



Protective effect of early prenatal stress on the induction of asthma in adult mice: Sex-specific differences



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HIGHLIGHTS

- Prenatal stress early in pregnancy programmed behavioral responses in adult mice.
- Prenatally stressed females presented attenuated allergic response in adulthood.
- The effects of prenatal stress on asthma development depend on the stage of pregnancy.

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ABSTRACT

Adversities faced during the prenatal period can be related to the onset of diseases in adulthood. However, little is known about the effects on the respiratory system. This study aimed to evaluate the effects of prenatal stress in two different time-points during pregnancy on pulmonary function and on the inflammatory profile of mice exposed to an asthma model. Male and female BALB/c mice were divided into 3 groups: control (CON), prenatal stress from the second week of pregnancy (PNS1) and prenatal stress on the last week of pregnancy (PNS2). Both PNS1 and PNS2 pregnant females were submitted to restraint stress. As adults, fear/anxiety behaviors were assessed, and animals were subjected to an asthma model induced by ovalbumin. Pulmonary function, inflammatory parameters in bronchoalveolar lavage (BAL) and histology were evaluated. There was a significant decrease in the number of entries and time spent in the central quadrant on the open field test for the PNS1 animals. Females (PNS1) showed improved pulmonary function (airway resistance, tissue damping and pulmonary elastance), significant increase in the percentage of neutrophils and lymphocytes and a decrease in eosinophils when compared to controls. There was a significant decrease in inflammatory cytokines in BAL of both males (IL-5 and IL-13) and females (IL-4, IL-5 and IL-13) from PNS1 and PNS2 when compared to the CON group. Prenatal stress starting from the beginning of pregnancy reduces the impact of asthma development in adult female mice, showing an improved pulmonary function and a lower inflammatory response in the lungs.

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1. Introduction

Adversities faced during the prenatal period might be implicated in fetal programming and in the development of diseases in adulthood, as explained by The Developmental Origins Theory [1,2]. Stress during the prenatal period can cause alterations in different organs and systems [3], such as changes in fear/anxiety behaviors [4], dysfunctions related to diabetes mellitus type 2 [5], predisposition to cardiovascular diseases [6] and impairment in the development and functioning of structures of the Central Nervous System (CNS) [7], among others.

The development of the respiratory system in rodents starts early in gestation (around day 9) [8], which allows changes in the prenatal period to program the development of this system [9]. This seems to be related to epigenetic modifications of genes encoding proteins that are involved in the regulation/activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to long-lasting effects [10]. Regardless of the precise mechanism, besides behavioral changes such as increased anxiety, prenatal stress can induce an enhancement in airway hyperreactivity, with augmented production of mucus in adult animals. Also, exposure to prenatal stress could lead to an increment in the recruitment of eosinophils to the lungs, which is a key feature in asthma [9]. On the other hand, the stimulatory role of glucocorticoids on prenatal lung development is well-known [11,12]. Hence, it is possible that exposure to continuous stress in this period could produce an increase in glucocorticoid secretion and result in protective effects on

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the development of the respiratory system. However, the complexity of long-term effects of prenatal stress on asthma is still poorly studied.

Asthma is a highly prevalent chronic disease [13] that presents scarcely known multifactorial (genetic and environmental) origins, which are related to atopy [14,15]. Thus, considering the impact of asthma in worldwide populations, it is important to study the mechanisms and effects that premature variations early in life, such as the ones caused by stress, can have on aspects related to the disease physiopathology.

Moreover, several studies have shown that the effects of prenatal stress may be different in males and females [16–19]. Also, it is known that asthma is more prevalent among males [20] and that early life stress effects can affect the response to asthma in a sex-dependent manner [21], which highlights the importance of studying the influence of sex on the interaction between prenatal programming and respiratory diseases.

Thus, considering the importance of the prenatal period in the development of numerous systems and the lack of studies on long-term effects of stress during this period on pulmonary development and on the expression of respiratory diseases, we evaluated the consequences of stress in different time-points of the gestational period and their connection to asthma development in adulthood, including the analysis of potential sex differences. Therefore, the goal of this study was to evaluate outcomes of prenatal stress induced by restraint in two different time-points of pregnancy on pulmonary function and on inflammatory profile of male and female mice exposed to a model of asthma induced by ovalbumin. Also, as a control of long-term programming effects induced by alterations in the prenatal environment, anxiety-like behavior (open field test) and HPA axis response (corticosterone measurement) were evaluated.

2. Material and methods

2.1. Animals

BALB/c mice were acquired from the Center of Biological and Experimental Models - CeMBE from PUCRS and maintained in a vivarium under light/dark cycles of 12 h with free access to food and water. All animal procedures followed the guidelines described in the *Guide for the Care and Use of Laboratory Animals* [22], and the study was previously approved under registry number 11/00270 by the University ethics committee in animal use (CEUA).

