensions of 30 cm in length, 20 cm in width, and 13 cm in height. They remained under a light/dark cycle of 12/12h, receiving a diet of CR1 Nuvilab (Quimtia, Colombo, Brazil) and water *ad libitum*. Their bedding was made of pine wood shavings (Inbrasfama, São José dos Pinhais, Brazil), which was changed twice weekly.

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Experimental design

Since the mice do not naturally develop asthma, it is necessary to sensitive their immune system, and thus, challenge their airways to elicit pulmonary inflammatory remodeling. In the present protocol, OVA was chosen as the allergen and airways were challenged through a tracheal instillation under anesthetia.

The 32 animals were randomnly divided into four groups: Control-A (n = 8), OVA-A (n = 8), Control-B (n = 8) and OVA-B (n = 8). The OVA groups were sensitized with an intraperitoneal injection (IP) of a 100 µL suspension containing 10 µg of OVA (Sigma-Aldrich, São Paulo, Brazil) on days 0, 2, 4, 7, 9 and 10, and airways were challenged by an intratracheal instillation (IT) of a 20 µL suspension with 20 µg of OVA on days 15, 18 and 21. On these same days, the control groups received the same volume of saline. On day 22, animals of the Control-A and OVA-A groups were euthanized to ensure the efficiency of the protocol. The mice of the Control-B and OVA-B groups were euthanized on day 29 to verify the persistence and intensity of inflammation over eight days from their last contact with the allergen. This model of asthma induction was adapted from Xisto *et al.* [11] (Figure 1).

Anesthetic protocol for intratracheal injection

To ensure standardization of our experimental model, the intratracheal procedures were performed by the same researcher (a veterinarian doctor). The animals were anesthetized by intraperitoneal injections of 5% ketamine hydrochloride (Vetanarcol; König, Santana de Parnaíba, Brazil) (100 mg/kg) and 2% xylazine hydrochloride (Anasedan; Vetbrands, Paulínia, Brazil) (10 mg/kg). The animals placed in a dorsal decubitus position with their necks extended in a heated bed (Master Digital SA-300; Champion Eletronics, Porto Alegre, Brazil) during anesthesia to avoid a sudden drop in body temperature due to the administered drugs. An incision of approximately 1 cm was made in the middle of the ventral cervical region, allowing tracheal exposition after the divulsion of the cervical muscles. The trachea was identified and then the sterile solution containing OVA (or only saline) was administered using a 0.3 mL ultrafine insulin syringe (Becton Dickson, Curitiba, Brazil). Furthermore, intratracheal instillation was timed, from the initial incision until completion of the surgery. This procedure was the same for all the allergenic challenges.

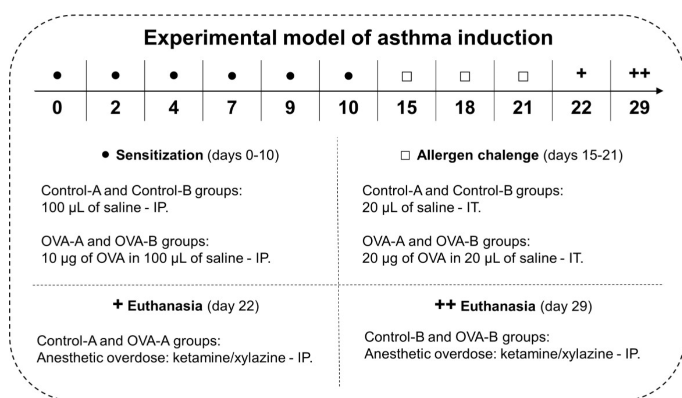

Figure 1. Protocol for experimental model of asthma induction using Balb/c mice. The animals in the OVA groups were sensitized with 10 µg of OVA on days 0, 2, 4, 7, 9, and with 10 and 20 µg the allergen challenge on days 15, 18, and 21. The control groups received only saline in these procedures. The Control-A and OVA- A groups were euthanized on day 22, while the Control-B and OVA-B groups were sacrificed on day 29

0-29: Days of procedure; IP: Intraperitoneal injection; IT: Intratracheal injection.

