

# The putative role of ovary removal and progesterone when considering the effect of formaldehyde exposure on lung inflammation induced by ovalbumin

Adriana Lino-dos-Santos-Franco,<sup>I,II</sup> Renata Midori Amemiya,<sup>I</sup> Ana Paula Ligeiro de Oliveira,<sup>III</sup> Amílcar Sabino Damazo,<sup>V</sup> Ana Cristina Breithaupt-Faloppa,<sup>IV</sup> Luana Beatriz Vitoretti,<sup>I</sup> Beatriz Golegã Acceturi,<sup>I</sup> Wothan Tavares-de-Lima<sup>I</sup>

<sup>I</sup> Universidade de São Paulo, Institute of Biomedical Sciences, Department of Pharmacology, São Paulo/SP, Brazil. <sup>II</sup> Universidade de São Paulo, Faculty of Pharmaceutical Sciences, Department of Clinical and Toxicological Analyses, São Paulo/SP, Brazil. <sup>III</sup> Nove de Julho University, Department of Biophotonics, São Paulo/SP, Brazil. <sup>IV</sup> Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo/SP, Brazil. <sup>V</sup> Universidade de Cuiabá (UNIC), Faculty of Medical Sciences, Department of Basic Science in Health, Cuiabá/MT, Brazil.

**OBJECTIVE:** Formaldehyde exposure during the menstrual cycle is known to affect the course of allergic lung inflammation. Because our previous data demonstrated that formaldehyde combined with an ovariectomy reduced allergic lung inflammation, we investigated the putative role of ovary removal and progesterone treatment when considering the effect of formaldehyde on allergic lung inflammation.

**METHOD:** Ovariectomized rats and their matched controls were exposed to formaldehyde (1%, 3 days, 90 min/day) or vehicle, and immediately after exposure, the rats were sensitized to ovalbumin by a subcutaneous route. After 1 week, the rats received a booster by the same route, and after an additional week, the rats were challenged with ovalbumin (1%) by an aerosol route. The leukocyte numbers, interleukin-10 (IL-10) release, myeloperoxidase activity, vascular permeability, *ex vivo* tracheal reactivity to methacholine and mast cell degranulation were determined 24 h later.

**RESULTS:** Our results showed that previous exposure to formaldehyde in allergic rats decreased lung cell recruitment, tracheal reactivity, myeloperoxidase activity, vascular permeability and mast cell degranulation while increasing IL-10 levels. Ovariectomy only caused an additional reduction in tracheal reactivity without changing the other parameters studied. Progesterone treatment reversed the effects of formaldehyde exposure on *ex vivo* tracheal reactivity, cell influx into the lungs and mast cell degranulation.

**CONCLUSION:** In conclusion, our study revealed that formaldehyde and ovariectomy downregulated allergic lung inflammation by IL-10 release and mast cell degranulation. Progesterone treatment increased eosinophil recruitment and mast cell degranulation, which in turn may be responsible for tracheal hyperreactivity and allergic lung inflammation.

**KEYWORDS:** Formaldehyde Exposure; Progesterone; Lung inflammation; Tracheal reactivity; Mast cells; Interleukin-10.

Lino-dos-Santos-Franco A, Amemiya RM, Oliveira AP, Damazo AS, Breithaupt-Faloppa AC, Vitoretti LB, et al. The putative role of ovary removal and progesterone when considering the effect of formaldehyde exposure on lung inflammation induced by ovalbumin. *Clinics*. 2013;68(12):1528-1536.

Received for publication on May 10, 2013; First review completed on May 30, 2013; Accepted for publication on June 5, 2013

E-mail: adrilino@usp.br

Tel.: 55 11 3091-2197

## INTRODUCTION

Formaldehyde (FA) is a pollutant that is widely employed in many industries and in anatomy, pathology and histology

**Copyright** © 2013 CLINICS – This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

No potential conflict of interest was reported.

**DOI:** 10.6061/clinics/2013(12)09

laboratories (1). FA is also emitted in tobacco smoke, burning fuel, urea-FA foam insulation, cosmetics, solvents and domestic disinfectants (2). FA exposure causes irritation of the eyes and mucous membranes and induces airway inflammation. Many people are exposed to FA, and its role as a risk factor in the development of asthma is still controversial. In previous studies, we reported that both male and female rats developed lung inflammation when exposed to FA inhalation (3,4). Interestingly, ovary removal caused a decrease in the inflammatory response induced by FA exposure (4).

Clinical and experimental data have both demonstrated a putative role of female sex hormones (FSHs) in lung



inflammation (4–9). Progesterone has been suggested to mediate eosinophilia, airway hyperreactivity and interleukin (IL)-5 production in murine models of allergic lung inflammation (10). Moreover, progesterone skews the differentiation of naïve T helper lymphocytes toward the Th<sub>2</sub> lineage *in vitro* (11).

Studies have shown that estradiol and progesterone both exert pro- and anti-inflammatory effects on lung inflammation, depending on the nature of the inflammatory agent (allergic or non-allergic) (4,7,12,13). In this regard, our group has demonstrated that ovariectomy (OVx) reduces lung inflammation when an inflammatory agent is related to ovalbumin (OVA), the allergic stimulus. Moreover, this effect is reversed by estradiol but not by progesterone (5,7). Conversely, pre-treatment with estradiol or progesterone re-established the inflammatory response when the stimulus was related to FA exposure (a non-allergic stimulus) (4).

In other studies, we also demonstrated that pre-exposure to FA in OVA-sensitized and OVA-challenged male rats blunted the allergic lung response (14). Therefore, both FA exposure and ovary removal suppressed the development of an allergic lung response induced by OVA. Considering these earlier results, we investigated the role of ovary removal when considering the effect of FA on lung inflammation induced by OVA. Moreover, because we observed in earlier studies that progesterone had pro-inflammatory effects in rats submitted to FA inhalation and that its effects were worse than those observed in rats treated with estradiol, we decided to investigate the involvement of progesterone in leukocyte migration into the lung, *ex vivo* tracheal reactivity to methacholine (MCh), IL-10 release in the lung tissue and mast cell degranulation.