2.2. Experimental design

Adult BALB/c primiparous females (6–8 weeks) were monitored for their estrous cycle and then were mated to a male (6–8 weeks). Pregnancy was identified by the presence of a vaginal plug, and females were then separated individually in boxes and divided in three groups: control, prenatal stress 1 (PNS1) and prenatal stress 2 (PNS2). After birth, the litter was culled to 5–7 animals and pups were weighed on days 1, 10 and 21, when they were weaned. Males and females were separated in boxes with a maximum of 5 animals. Mice were kept until adulthood for experiments without any type of intervention except for routine cleaning. Fear/anxiety behavior analyses were performed between days 50–54 through the open field test. On the 56th day of life, males and females were sensitized by two subcutaneous (s.c) injections with ovalbumin (OVA) on days 1 and 7. Then, an intranasal (i.n) challenge was performed on days 14, 15 and 16. On the 17th day, animals were anesthetized and a pulmonary function test was performed for the evaluation of airway resistance, tissue damping and elastance. Bronchoalveolar lavage (BAL) was collected and used for total and differential cell count and for the evaluation of inflammatory cytokines. After animals were euthanized, the left lung was removed and processed for histological analysis. The experimental design as described is shown on Fig. 1.

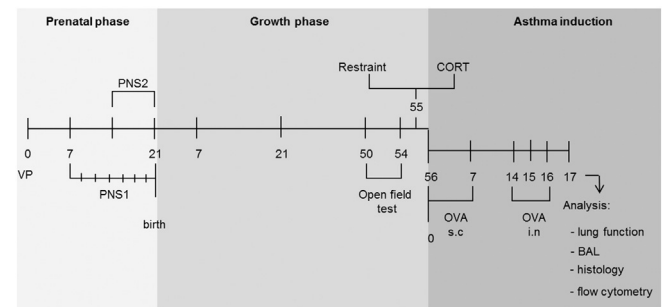


Fig. 1. Experimental design used in the study. VP: vaginal plug; PNS1: prenatal stress from the second week of pregnancy; PNS2: prenatal stress at the last week; Restraint: 30 min restraint stress; CORT: corticosterone measurement; OVA: ovalbumin; s.c: subcutaneous; i.n: intranasal; BAL: bronchoalveolar lavage.

2.3. Maternal exposure to stress

Stress induction was performed by restraint in a closed cylinder (Insight, Brazil) for 30 min. Pregnant females from PNS1 group were subjected to stress early in the gestational period, from the 8th day of pregnancy until birth, every other day. Females from PNS2 group were subjected to stress later in the gestational period (from day 14 to 21), everyday during the last week of pregnancy. The control group did not undergo any intervention during the gestational period.

2.4. Behavioral analysis

Behavioral analysis was performed as a control of long-term programming effects induced by alterations in the prenatal environment. For that, an open field test was performed to evaluate fear/anxiety behavioral responses in adulthood (50–54 days), as previously described [23]. Briefly, this test consists in a squared black box/arena (45 cm × 45 cm × 15 cm of height) divided in central quadrant and periphery. The test consists of placing the animal in a corner of the open field (randomly chosen) and recording the activity of the mouse on video for 10 min. Two specific behaviors were determined: number of entries in the central quadrant and time spent in the central quadrant (measured in seconds). Video analyses of the behaviors recorded were performed using ANY-maze software (version 4.7).

2.5. Corticosterone measurement

In order to evaluate the HPA axis response to a second hit stress, the circulating corticosterone level was measured. When animals (males and females) reached adulthood they were subdivided in two groups (i) decapitated under basal conditions or (ii) subjected to a new restraint stress (as previously described) of 30 min and immediately decapitated for blood collection. Blood was then centrifuged at 13,000 rpm and serum was stored at -80°C for further analysis. Corticosterone concentration was measured by ELISA (Corticosterone EIA Kit, Cayman Chemical Company, USA) according to the manufacturer. Final results are expressed as pg/mL. The lower limit of detection was 30 pg/mL.

2.6. Asthma induction protocol

On the 56th day of life, males and females were again divided into groups: OVA and phosphate-buffered saline (PBS). Animals on the OVA group were sensitized with ovalbumin (grade VI; Sigma-Aldrich). Two subcutaneous injections (s.c) were performed with 20 μg of OVA diluted in total volume of 200 μL (PBS) per animal (days 1 and 7). Animals were sedated with isoflurane in an anesthetic chamber and then an intranasal challenge was performed, with 100 μg of OVA in 50 μL of PBS applied on each animal (days 14, 15 and 16). Animals on

the PBS group were exposed to the same protocol, but saline solution (PBS) was administered instead.