Weight monitoring and physiological parameters

In all procedures, animal weight was monitored on a digital scale (Toledo do Brasil, Pinhais, Brazil). In addition, on days 22 and 29, moments before euthanasia, the following cardiopulmonary parameters were measured using an multiparametric monitor (Instramed, Porto Alegre, Brazil): Blood Oxygen Saturation (SPO2), Respiratory Rate (RR), and Heart Rate (HR).

Lungs collection and histopathologic evaluation

Euthanasia was performed in an ethical and painless manner. The procedure involved individualized overdoses of anesthesia (400 mg/kg of 5% ketamine hydrochloride with 50 mg/kg of 2% xylazine) by intraperitoneal administration. The mice were maintained in a supine position for the thoracotomies, which were performed to collect their lungs. The lungs were washed with saline to remove blood present on the surface, fixed in 10% buffered formalin (Biotec, Pinhais, Brazil) for 48 hours, and then transferred to cassettes for histopathological evaluation.

To analyze pulmonary inflammation, lung morphology, inflammatory cells, smooth muscle thickening, and epithelium desquamation, histological sections were stained with Hematoxylin-Eosin (H&E); Periodic Acid-Schiff (PAS) was used for the observation of goblet cells and mucus production; Masson's Trichrome (MT) was used for collagen deposition.

Images of 30 bronchioles (15 from each lung) were randomly selected from the histological sections using the Olympus DP25 digital camera (Olympus, São Paulo, Brazil) coupled to an Olympus CX41 optical microscope (Olympus, São Paulo, Brazil). The images were acquired by the DP2-BSW software (Olympus, São Paulo, Brazil) in 200× and 400× magnifications. The histopathological evaluation was performed by two researchers, using a semi-quantitative assessment with scoring system. The scoring system was adapted from Sun *et al.* [12] and ranged from 0 to 3, where 0 represents healthy bronchioles (0%: preserved architecture and collagen deposition according to normality), 1 indicates mild inflammation (1-33%: small number of inflammatory cells, mild peribronchiolar collagen deposition, discrete epithelial and muscle thickening, and slight epithelial desquamation), 2 demonstrates the presence of moderate inflammation (34-66%: regions with a considerable number of inflammatory cells, an increased presence of smooth muscle, epithelium and collagen surrounding the bronchioles, and presence of desquamated epithelial cells in the bronchiole lumen), and 3 indicates intense-inflammation lung remodeling with significant structural changes (67-100%: significant structural changes, diffuse peribronchiolar region with intense inflammatory cells and accentuated collagen deposition, significant thickening of the epithelial and smooth muscle layers, and frequent epithelium desquamation). The mucus production was estimated by the presence of positive cells in mucin-stained lung sections from the superposition of a circle divided into eight regions with congruent central angles, as adapted Firinci *et al.* [13], and categorized according to the scoring system mentioned above.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test for the comparison between groups (Control-A vs. OVA-A; Control-B vs. OVA-B; Control-A vs. Control-B; OVA-A vs. OVA-B), and a paired t-test to compare the initial and final animal weights (mean ± SD). Analysis was performed using the software GraphPad version 5.03 for Windows. Values of $p < 0.05$ were considered to be statistically significant.

Results

Survival

We submit to protocol 37 animals, of which five died because of hypoxemia after intratracheal instillation during the allergen-challenge step of the procedure. As a result, only 32 mice participated in the study. This represented a survival rate of 86.5% for this murine model of asthma.

Anesthetic protocol

The mean duration for the anesthetic procedures used for both the intratracheal instillations and euthanasia are demonstrated in Table 1.