## ■ MATERIALS AND METHODS

### Animals

Female Wistar rats (160–180 g) were obtained from the Institute of Biomedical Sciences, University of São Paulo. The rats were housed in groups (5 rats per cage) in a light-and temperature-controlled room (12/12-h light-dark cycle, 21 ± 2°C) with free access to food and water. The experiments were approved by the Institutional Animal Care Committee.

### Ovariectomy (OVx)

Female rats were anesthetized with intraperitoneal ketamine-xylazine (Konig, São Paulo, Brazil) (100 and 20 mg/kg, respectively), and their ovaries were removed. Following the surgery, the rats received a single dose of Pentantibiotic® (Fort Dodge, IA) (570 mg/kg, intramuscular). Vaginal smears and uterine weight measurements were used to assess the effectiveness of the OVx. Rats that underwent a similar operation, but without ovary removal, were used as the sham-operated controls (Sham-OVx group). A non-manipulated group of rats (Naïve group) was used to obtain the basal parameters.

### Exposure to formaldehyde (FA)

Rats (5/chamber) were exposed to daily 90-min sessions of FA or vehicle (water + methanol) inhalation for 3 consecutive days (14). For this procedure, a standard glass chamber (20 l) coupled to an ultrasonic nebulizer device (Icel®, Brazil) was used to generate a constant airstream in an aqueous solution of formalin diluted to 1% FA by weight.

This concentration and duration of FA exposure were found to cause a neutrophilic lung inflammation (14).

### Experimental design

The rats in the study were randomly assigned to 5 groups: 1) FA/OVA Sham OVx, 2) FA/OVA OVx, 3) OVA/OVA Sham OVx, 4) OVA/OVA OVx and 5) FA/OVA OVx + P.

Immediately after the last session of FA or vehicle inhalation (day 10), the OVx or Sham-operated rats were sensitized with 10 µg of OVA and 10 mg of aluminum hydroxide by a subcutaneous route. Seven days after the first OVA sensitization (day 17), the rats subcutaneously received a second sensitization (booster) with OVA. At day 24, a challenge was performed by OVA (1%) inhalation for 15 min.

In parallel, the OVx rats were treated with progesterone before each FA inhalation and were sensitized with OVA (day 10), boosted 1 week later (day 17) and challenged with OVA 7 days later (day 24), as described above.

Rats from all of the study groups were euthanized by exsanguination of the abdominal aorta under deep anesthesia (choral hydrate, >400 mg/kg ip) 1 day after the OVA challenge. The rats were submitted to FA inhalation 7 days after ovary removal because we had previously demonstrated that the levels of female sex hormones in the serum and the weight of the uterus were both significantly reduced at this time (Figure 1) (4,5,7).

### Evaluation of leukocyte recruitment into the lungs

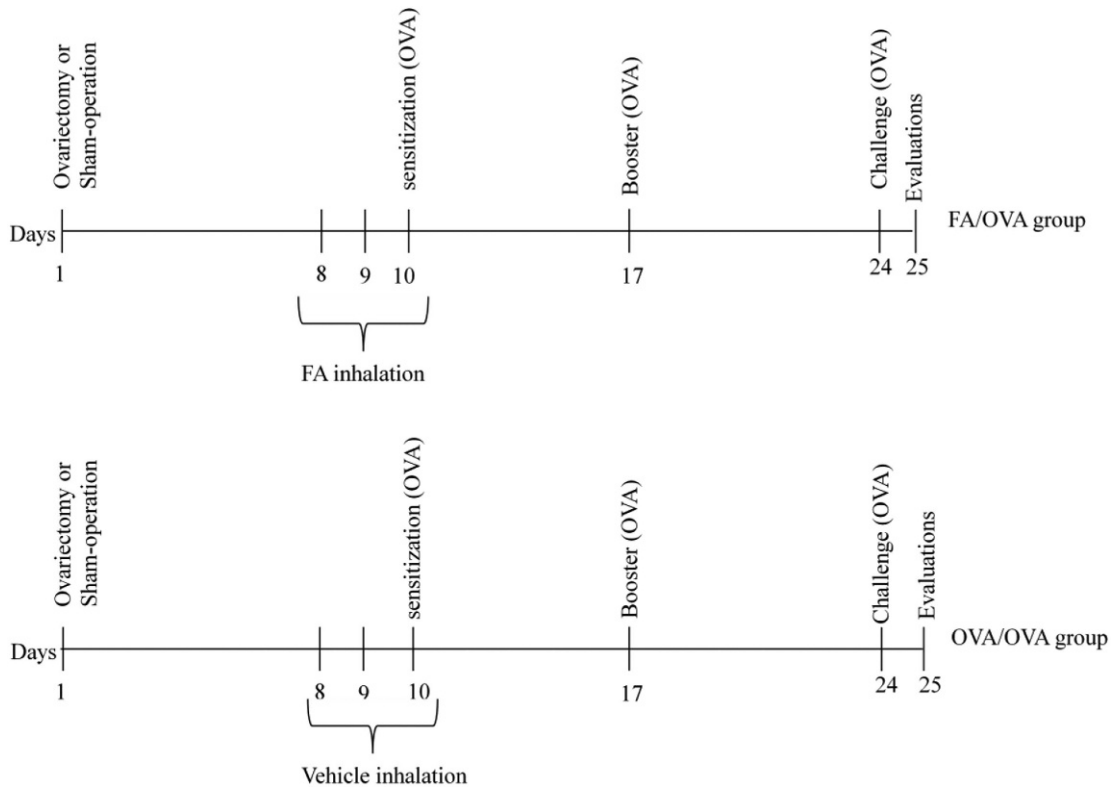
Bronchoalveolar lavage (BAL) was performed according to De Lima et al. (15). Polyethylene tubing (1 mm inner diameter) was inserted into the trachea, and the alveolar space was washed by flushing with PBS (20 ml total volume). The recovered BAL fluid was centrifuged (170 × g for 10 min at 20°C), and the resulting cell pellet was resuspended in 1 ml of PBS. Cell suspensions (90 µl) were stained with 10 µl of crystal violet (0.2%), and the total cell number was determined in Neubauer chambers. Neutrophils, eosinophils and mononuclear cells were quantified by morphological criteria after cytopspin processing and staining with May-Grünwald-Giemsa.