2.7. Pulmonary function

One day after the last intranasal challenge (day 17), animals were anesthetized with xylazine (0.2 mg/g body weight) and ketamine (0.4 mg/g body weight). Following anesthesia, a pulmonary function test using the forced oscillation technique was performed after cannulation of the trachea by tracheostomy while the animal was on a ventilator (Harvard Minivent, Hugo Sachs Elektronik, USA) for 5 min, with a PEEP of 3 cmH₂O, frequency of 450 (breaths/min) and volume of 10 mL/kg of body weight before the beginning of the test. Data were analyzed on a specific software (WinLung, Hungary) for the evaluation of airway resistance, tissue damping (which represents the resistance of the upper airways) and pulmonary elastance (which assesses the rigidity of lung parenchyma) [24].

2.8. Bronchoalveolar lavage (BAL)

BAL was performed right after the pulmonary function test, through the previously inserted tracheal cannula. 1 mL of PBS was injected in the cannula and then the same volume was aspirated twice. After the BAL procedure was completed, animals were euthanized via exsanguination by cardiac puncture. Each BAL sample was centrifuged (2000 rpm for 4 min) and the precipitate was suspended with 350 μ L of PBS. Total cell count (TCC) and calculation of cell viability in the suspension were carried out in all samples by trypan blue exclusion method on a Neubauer chamber (Boeco, Germany). Slides for differential cytology were prepared with 80 μ L from the suspension in a cytospin centrifuge (FANEM, São Paulo, Mod. 218), 500 rpm for 5 min. The slides were fixed in air and stained with Diff-Quick stain (Laborclin Ltda, Brazil). Cell morphology was assessed. Cell types as observed under optical microscope are expressed as percentage of a total of 400 cells counted.

2.9. Histological analyses

Lungs were connected to a gravity column (20 cm) and filled with 10% formaldehyde. Slides were prepared from lung tissue paraffin blocks and stained with hematoxylin-eosin (H&E, Soltan, Brazil) and Alcian Blue (InLab, Brazil) for the analysis of inflammatory infiltrate and presence of goblet cells in the lower airways, respectively. A qualitative analysis of the slides was performed by an examiner that was blinded to the experimental groups.

2.10. Inflammatory cytokine analyses

Considering the classic immune response in asthma, the following cytokines were measured: Th1 response (IFN- γ), Th2 response (IL-4 and IL-5) and the anti-inflammatory (regulatory) IL-10. Cytokines were analyzed on the supernatant from the BAL, on a flow cytometer through the cytometric bead array (CBA) kit. IL-4, IL-5, IL-10, IL-13 and INF- γ were analyzed by simultaneous detection with CBA Flex Set kit (BD Pharmingen, USA) for mice. Briefly, 50 μ L of each sample was mixed to 50 μ L of mixed capture beads, followed by incubation for 2 h at room temperature in the dark. After that, 50 μ L of detection reagent PE was added and another round of incubation for 2 h in the dark was performed. Samples were washed once and resuspended in 300 μ L of washing buffer prior to FACSCalibur cytometer reading (BD Biosciences, USA). Data were analyzed on FCAP Array 3.0 software (BD Biosciences, USA). Standard curves were generated for each cytokine based on the mixed standard of cytokines supplied in the kit. The concentration of each cytokine on the supernatant of BAL samples was determined by interpolation in the corresponding standard curve.

2.11. Statistical analysis

Data were analyzed on GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Data normality was assessed through Shapiro-Wilk test and expressed as mean and standard error of the mean, shown on bar graphs. A one-way analysis of variance (ANOVA), followed by the Tukey post-hoc test, was chosen in order to identify differences between experimental groups (CON, PNS1 and PNS2), since only comparisons of factor “group” in OVA-sensitized animals were used to evaluate stress-induced effects. In all cases, significance level was established when $p \leq 0.05$.

3. Results

3.1. Prenatal stress effect on animal weight

There was no significant difference in animal weight on the three time points measured: birth (CON: 1.69 ± 0.12 ; PNS1: 1.80 ± 0.09 ; PNS2: 1.59 ± 0.09), 10 days old (CON: 6.92 ± 0.20 ; PNS1: 6.54 ± 0.15 ; PNS2: 6.93 ± 0.30) and weaning, males (CON: 10.72 ± 0.28 ; PNS1: 10.30 ± 0.43 ; PNS2: 10.86 ± 0.26) and females (CON: 10.23 ± 0.35 ; PNS1: 10.31 ± 0.52 ; PNS2: 10.72 ± 0.41).