Weight monitoring and physiological parameters

The animals were weighed at all steps of the asthma induction model until the day of euthanasia. There was a significant weight increase observed in the Control-A (31.18 ± 3.0 vs. 29.27 ± 2.45, $p = 0.015$), OVA-A (31.82 ± 2.14 vs. 28.55 ± 2.02, $p = 0.0003$), and OVA-B (33.21 ± 2.29 vs. 30.71 ± 2.78, $p = 0.001$) groups. There was no difference in weight observed in the group Control-B (32.73 ± 3.41 vs. 31.82 ± 3.09, $p = 0.488$).

For the HR analysis, which was obtained at the end of the study (with the animals under anesthesia), we observed that, usually, HR was lower in the asthmatics versus controls. Furthermore, HR was significantly lower in the OVA- B group compared with the Control-B group (151.6 ± 27.91 vs. 227.0 ± 28.12, p=0.019). There was no difference in the RR or SPO2 between any of the groups (Table 2).

Lung histopathological evaluation

The histopathological lung tissue modifications resulted from the allergic asthma model using OVA. The presence of airway remodeling was observed in both the OVA-A and OVA-B groups. Little or no tissue changes were observed in animals of the Control-A and Control-B groups (Figure 2). These results demonstrated that inflammation was enhanced (in all criteria analyzed) when experimental groups were compared ($p < 0.001$) with their respective controls (Figure 3).

Discussion

In this study, we used the Balb/c mouse as our animal model and an asthma-induction protocol using adjuvant-free OVA as the allergen. Histopathological modifications were evident at 24 hours and 8 days following the last airway challenge, thus proving that this method is an effective asthma model for use in different studies of lung inflammation.

It should be noted that the absence of an adjuvant did not interfere with the process of lung remodeling. Previously, a study showed that an adjuvant of Al(OH)3 in conjunction with OVA was used to potentiate pulmonary inflammation [5]. However, in other studies, it was shown that an adjuvant-free asthma- induction protocol triggered an adjuvant-independent inflammatory response [11] and, that its use may actually interfere with the results of studies on therapeutic strategies [14].

The methodology of the present study could provoke structural lung modifications, which could lead to the development of human asthma. To assess the impact of the histological findings, we analyzed a considerable number of bronchioles using two evaluators. In literature, there are different practices used in histological evaluation, but no standards have been reported on the adequate number of bronchioles that should be evaluated. Indeed, Sun *et al.* [12] used 5 cuts per animal, Jeon et al. analyzed 5 bronchioles [15], and Goodwin *et al.* [16] studied 4 airways per animal. Thus, our analysis was performed efficiently and allowed for the demonstration of pulmonary remodeling during the initial stage of the disease at two different moments in time. In fact, results were observed on two separate days of euthanasia and demonstrated the presence of diffuse inflammation and remodeling.

The present protocol emphasizes the changes that occur in the initial period of asthma development, and differs from most which investigates the later phases of the disease [13,17]. It is important to understand the immune response at an early stage of asthma so that therapeutic treatments for, specifically, the young population can be developed.

The observed pulmonary alterations correspond to a TH2-type immunological response that is highly prevalent in cases of asthma [18]. During this response, the interleukins IL-4, IL-5, and IL-13 are involved and responsible for the histopathological changes of marked

Table 1. Mean time for the first, second, and third intratracheal instillations (IT) for four different groups (controls and asthmatics; 8 animals/group) applied in a mouse model of asthma induction. In addition, the table shows, for each of the procedures, the time spent for conducting the allergenic challenge (AChal), time for the anesthetic effect to start (StTime), and the total anesthetic period until mice recovery (AnPer), in a protocol using intraperitoneal ketamine and xylazine. Data are expressed as hours:minutes:seconds (h:min:sec). The duration of the euthanasia procedure is also shown. Mean and standard deviation for all groups was calculated