### Analysis of IgE antibodies

IgE antibodies were detected by the Passive Cutaneous Anaphylaxis (PCA) reaction (Mota and Wong, 1969). The skin of non-manipulated rats (recipients) was sensitized with an intradermal injection (100 µl/site) of serially diluted (1:2 up to 1:256) sera from FA/OVA and OVA/OVA rats. After 24 h, the recipient rats received (by an intravenous route) a solution containing 500 µg of OVA plus 2.5 mg of Evans blue (EB) dye, dissolved in NaCl (0.9%). After 30 min, the rats were killed, and the skin was removed. The diameter of the dye stain was measured on the inner surface of the skin. The PCA titers were represented by the highest dilution of the serum that resulted in a dye stain of >5 mm in diameter.

### Lung myeloperoxidase (MPO) activity

The lungs were removed after perfusion through the pulmonary artery with phosphate-buffered saline (PBS). To normalize the MPO activity among the different groups, the lung tissues were homogenized with 3 ml/g PBS containing 0.5% hexadecyltrimethylammonium bromide and 5 mM



**Figure 1** - Protocol of study. The rats were ovariectomized or not and after 7 days submitted or not to FA inhalation. Subsequently the animals were sensitized and challenged with OVA.

EDTA and were centrifuged at  $37,000 \times g$  for 15 min. Samples of the tissue homogenates (10  $\mu$ l) were incubated for 15 min with  $H_2O_2$  and o-dianisidine (Sigma, St Louis, MO). The reaction was stopped by the addition of 1%  $NaN_3$ . The absorbance was determined at 460 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT).

### Lung microvascular leakage

Lung vascular permeability was assessed using the EB dye extravasation procedure. Briefly, immediately after the OVA challenge, the EB dye was injected (20 mg/kg, iv), and the rats were killed 15 min later. The lungs were perfused through the pulmonary artery with PBS, pH 7.0 containing 5 IU/ml heparin. At this time, a fragment of the lung parenchyma was removed, weighed and incubated overnight in formamide (4 ml/g wet weight) at room temperature. The concentration of EB dye extracted by the formamide was determined by spectrophotometry at 620 nm (Bio-Tek instruments) using a standard curve of EB in formamide (0.3–100  $\mu$ g/ml). The extravasate dye was expressed as  $\mu$ g/g of dry tissue weight.

### Determination of ex vivo tracheal reactivity to methacholine (MCh)

Tracheal rings were mounted for the measurement of isometric force using 2 steel hooks in a 15-ml organ bath (16). The force contraction was registered using a force displacement transducer and a chart recorder (Powerlab®, Labchart, AD Instruments). The tracheal rings were suspended in an organ bath filled with Krebs-Henseleit (KH) buffer at 37°C and continuously aerated (95%  $O_2$  and 5%

$CO_2$ ). After the equilibrium period (40 min), the tracheal tension was adjusted to 1 g and the tissue viabilities were assessed by replacing the KH solution with KCl buffer. Subsequently, cumulative dose-response curves to methacholine (MCh) were constructed according to Van Rossum (1963).

### Determination of IL-10 in lung explants

According to the methods of Proust et al. (17), the IL-10 levels were determined in samples of the supernatant from lung explants in culture. To remove the intravascular blood, the lungs were flushed through the right heart with 5 ml of PBS. Next, the remaining parenchyma was chopped randomly into 4 small pieces per well distributed in 24-well plates and cultured in 1 ml of Dulbecco-modified Eagle's medium (DMEM) for 4 h at 37°C with 5%  $CO_2$  and 0.5% penicillin-streptomycin (10,000 UI-10 mg/ml). The results were expressed as pg of interleukin produced per mg of lung tissue dry weight. The IL-10 levels were obtained using standard curves via ELISA kits purchased from R&D Systems (Minneapolis, MN).

### Evaluation of mast cell degranulation

To investigate the role of progesterone and ovary removal on mast cell degranulation, lung fragments were removed from rats and fixed in paraformaldehyde (4%) containing 0.1 M Sørensen phosphate buffer (SPB), pH 7.4, at 4°C for 2 h. Next, the fragments were washed with SPB, dehydrated through a graded ethanol series and finally embedded in Paraplast® (Sigma, USA). Sections (3- $\mu$ m thick) were stained with 1% toluidine blue in a 1% borax solution. The lung mast cells were quantified using 10 serial histological



sections for every animal ( $n=3$ ) (30- $\mu\text{m}$  space between 2 consecutive sections). The analyses were performed by 2 blinded observers using a Zeiss Axioskop II mot plus a high-power objective (x40) and the Axiovision® software (Carl Zeiss, Germany).

### Pharmacological studies

Four hours before each FA inhalation session, the groups of OVx rats were subjected to a subcutaneous injection of water-soluble progesterone (200  $\mu\text{g}/\text{kg}$ ). The controls consisted of OVx rats injected with corresponding volumes of the progesterone vehicle (distilled water). The levels of progesterone in the serum were significantly increased compared with the levels in non-treated OVx rats and did not differ from those in the naïve rats (5).