3.2. Prenatal stress effect on fear/anxiety behavior and corticosterone secretion

Fear/anxiety behavior was estimated based on the number of entries and the time spent on the central quadrant of the open field (Fig. 2). There was a significant decrease ($p < 0.05$) in the number of entries and in the time spent on the central quadrant for both males and females from PNS1 OVA group when compared to control OVA. These data indicate that prenatal stress experienced since the second week of pregnancy has a long-term effect on fear/anxiety behavior. Also, when analyzing corticosterone (pg/mL) response to stress (difference between basal and 30-min restraint) both adult PNS1 males ($\Delta = 4448$) and females ($\Delta = 4972$) presented and higher response when compared to the control group (males: $\Delta = 3166$ and females: $\Delta = 3292$). PNS2 males ($\Delta = 3639$) and females ($\Delta = 2542$) did not show the same response. These data corroborate behavioral findings that

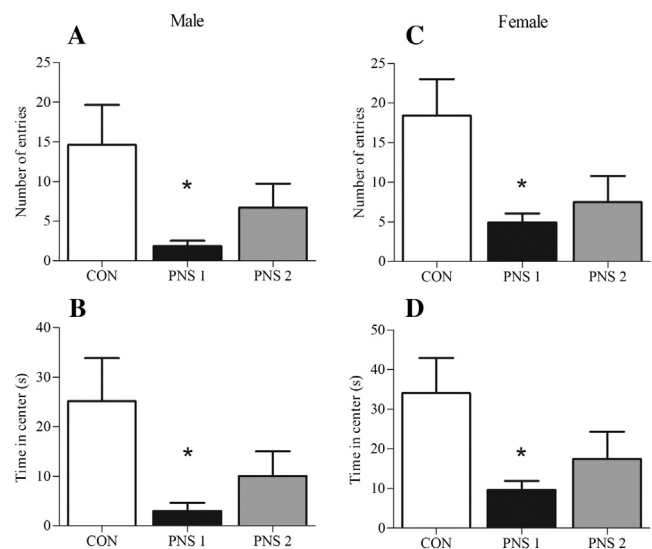


Fig. 2. Effects of prenatal stress on fear/anxiety behaviors during the open field test. (A) Number of entries on the central quadrant, and (B) time spent on the center for males. (C) Number of entries on the central quadrant, and (D) time spent on the center for females. Values are shown as mean and standard error of the mean. * $p < 0.05$ when compared to control group, $n = 12$ –14 animals per group. CON: control; PNS1: prenatal stress induced from the second week of pregnancy until birth; PNS2: prenatal stress induced on the last week of pregnancy.

suggest an increased response to stress in animals that experience prenatal stress early in pregnancy.

3.3. Prenatal stress effect on pulmonary function in animals exposed to the OVA protocol

For pulmonary function assessment, airway resistance, tissue damping and pulmonary elastance were analyzed (Fig. 3). Males on PNS1 OVA group showed an increase in tissue damping ($p < 0.05$) and in lung elastance ($p < 0.01$) when compared to control OVA (Fig. 3B and C). PNS1 OVA females, on the other hand, showed a decrease in airway resistance ($p < 0.05$), in tissue damping ($p < 0.05$) and in lung elastance ($p < 0.01$) when compared to control OVA (Fig. 3D, E and F). No significant differences were found for PNS2 OVA animals.

3.4. Prenatal stress effect on BAL cell profile and on lung histology of animals exposed to OVA

There was an increase ($p < 0.05$) in the absolute values of neutrophils in PNS2 OVA males when compared to control OVA. On the other hand, no significant differences were found among males of the two stressed groups (PNS1 OVA and PNS2 OVA) when compared to the control OVA group in terms of absolute values of TCC and of macrophages, lymphocytes and eosinophils (Table 1). Also, on males, the percentage values of differential cytology did not differ between groups (Fig. 4A). Lung histology shows an increase in peribronchovascular infiltrate from animals that were subjected to the OVA protocol when compared to the saline (PBS) group (Fig. 4B). Alcian Blue dye showed an increase in goblet cells between animals in the OVA group when compared to animals in the PBS group (data not shown). In females, on the other hand, there was a significant increase ($p < 0.05$) in absolute