Groups	AChal	1st IT StTime	AnPer	AChal	2nd IT StTime	AnPer	AChal	3rd IT StTime	AnPer	EUT StTime
Control-A	00:07:11	00:03:00	01:03:24	00:04:38	00:03:05	00:59:55	00:07:27	00:03:16	00:51:55	00:04:05
OVA-A	00:03:22	00:04:38	01:08:05	00:02:42	00:03:00	00:58:12	00:05:05	00:02:33	01:06:27	00:02:49
Control-B	00:03:50	00:03:40	00:33:40	00:05:40	00:04:20	01:02:07	00:06:00	00:08:00	00:57:07	00:04:20
OVA-B	00:03:43	00:02:51	01:01:17	00:04:12	00:03:12	00:58:27	00:06:36	00:05:18	01:16:48	00:02:51
Mean	00:04:31	00:03:32	00:56:36	00:04:18	00:03:24	00:59:40	00:06:17	00:04:46	01:03:04	00:03:31
SD	00:01:47	00:00:48	00:15:33	00:01:13	00:00:37	00:01:48	00:00:59	00:02:26	00:10:57	00:00:48

1st IT: First intratracheal instillation; 2nd IT: Second intratracheal instillation; 3rd IT: Third intratracheal instillation; EUT: Euthanasia; AChal: Allergenic challenge; StTime: Start time of the anesthetic effect; AnPer: Anesthesia period; SD: Standard Deviation

Table 2. Cardiopulmonary parameters evaluated in Balb/c mice, 24 hours (Control-A and OVA-A) and 8 days (Control-B and OVA-B) after the last allergen challenge, at the end of the study

Groups	SPO2	RR	HR
Control-A	75.91 ± 10.50	140.7 ± 19.42	199.7 ± 33.95
Control-B	78.50 ± 5.99	134.0 ± 19.22	227.0 ± 28.12*
OVA-A	77.70 ± 12.38	131.2 ± 31.60	179.4 ± 46.36
OVA-B	79.29 ± 6.53	121.1 ± 18.58	151.6 ± 27.91*

SPO2: Blood oxygen saturation; RR: Respiratory rate; HR: Heart rate; $^{*}p = 0.019$ Control- B vs. OVA-B. *Significant statistical difference