### Statistical analysis

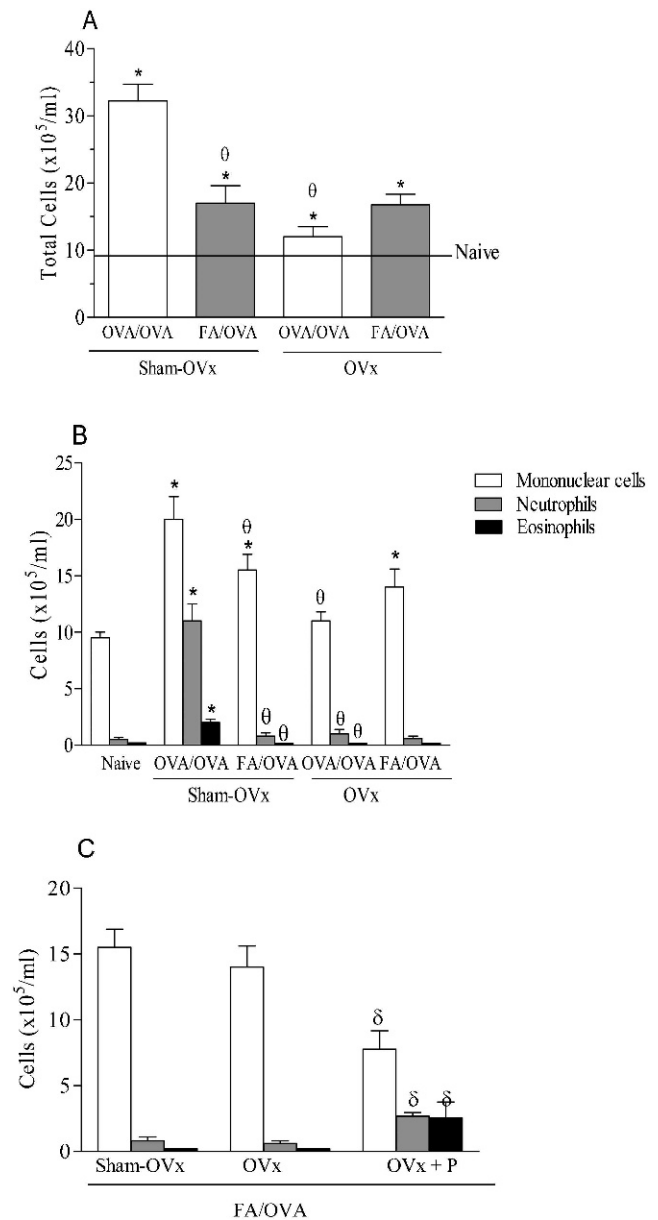
The data are expressed as the mean  $\pm$  SEM, and comparisons among the experimental groups were analyzed by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons using GraphPad software V.2.01, GraphPad InStat™ (1990–1993). Statistically significant differences were considered for  $p$ -values less than 0.05.

## RESULTS

### The role of OVx and progesterone when considering the effect of formaldehyde inhalation on leukocyte migration into the lung

Figure 2 (panel A) shows that previous FA inhalation in allergic rats (FA/OVA) with an intact ovary (Sham-OVx) caused a significant decrease in the number of leukocytes recovered in the BAL fluid compared with the allergic group (OVA/OVA). Similarly, an OVx in allergic rats (OVA/OVA) caused a significant decrease in the number of leukocytes detected in the BAL fluid compared with the allergic rats with an intact ovary (Sham-OVx OVA/OVA group). However, an OVx in the FA/OVA group did not cause an additional reduction in the number of leukocytes in the BAL relative to the number detected in the FA/OVA group with an intact ovary (Sham-OVx FA/OVA). We also observed that all of the groups in this study produced an increased number of leukocytes in the BAL fluid compared with the naïve group.

As shown in panel B, relative to the naïve rats, a significant increase in the number of mononuclear cells, neutrophils and eosinophils is observed in the Sham-OVx OVA/OVA rats. In contrast, an OVx significantly decreased the number of mononuclear cells, neutrophils and eosinophils in allergic rats (OVx OVA/OVA) compared with the animals with an intact ovary. Additionally, previous FA inhalation in allergic rats with an intact ovary (FA/OVA group) reduced the number of mononuclear cells, neutrophils and eosinophils in the BAL compared with the OVA/OVA group with an intact ovary. An OVx in animals from the FA/OVA group did not cause an additional reduction in the number of mononuclear cells, neutrophils and eosinophils compared with the FA/OVA group with an intact ovary. Interestingly, the OVA/OVA group without an ovary and the FA/OVA group with or without an ovary all presented a leukocyte profile in the BAL that was similar to the one observed in naïve rats.



**Figure 2** - The effect of ovariectomy (OVx) and progesterone treatment (P) when considering the results of formaldehyde inhalation on the number of cells recruited in the bronchoalveolar lavage (BAL). Seven days after OVx or Sham-operation (Sham-OVx), the rats were subjected or not to FA inhalation. Subsequently, the rats were sensitized and challenged with OVA. In a parallel study, 7 days after the OVx, the rats were treated with progesterone. Lung inflammation was assessed by quantification of the total number of cells (A) and the total number of differential cells (B and C) present in the BAL 24 h after the OVA challenge. Basal parameters were obtained from non-manipulated rats (naïve). The data are the mean  $\pm$  SEM of 6 animals per group. \* $p < 0.05$  relative to the naïve group; <sup>θ</sup> $p < 0.05$  relative to the OVA/OVA Sham-OVx group; <sup>δ</sup> $p < 0.05$  relative to the FA/OVA OVx group. (ANOVA followed by the Student-Newman-Keuls test).

In Panel C, we observe that the treatment of OVx allergic rats with progesterone (P) before FA inhalation increased the number of neutrophils and eosinophils recruited into the

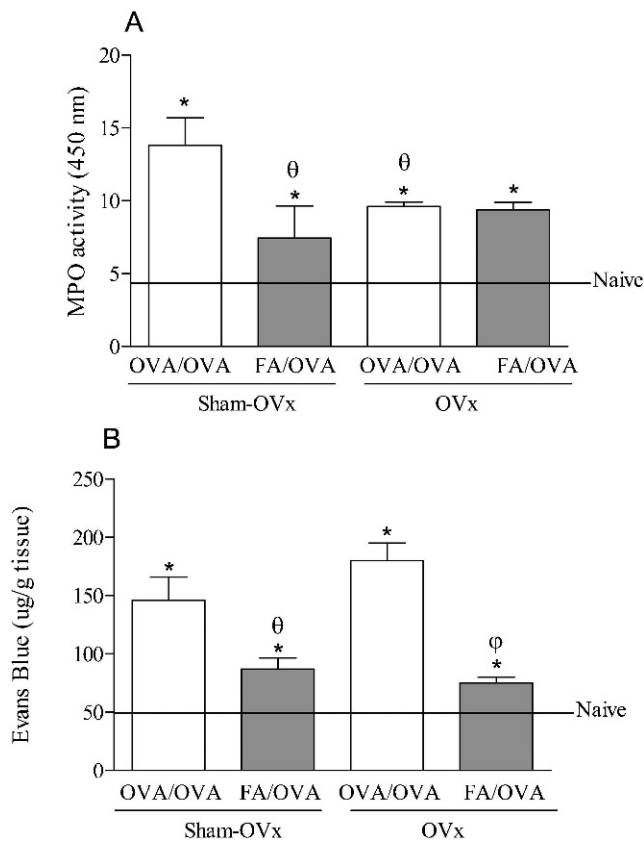


lung compared with the untreated rats. However, this treatment decreased the number of mononuclear cells relative to that in untreated FA/OVA OVx rats.