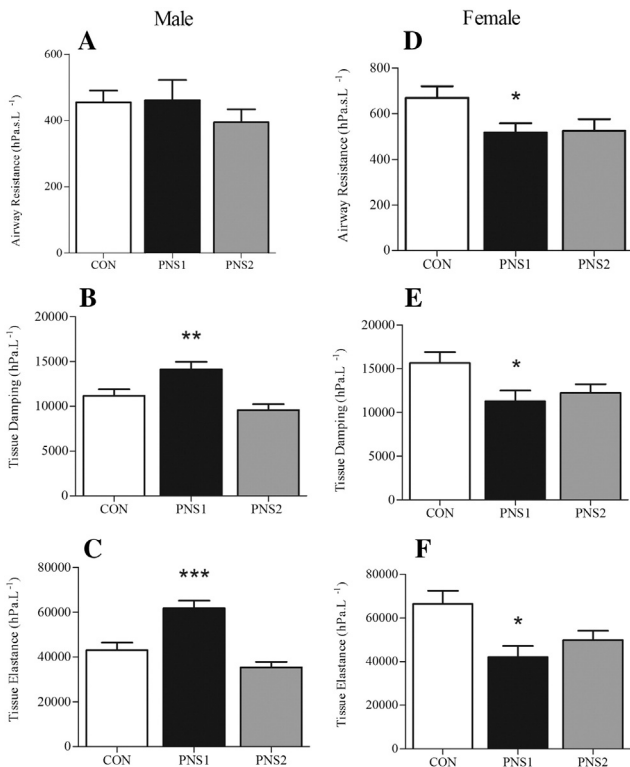


Fig. 3. Pulmonary function parameters in animals exposed to an ovalbumin-induced asthma protocol. Males: (A) airway resistance; (B) tissue damping; (C) pulmonary elastance. Females: (D) airway resistance; (E) tissue damping; (F) pulmonary elastance. Values are shown as mean and standard error of the mean. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared to control group, $n = 6-9$ animals per group. CON: control; PNS1: prenatal stress induced from the second week of pregnancy until birth; PNS2: prenatal stress induced on the last week of pregnancy.

Table 1
Differential cell count from bronchoalveolar lavage (absolute values).

	Male						Female					
	CON			PNS1			CON			PNS1		
	PBS	OVA	TCC	PBS	OVA	TCC	PBS	OVA	TCC	PBS	OVA	TCC
($\times 10^4$ /mL)												
TCC	4.3 $\pm$ 1.3	48.3 $\pm$ 11.0	4.3 $\pm$ 1.3	3.0 $\pm$ 0.8	54.5 $\pm$ 10.8	4.3 $\pm$ 1.3	2.8 $\pm$ 0.5	50.2 $\pm$ 7.5	2.6 $\pm$ 0.4	4.9 $\pm$ 1.5	67.1 $\pm$ 13.0	4.9 $\pm$ 1.5
Macrophages	4.0 $\pm$ 1.2	13.0 $\pm$ 3.7	4.0 $\pm$ 1.2	2.4 $\pm$ 0.6	13.4 $\pm$ 3.1	4.0 $\pm$ 1.2	2.1 $\pm$ 0.4	10.1 $\pm$ 2.0	2.3 $\pm$ 0.4	3.4 $\pm$ 0.8	11.8 $\pm$ 1.5	3.4 $\pm$ 0.8
Neutrophils	0.3 $\pm$ 0.05	5.3 $\pm$ 0.5	0.3 $\pm$ 0.05	0.3 $\pm$ 0.1	3.9 $\pm$ 1.3	0.3 $\pm$ 0.05	0.7 $\pm$ 0.2	2.4 $\pm$ 0.7	0.21 $\pm$ 0.05	12.9 $\pm$ 3.7*	4.0 $\pm$ 0.4	0.9 $\pm$ 0.3
Lymphocytes	0.01 $\pm$ 0.0	2.6 $\pm$ 0.7	0.01 $\pm$ 0.0	0.12 $\pm$ 0.06	3.5 $\pm$ 0.7	0.01 $\pm$ 0.0	0.01 $\pm$ 0.00	1.8 $\pm$ 0.3	0.05 $\pm$ 0.02	4.3 $\pm$ 0.8	4.4 $\pm$ 1.6	0.6 $\pm$ 0.4
Eosinophils	0.01 $\pm$ 0.0	27.5 $\pm$ 7.3	0.01 $\pm$ 0.0	0.12 $\pm$ 0.09	33.7 $\pm$ 6.8	0.01 $\pm$ 0.0	0.01 $\pm$ 0.00	35.9 $\pm$ 5.5	0.02 $\pm$ 0.01	29.9 $\pm$ 5.6	47.0 $\pm$ 10.9	0.03 $\pm$ 0.02

Values are shown as mean and standard error of the mean. * $p < 0.05$ and ** $p < 0.01$ when compared to CON OVA group. TCC: total cell count; OVA: ovalbumin; PBS: phosphate buffer saline; CON: control; PNS1: prenatal stress from the second week of pregnancy until birth; PNS2: prenatal stress on the last week of pregnancy.

values for neutrophils and lymphocytes on PNS1 OVA group when compared to control OVA (Table 1). Differential cell counting pointed to a significant percentage increase in neutrophils and lymphocytes ($p < 0.01$) and a significant decrease in eosinophils ($p < 0.01$) when compared to control OVA (Fig. 4C). Lung histology in females showed a decrease in the recruitment of inflammatory cells on PNS1 OVA group when compared to control OVA (Fig. 4D). Alcian Blue staining also showed a clear decline in mucus production in PNS1 OVA group when compared to control OVA (data not shown).