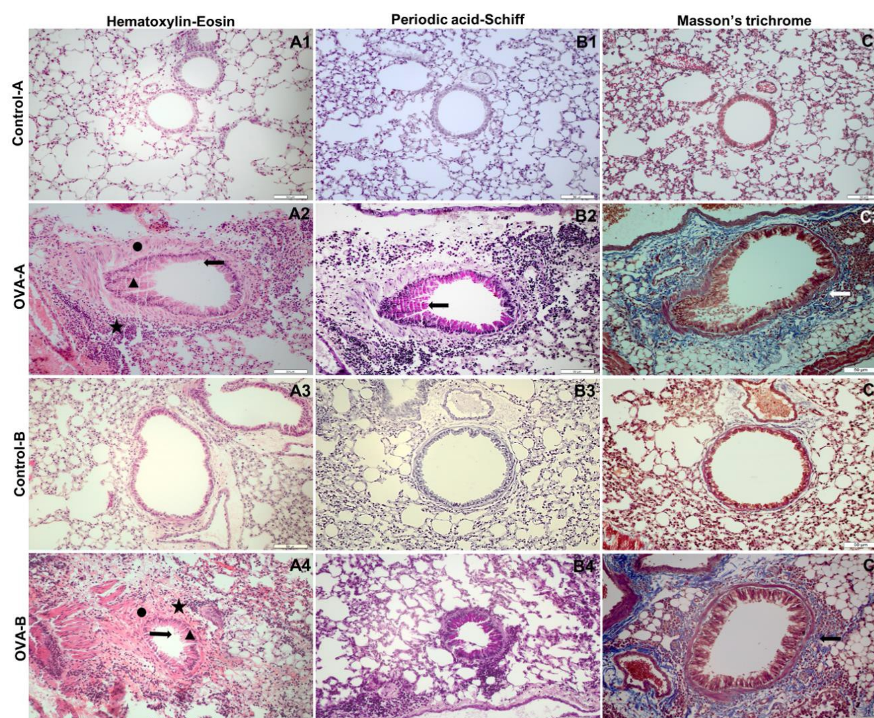

Figure 2. Histological evaluation in lungs of Balb/c mice stained with H&E (A), PAS (B), and Masson's trichrome (C), 24 h (Control-A and OVA-A) and eight days (Control-B and OVA-B) after the last allergen challenge. A. Intense presence of inflammation in the bronchial region of animals in OVA-A and OVA-B groups: inflammatory infiltration (star), thickening of muscle layer (circle), thickening of the epithelium (triangle) and epithelium desquamation (arrow). B. Evidence of mucus production and goblet cells (arrow) in the groups exposed to ovalbumin. C. Collagen deposition (arrow) increased in the peribronchial area of groups with asthma. Representative images of the Control-A (A1, B1, and C1), OVA-A (A2, B2, and C2), Control-B (A3, B3, and C3) and OVA-B groups (A4, B4, and C4). Magnification 200×

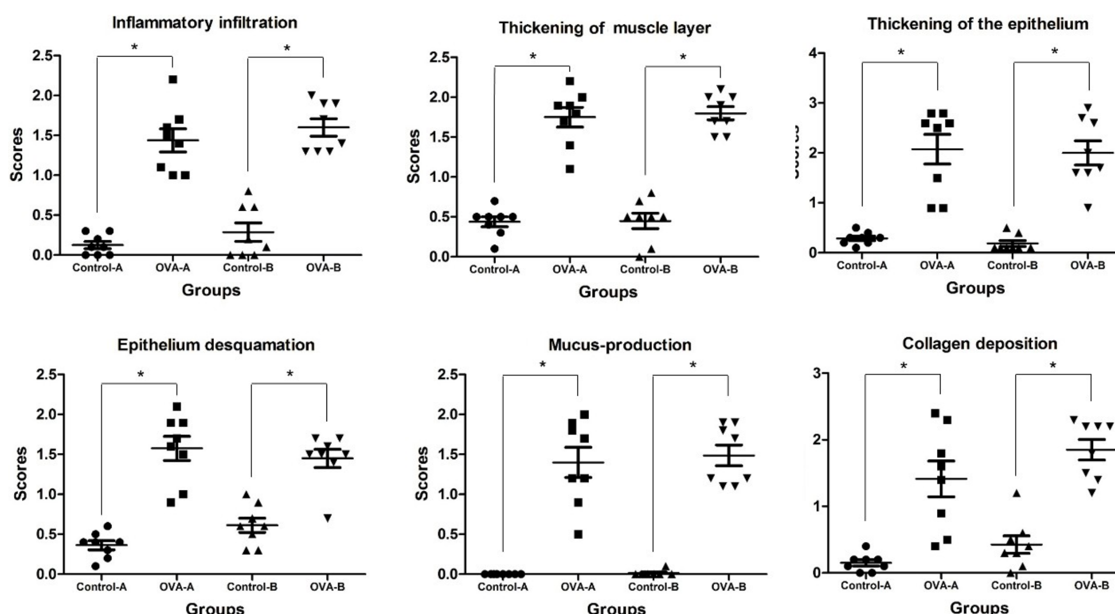

Figure 3. Histopathological changes in the regions of the bronchioles of Balb/c mice, 24 hours (Control-A and OVA-A) and 8 days (Control-B and OVA-B) after the last allergen challenge. OVA-A and OVA-B groups exhibited lung remodeling, as a result of inflammatory infiltration, thickening of muscle layer, thickening of the epithelium, epithelium desquamation, mucus production by goblet cells and collagen deposition. These measurements of lung remodeling in the OVA-A and OVA-B groups were statistically different (*p < 0.001) vs. Control-A and Control- B, respectively

inflammatory infiltrate, muscle and epithelial thickening, epithelial desquamation, goblet cell metaplasia, and the collagen deposition [19].