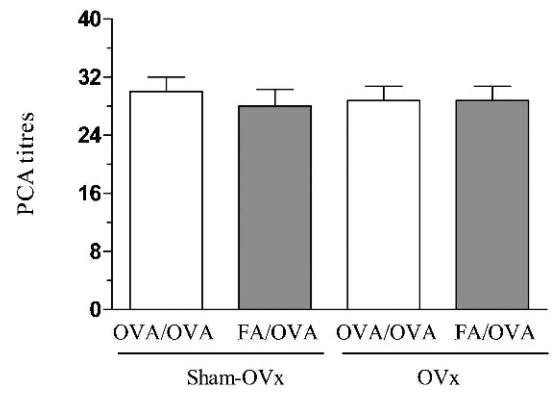
**The influence of OVx on the effect of formaldehyde on MPO activity and lung microvascular leakage in allergic rats**

Figure 3 (Panel A) shows that both OVx and previous FA exposure reduced MPO activity in the OVA/OVA group compared with the Sham-OVx group. In contrast, ovary removal did not affect MPO activity in the FA/OVA group. We also observed that all of the groups in this study showed increased MPO activity compared with the naïve group.

As shown in panel B, OVx did not interfere with EB extravasation in the OVA/OVA rats, whereas previous FA inhalation reduced EB extravasation compared with the OVA/OVA rats with an intact ovary. In addition, we observed that ovary removal in the FA/OVA group did not alter EB extravasation compared with the animals with an intact ovary.



**Figure 3** - The effect of ovariectomy (OVx) when considering the results of FA exposure on myeloperoxidase activity (MPO) and vascular permeability in the lung tissue. Seven days after the OVx or Sham-OVx, the rats were submitted or not to FA inhalation and then sensitized and challenged with OVA. Either 24 h (A) or immediately after the OVA challenge, the MPO activity and vascular permeability were measured. The data are the mean  $\pm$  SEM of 5 animals. \* $p < 0.05$  relative to the naïve group;  $\theta p < 0.05$  relative to the OVA/OVA Sham-OVx group;  $\phi p < 0.05$  relative to the OVA/OVA OVx group. (ANOVA followed by the Student-Newman-Keuls test).



**Figure 4** - The effect of ovariectomy (OVx) and FA exposure on the synthesis of anti-OVA IgE. Seven days after the OVx or Sham-OVx, the rats were submitted or not to FA inhalation and then sensitized and challenged with OVA. The IgE titers were determined by passive cutaneous anaphylaxis. The data are the mean  $\pm$  SEM of 5 animals. (ANOVA).

**The effect of OVx and FA inhalation on the synthesis of IgE antibodies**

Figure 4 shows that the titers of anaphylactic antibodies were not modified by OVx or FA inhalation.

**The effect of OVx and progesterone on the IL-10 levels in lung explants**

Figure 5 (Panel A) shows increased IL-10 levels in the supernatant of lung tissue from the OVA/OVA OVx, FA/OVA Sham-OVx and OVx rats compared with the naïve group. We also observed that the OVx increased the levels of IL-10 in the OVA/OVA group compared with the Sham-OVx group. In addition, we did not observe a difference in the IL-10 levels between the FA/OVA Sham-OVx and OVx groups.

As shown in panel B, progesterone treatment of the FA/OVA OVx group did not alter the levels of IL-10 in the lung tissue supernatant compared with the Sham-OVx and OVx untreated rats.

Panel C shows that both FA inhalation and OVx *per se* presented IL-10 levels similar to those obtained in the naïve group.

**The role of OVx and progesterone treatment (P) on the effect of formaldehyde exposure on tracheal reactivity in allergic rats**

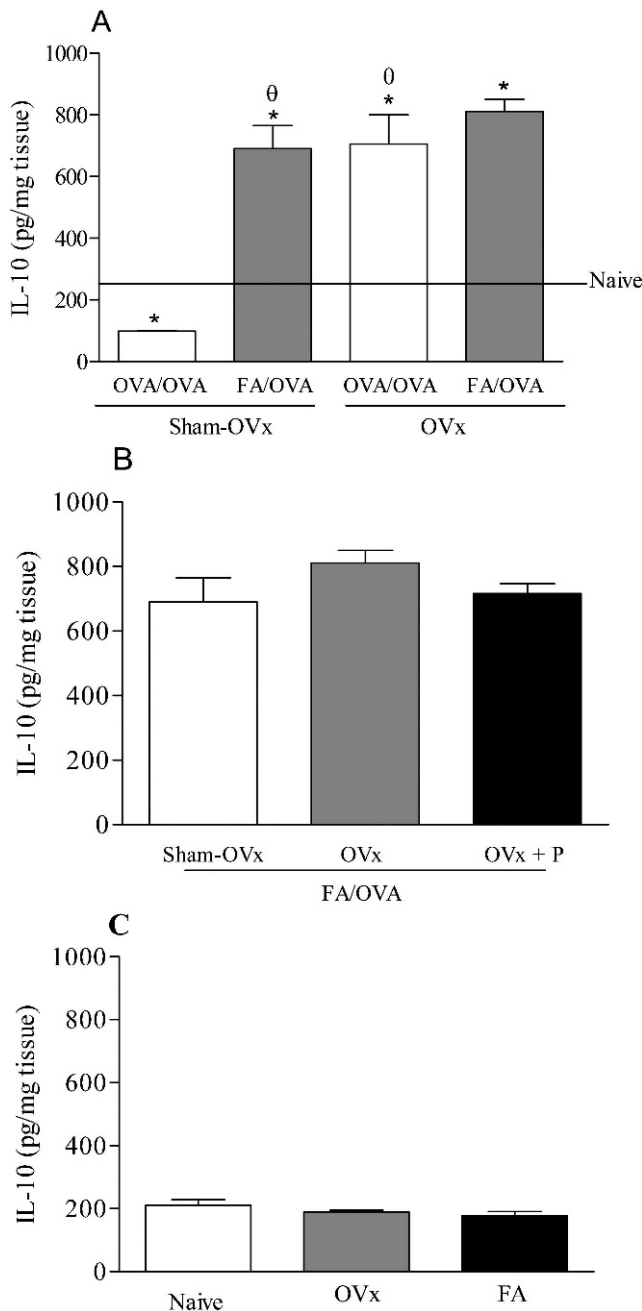
As shown in Figure 6 (Panels A and B), previous FA inhalation in animals of the OVA/OVA group with or without an ovary prevented tracheal hyperreactivity to MCh.