3.5. Prenatal stress effect on inflammatory cytokines in BAL from animals exposed to OVA

There was a significant decrease in IL-5 and IL-13 secretion on BAL from males of PNS1 OVA ($p < 0.001$ and $p < 0.05$) and PNS2 OVA ($p < 0.05$ and $p < 0.01$) groups when compared to the control OVA group (Fig. 5B and C). INF- γ secretion was found reduced ($p < 0.05$) only in PNS1 OVA group when compared to control OVA (Fig. 5E). In females, there was a significant decline in IL-4 and IL-5 secretion on PNS1 OVA ($p < 0.05$ and $p < 0.001$) and PNS2 OVA ($p < 0.01$ and $p < 0.001$) groups (Fig. 5F and G). IL-13 secretion was reduced ($p < 0.001$) only on PNS1 OVA group, and IL-10 was decreased ($p < 0.01$) only on PNS2 OVA group when compared to control OVA.

4. Discussion

Asthma is a chronic disease that often manifests early, with important epigenetic mechanisms involved on its onset. These mechanisms are very complex and yet not very clear. Protective factors in the disease development have been extensively studied. Our data suggest that prenatal stress beginning in the second gestational week can reduce

the impact of asthma in mice at adult age, in a sex-dependent effect. Increasing evidence suggests that this effect may be related to the developmental origins of health and disease concept, as prenatal stress affects early environment leading to the development of physiological adaptive changes that become permanently programmed and impact later health [1,2].

Our findings indicate that females exposed to stress since the second week of pregnancy (PNS1 group) showed an improved pulmonary function and cell profile in the inflammatory response, which suggests a long-term protective effect of prenatal stress. Nevertheless, evidence in humans show that prenatal stress is connected to an increased risk of developing asthma [25], eczema and chronic obstructive pulmonary disease during childhood [21,26]. Still, there is little evidence from studies with prenatal stress and asthma in experimental models. Two studies have shown that prenatal stress increases the percentage of eosinophils in BAL and airway hyperresponsiveness in females in an asthma model where increasing doses of methacholine were used, but no other parameters of pulmonary function were measured [9,21].

We also show here that prenatal stress altered the inflammatory cytokine profile in BAL fluid, reducing inflammation in both males and females. This response, however, seems to be more pronounced in females that are exposed to stress since the second week of pregnancy (PNS1 group). Different results were found on another study in which prenatal stress increased inflammatory cytokine levels in females and decreased it in males [21]. Such alterations could be related to differences in the type of stress used, its intensity and the specific period of exposure during pregnancy [10]. It is possible that prenatal stress, when applied repeatedly and with moderate intensity and duration during early pulmonary development (which begins around the 9th day of pregnancy) [8], could modulate the development of the respiratory system in a distinct manner. Stress during this period