The route of allergen administration to provoke an immune system reaction varies and may influence the results. Here, sensitization was performed via intraperitoneal injection and the allergenic challenge occurred by intratracheal instillation to ensure the direct access of the allergen to the airways. Other protocols challenge the airways by aerosolization [5,20] or intranasal instillation [21], which can result in a greater quantity of OVA being administered or even lost. Intratracheal instillation allows a strict control and standardization of the volume

of administered OVA and, consequently, a reduction in the cost of the procedure. Moreover, the inflammatory response is more directly focused on the lungs in this procedure.

Studies investigating asthma usually describe the main objectives without describing the associated protocols in detail. The detailed description of the procedures performed in a protocol is important for future studies to efficiently reproduce useful methodology. The detailed information on anesthetic protocols and monitoring of physiological parameters are presented in the present study.

In terms of survival rate, it was slightly higher than the 83.33% achieved by Xisto *et al.* [11]. This result is not surprising as the animals used in our study underwent consecutive anesthetic procedures for intratracheal instillations and each procedure was performed by an anesthetist veterinarian that could identify respiratory difficulties and rapidly initiate artificial ventilation and oxygen therapy using a facial mask whenever necessary, thus preventing the death of animals.

We monitored the administration of drugs used for anesthesia (a combination of ketamine hydrochloride and xylazine hydrochloride) and noted an average time of 03:48 (min: sec) for the animals to demonstrate an absence of signs and reflexes. After the intratracheal procedures, the mice were kept in a warm bed to avoid hypothermia until they had recovered from anesthesia as observed by the restoration of normal parameters. The mean duration of the anesthetic effect for all the groups at the three moments of allergen challenge was 00:59:47 (h: min: sec). In this study, we used ketamine hydrochloride, a general anesthetic that is rapidly distributed throughout the body where it remains for up to two hours, and xylazine hydrochloride, which causes sedation and analgesia to complement the observed effects of ketamine. Xylazine hydrochloride remains in the animal for a varied 23 to 50 min. The combination of these drugs allows for experiments with fast recovery times, efficient pain control and safe anesthetic procedures [22].

Data on the physiological parameters (HR, RR, and SPO2) in the study of asthma are scarce in the literature, especially in preclinical models, probably because they are difficult to evaluate. According to the Brazilian Society of Pneumology and Tisiology [23], patients with 1) mild and moderate asthma present with an SPO2: > 95%, RR: normal or increased, HR: ≤ 110%; 2) severe asthma present with SPO2: from 91 to 95%, RR: increased, HR: > 110; and 3) very severe asthma present with SPO2: ≤ 90%, RR: increased, HR: > 140 or bradycardia. This information has not been described in mice. In the present study, asthmatic mice had a lower HR than controls, which was significant in the group euthanized eight days after the last airways challenge. In children up to five years of age, the progression of alveolar hypoxia due to imbalance in the ventilation-perfusion relationship may lead to changes in consciousness, cardiovascular response with initial tachycardia, and subsequent bradycardia and hypotension with consequent shock and cardiorespiratory arrest [24]. In this context, we believe that these are important considerations to help assess disease progression and the effectiveness of new therapies.

Finally, all animals gained weight during the study, including the Control-B group, without any differences between groups; this was also observed by Xisto *et al.* [11] and Burburan *et al.* [25].

Conclusion

In conclusion, the experimental asthma-inducing protocol used in this study caused the airway remodeling characteristic of the diffuse inflammatory process of asthma. The presented model proved to be effective, contributing to the research of new therapeutic modalities as well as the pathophysiological events of asthma.

Acknowledgments

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Ethical approval

The study was approved by the Ethics Committee on Animal Use (724) of the PUCPR (Curitiba, Brazil). All procedures performed in this study were in accordance with the ethical standards of the institution.

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