Similarly, ovary removal in animals from the OVA/OVA and FA/OVA groups reduced tracheal reactivity to MCh (Panels C and D).

Panel E shows that treatment with progesterone in FA/OVA OVx rats caused a tracheal hyperreactivity to MCh compared with the untreated group. We also observed that progesterone *per se* did not alter tracheal reactivity to MCh.

**The effects of OVx and progesterone on mast cell degranulation in lung tissue**

As show in Table 1, the histological analysis indicated that in the OVA/OVA Sham-OVx group, the mast cells had



**Figure 5** - The effect of FA inhalation and OVx on IL-10 release in the lung explants. Seven days after the OVx or Sham-OVx, the rats were submitted or not to FA inhalation and then sensitized and challenged with OVA. In a parallel study, the rats 7 days after the OVx were treated with progesterone (P). IL-10 quantification was performed on lung tissue. The data are the mean  $\pm$  SEM of 5 animals. \* $p < 0.05$  relative to the naïve group; <sup>θ</sup> $p < 0.05$  relative to the OVA/OVA Sham-OVx group (ANOVA followed by the Student-Newman-Keuls test).

a high percentage of degranulation compared with the naïve group. In contrast, ovary removal in the OVA/OVA rats and FA/OVA rats resulted in a reduction in mast cell degranulation compared with their counterpart animals with an intact ovary. Similarly, previous FA exposure in animals of the OVA/OVA Sham-OVx group reduced the

percentage of mast cell degranulation compared with the OVA/OVA Sham-OVx group.

Finally, we observed that treatment with progesterone increased mast cell degranulation compared with the FA/OVA Sham-OVx and OVx groups.

## DISCUSSION

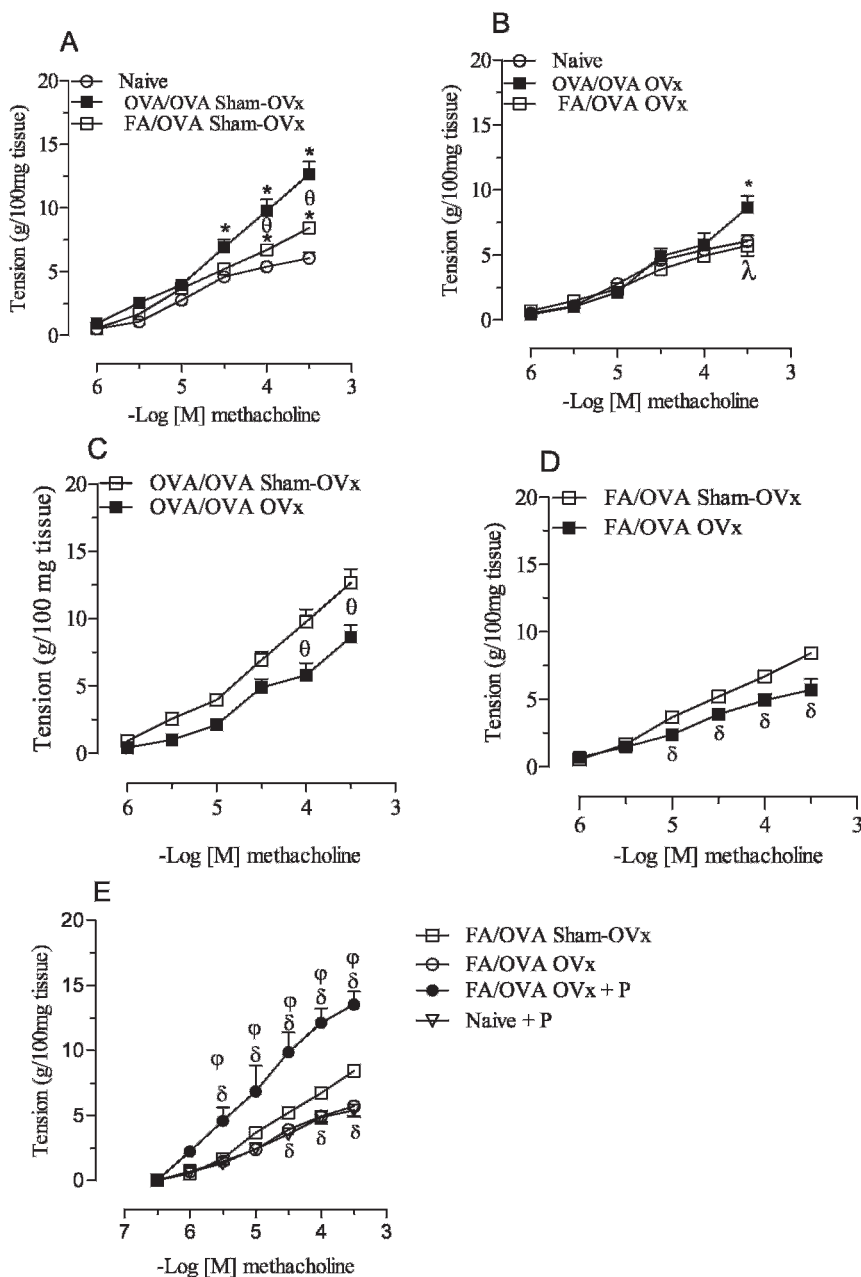
In this study, we investigated the putative role of ovary removal (OVx) and progesterone when considering the effect of FA exposure on lung inflammation induced by OVA. The justification for this study arose from the observation that OVx and FA inhalation reduced leukocyte migration into the lung following an allergic challenge (5,7,14). Here, we confirmed that OVx reduces allergic lung inflammation and added information about the influence of OVx on the effect of FA exposure during an allergic response in the lung. Our data showed that previous FA exposure in allergic female rats reduced cellular recruitment to the lung, MPO activity and lung microvascular permeability and that ovary removal did not modify these parameters. However, we recently observed that OVx prevented lung inflammation induced by FA inhalation in non-allergic rats and that this association increased tracheal reactivity and mast cell degranulation (4).