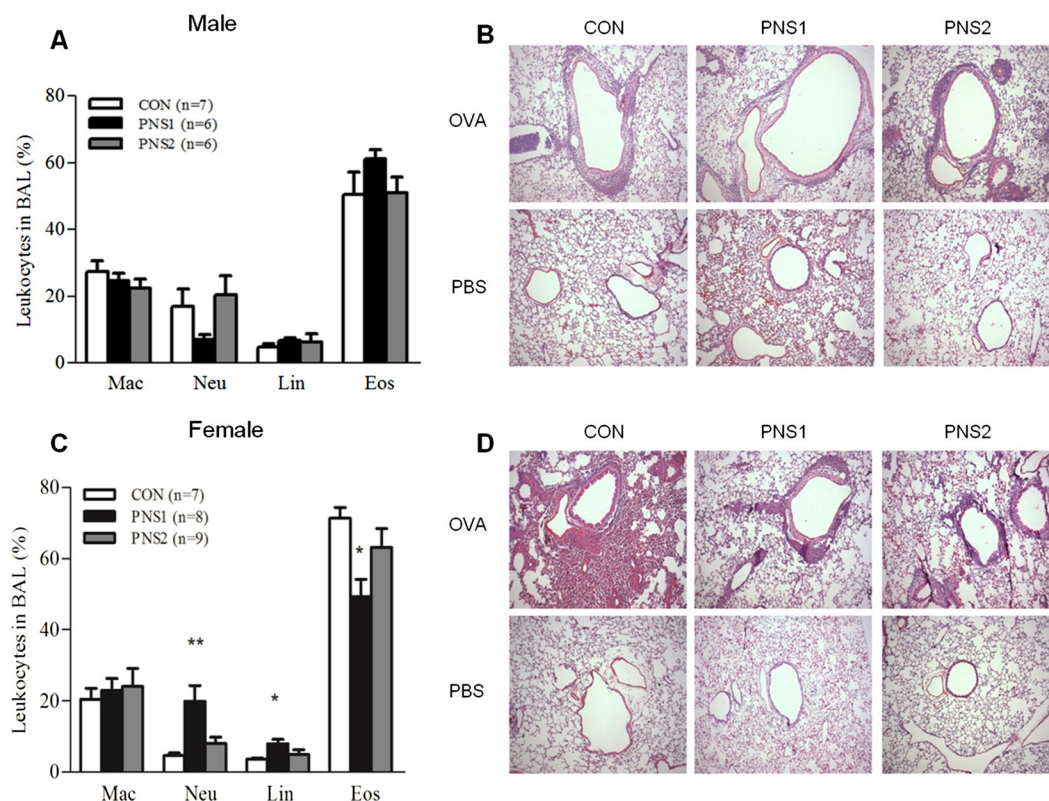


Fig. 4. Differential cell count in the bronchoalveolar lavage for (A) males and (C) females exposed to an ovalbumin-induced asthma protocol, stained with hematoxylin/eosin, and expressed as a percentage of macrophages (Mac), neutrophils (Neu), lymphocytes (Lin) and eosinophils (Eos). Values are shown as mean and standard error of the mean. * $p < 0.05$ and ** $p < 0.01$ when compared to control group. In (B) and (D), qualitative analysis of cellular infiltrate in histological sections of male and female lungs, respectively, when exposed to an asthma-induction protocol (OVA) or (PBS) and stained with hematoxylin/eosin. Photomicrographs were taken under an optical microscope, in a 200 $\times$ zoom. OVA: ovalbumin; PBS: phosphate buffer saline; CON: control; PNS1: prenatal stress induced from the second week of pregnancy until birth; PNS2: prenatal stress induced on the last week of pregnancy.

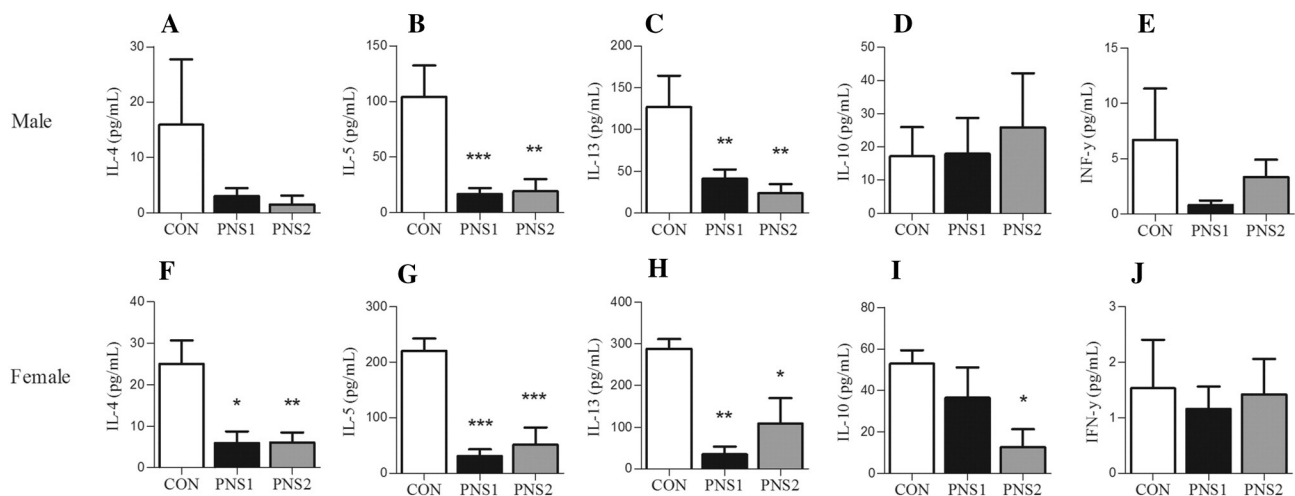


Fig. 5. Inflammatory cytokines on bronchoalveolar lavage (BAL) from animals that were exposed to a protocol of asthma induction with ovalbumin. Males: (A) IL-4; (B) IL-5; (C) IL-13; (D) IL-10; (E) INF-γ. Females: (F) IL-4; (G) IL-5; (H) IL-13; (I) IL-10; (J) INF-γ. Values are shown as mean and standard error of the mean. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared to control group, $n = 5-8$ animals per group. CON: control; PNS1: prenatal stress induced from the second week of pregnancy until birth; PNS2: prenatal stress induced on the last week of pregnancy.