However, in the present work, OVx did not modify the inflammatory parameters in allergic rats previously exposed to FA, and the female sex hormones (FSHs) appeared to play an important role in the ability of FA to induce a lung inflammatory response (4).

Because OVx and FA inhalation reduced leukocyte migration into the lung and MPO activity in allergic rats, we hypothesized that FA and the lack of FSHs may act on the same target. Considering that FA inhalation and OVx were performed before the OVA sensitization, we decided to investigate whether FA or OVx could reduce the synthesis of anti-OVA IgE because the OVA response is dependent on IgE synthesis. Our results showed that FA and OVx did not modify IgE synthesis. Therefore, we can infer that the effects of FA and OVx do not correlate with an interference in the induction of an allergic response because IgE synthesis was not modified.

In the evaluation of other common pathways involved in the response to both FA and OVx, mast cells have emerged as an important factor because these cells are involved in the actions of FA and OVA in lung tissue (3-5,14). Our results showed that, in fact, FA or OVx reduced mast cell degranulation in allergic rats. Therefore, we can infer that both FA inhalation and the lack of FSHs modulate mast cell degranulation and that the reduced allergic lung inflammation observed in these groups can be attributed to a lower percentage of mast cell degranulation. In addition, we observed that no differences were found between the FA/OVA OVx and FA/OVA Sham-OVx groups because in the first group, mast cell degranulation was elicited by OVx, but in the second group, mast cell degranulation was triggered by FA exposure.

IL-10 has been well established to be an important anti-inflammatory interleukin. Increased levels of IL-10 may suppress eosinophil activity (18). Moreover, IL-10 can also modulate allergic lung inflammation through the activation of adhesion molecule expression (19). In the present work, we observed elevated levels of IL-10 in the FA/OVA Sham-OVx and OVx groups, a phenomenon that coexisted with a



**Figure 6** - The effects of OVx and FA exposure on tracheal reactivity in allergic rats. Seven days after the OVx or Sham-OVx, the rats were submitted or not to FA inhalation and then sensitized and challenged with OVA. In parallel, the rats 7 days after the OVx were treated with progesterone 4 h before each inhalation of FA. The dose response curve was constructed by the administration of a cholinergic agonist (methacholine). The data are expressed as the mean  $\pm$  SEM of 6 animals. \* $p < 0.05$  relative to the naïve group;  $^{\circ}p < 0.05$  relative to the OVA/OVA Sham-OVx group;  $^{\delta}p < 0.05$  relative to the OVA/OVA OVx group;  $^{\square}p < 0.05$  relative to the FA/OVA Sham-OVx group;  $^{\circ}p < 0.05$  relative to the FA/OVA OVx group. (ANOVA followed by the Student-Newman-Keuls test).

decrease in the number of cells recruited in the BAL, reduced MPO activity and EB extravasation. Similarly, the OVA/OVA OVx group presented increased levels of IL-10 in the lung tissue supernatant. Therefore, we suggest that FA and OVx downregulated the allergic lung inflammation, at least in part, by an IL-10 release mechanism that was mediated in the lung tissue. These results were confirmed in naïve rats that were exposed to FA or submitted to OVx and did not demonstrate increased IL-10 levels in lung explants. Therefore, our data suggest that the effects of FA and OVx during an allergic response are blunted by IL-10 release.

Although we did not quantify the expression of adhesion molecules in the present study, elevated levels of IL-10 can modulate the expression of adhesion molecules, thereby contributing to the suppressed allergic lung response. In another study, we demonstrated that FA inhalation in female rats reduced the expression of ICAM-1 and Mac-1 in granulocytes (4).

In the present study, we decided to investigate the impacts of progesterone because it causes increased IL-4 levels and airway hyperresponsiveness (10,20,21). In addition, progesterone exacerbates the airway inflammation



**Table 1** - Mast cell degranulation in samples from the lungs of allergic rats, either ovariectomized (OVx) or sham-operated (Sham-OVx), that were subjected or not to formaldehyde (FA) inhalation (1%, 90 min daily, 3 days). A naïve group was used as the control. In a parallel set, the rats were treated with progesterone before the FA inhalation. The data are the mean  $\pm$  SEM of 5 rats per group.

Groups	Mast cell number/mm <sup>2</sup>		%
	Intact	Degranulated	
Naive	2.15 $\pm$ 0.03	0.04 $\pm$ 0.04	0.0
OVA/OVA Sham-OVx	0.36 $\pm$ 0.06*	1.72 $\pm$ 0.07*	82.5*
OVA/OVA OVx	1.40 $\pm$ 0.07*#	0.79 $\pm$ 0.06*#	36.1*#
FA/OVA Sham-OVx	1.62 $\pm$ 0.04*#	0.12 $\pm$ 0.03*#	26.5*#
FA/OVA OVx	1.60 $\pm$ 0.07* $\theta$	0.30 $\pm$ 0.07* $\theta$	17.2* $\theta$
FA/OVA OVx + P	1.10 $\pm$ 0.03* $\theta\gamma$	1.10 $\pm$ 0.20* $\theta\gamma$	50.0* $\theta\gamma$

\* $p < 0.05$  vs. Naïve group; # $p < 0.05$  vs. OVA/OVA Sham-OVx group;  
 $\theta$   $p < 0.05$  vs. FA/OVA Sham-OVx group;  $\gamma$   $p < 0.05$  vs. FA/OVA OVx group.

and remodeling induced by environmental tobacco smoke (22).