could, for instance, induce a decline in the expression of the enzyme 11β -hydroxysteroid dehydrogenase type 2 (11β -HSD2), diminishing the enzymatic barrier against glucocorticoids and allowing the effects of this hormone in the maturation and development of the lungs in the fetuses [27]. Several evidences show that the use of exogenous therapy (corticosteroid therapy) improves lung maturation [28,29]. However, these effects, on long-term, depend on the stage of pregnancy in which the exposure to glucocorticoids occurs and on the sex of the developing fetus. Anyway, it seems to be well-established that effects of fetal programming induced by either glucocorticoids or by disruptions in nutrient supply or in maternal behavior are mediated by epigenetic mechanisms that are capable of programming the fetus for the rest of its life [11], which could help explain the protective effect of stress on the respiratory system response. Regardless of its precise origin, the results presented in this study demonstrate that prenatal stress improved lung function, decreased the percentage of eosinophils and reduced the production of BAL inflammatory interleukins in females.

Data shown here also reveals specific and distinct effects for males and females. Regarding pulmonary function, unlike females, males showed a worsening in tissue damping and elastance in response to stress. Studies in humans have previously shown that there is a connection between gender and several factors related to asthma, indicating that males have worse symptoms of the disease, such as eczema and wheezing [30,31]. Also, males have a wider variety of phenotypes related to the disease, including exposure to house dust (mites), pollen and others, causing an increased risk of exacerbations [30]. In our study, prenatal stress in males had no influence on the percentage of inflammatory cells on the BAL, while females showed a reduction in eosinophils. Studies have shown that the effect of programming and its underlying mechanisms are influenced in a sex-dependent manner [10,32]. Sex differences are widely studied in experimental models and are well-known, although the mechanisms involved in these differences remain to be better understood [33]. In this context, the protective effects of prenatal stress found only in females may suggest hormonal influences in the programming of the respiratory system. As a whole, apart from an increase in tissue damping and elastance, but without alterations in airway resistance, differential cytology, inflammatory cytokines and histology, data presented here for males does not seem to support a consistent long-term deleterious effect on the respiratory system. Still, the prenatal period and the intensity/duration of the stress seem to be decisive in generating these changes.

We have also shown that prenatal stress from the second week on (PNS1) caused an increased corticosterone response to stress and a

rise in fear/anxiety (number of entries and the time spent in the center on the open field test) both for males and females when compared to the control group. Prenatal stress and behavioral changes have been the focus of numerous studies since prenatal exposure to a stressor was associated to developmental delays, behavioral abnormalities and alterations in the functioning of several systems in adults [4,34,35]. These changes can be related to excess glucocorticoid secretion by the mother, that could induce structural and functional alterations in the fetal brain, which predisposes to increase in fear/anxiety and to learning deficits in males and females in addition to the deregulation of the HPA axis described previously [4,33]. On the other hand, prenatal stress on the last gestational week (PNS2) did not alter the hormonal and behavioral response to fear/anxiety in the offspring. However, previous studies have shown evidences that prenatal stress during this period could indeed alter fear/anxiety behavior in rats [36] and Swiss mice [35]. For our mouse line (BALB/c) and stress model, it is the first time to our knowledge that these effects were observed. Either way, data presented here indicates that the stress model from the second week on (PNS1) was capable of programming behavioral responses in adulthood.

Knowing that lung development in mice begins around the 8th–9th to the 16th gestational day and is divided in four stages (pseudoglandular, canicular, saccular and alveolar), as formerly described [8], this study aimed to evaluate prenatal stress in two distinct time-points during pregnancy (PNS1 and PNS2). Thus, stress on PNS1 group began on the second gestational week and was responsible for the most striking results in this study. In PNS2 group, stress was induced only during the last week of pregnancy, a widely used model in the literature. However, the alterations seen in the latter were not as significant for the outcomes evaluated. It is well-documented in the literature that stressful factors in the last week of pregnancy can lead to important alterations in other systems, including the CNS and behavioral regulatory mechanisms [36–38].

One limitation of this study is the lack of assessment of airway hyperreactivity (bronchial provocation with methacholine), which could not be performed due to the equipment used. However, other parameters were employed to evaluate pulmonary function, which allowed the identification of differences in the experimental model used.

5. Conclusions

In conclusion, data from our study points to a reduced impact of prenatal stress, from the second week of pregnancy, on asthma development

for females in adulthood, showing improved pulmonary function and lower allergic response in the lungs. The mechanisms underlying this protective effect are not clear, although sex differences seem to play an important role. Stress early in life is not free of long-term effects, but our findings seem to show that they are not always harmful.

Conflicts of interests

All authors declare that there are no conflicts of interest involved.

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