In previous studies, we showed that the administration of progesterone did not affect the tracheal hyperresponsiveness of OVx rats after exposure to FA inhalation. In contrast, progesterone was effective in increasing leukocyte recruitment into the lung of OVx rats exposed to FA inhalation (4). In the present study, using an allergic model of lung inflammation, we found a similar impact of progesterone on the effect of FA on cellular recruitment, but we detected an opposite effect on tracheal responsiveness.

Treatment with progesterone before each FA inhalation session did not modify the number of total leukocytes recovered in the BAL. However, an increased number of eosinophils and neutrophils as well as an elevated percentage of degranulated mast cells were found in animals treated with progesterone. Interestingly, the levels of IL-10 were not modified by progesterone treatment. Our data agree with the results of Hellings et al. (10), which demonstrated that progesterone exogenously induces eosinophilic airway inflammation and increases bronchial reactivity by enhancing systemic IL-5 production.

Eosinophils have been known to mediate allergic airway hyperreactivity (23,24). It is well established that the mediators released by eosinophils contribute to airway hyperresponsiveness, and progesterone has been demonstrated to play a role in the degranulation process of eosinophils (22,25). Moreover, the induction of IL-5 by progesterone appears to be a pivotal event by which progesterone mediates the recruitment of eosinophils into the airways (10). Taking these data into account, we might infer that progesterone increased mast cell degranulation and eosinophil recruitment into the lung and that these cells, once activated, could mediate tracheal hyperresponsiveness.

Moreover, our data demonstrated that tracheal hyperresponsiveness in the OVA/OVA Sham-OVx group was partially reverted when the rats were submitted to FA inhalation and that OVx caused an additional reduction in the tracheal responsiveness of FA/OVA rats.

Data from previous studies showed opposite effects of OVx compared with the results of the present study. OVx in non-allergic rats exposed to FA inhalation determined

tracheal hyperresponsiveness (4), whereas in the present study, OVx caused tracheal hyporesponsiveness in allergic rats submitted to FA inhalation. This discrepancy can be explained by a difference between the protocols in each study. In previous studies, the analyses were performed 24 h after the last exposure to FA, but in the present study, the analysis was performed 24 h after the OVA challenge (14 days after FA inhalation). It is important to mention that the relationship among FA, OVx and OVA is the main reason for the results observed in this study.

In conclusion, our study revealed that FA and OVx downregulated the allergic lung inflammation through IL-10 release and mast cell degranulation mediated in the lung tissue. We observed that reduced allergic lung inflammation induced by FA was not affected by a lack of FSHs but that tracheal hyperresponsiveness was influenced by a lack of FSHs. We also demonstrated that progesterone increased mast cell degranulation, eosinophil recruitment and tracheal responsiveness. Overall, our data have made it possible to understand the role of pollutants on asthma deterioration in women undergoing progesterone therapy.

## ACKNOWLEDGMENTS

This study was sponsored by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP no. 09/51886-3) and the Conselho Nacional de Pesquisa (CNPq). Adriana Lino dos Santos Franco is a scholarship holder from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP no. 2011/51711-9), and Renata Midori Amemiya is a scholarship holder from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP no. 2008/58108-3). Wothan Tavares de Lima is a research fellow from CNPq.

## AUTHOR CONTRIBUTIONS

Lino-dos-Santos-Franco A performed the airway reactivity study and wrote the manuscript. Amemiya RM and Vitoretti L performed the OVx and cellular analyses. Acceturi BG performed the OVx. Oliveira AP quantified the IL-10 cytokine results. Breithaupt-Faloppa AC evaluated the MPO activity and helped write the manuscript. Damazo AS performed the mast cell analysis. Lima WT revised the manuscript.

## REFERENCES

- Fló-Neyret C, Lorenzi-Filho G, Macchione M, Garcia MLB, Saldiva PHN. Effects of formaldehyde on the frog's mucociliary epithelium as a surrogate to evaluate air pollution effects on the respiratory epithelium. *Braz J Med Biol Res.* 2001;34(5):639-43. <http://dx.doi.org/10.1590/S0100-879X2001000500012>.
- Carlson RM, Smith MC, Nedorost ST. Diagnosis and treatment of dermatitis due to formaldehyde resins in clothing. *Dermatitis.* 2004;15(4):169-75. <http://dx.doi.org/10.2310/6620.2004.04021>.
- Lino dos Santos Franco A, Damazo AS, Beraldo de Souza HR, Domingos HV, Oliveira-Filho RM, Oliani SM, et al. Pulmonary neutrophil recruitment and bronchial reactivity in formaldehyde-exposed rats are modulated by mast cells and differentially by neuropeptides and nitric oxide. *Toxicol Appl Pharmacol.* 2006;214(1):35-42. <http://dx.doi.org/10.1016/j.taap.2005.11.014>.
- Lino-dos-Santos-Franco A, Amemiya RM, Ligeiro de Oliveira AP, Breithaupt-Faloppa AC, Damazo AS, Oliveira-Filho RM, et al. Differential effects of female sex hormones on cellular recruitment and tracheal reactivity after formaldehyde exposure. *Toxicol Lett.* 2011;205(3):327-35. <http://dx.doi.org/10.1016/j.toxlet.2011.06.023>.
- De Oliveira AP, Domingos HV, Cavriani G, Damazo AS, Dos Santos Franco AL, Oliani SM, et al. Cellular recruitment and cytokine generation in a rat model of allergic lung inflammation are differentially modulated by progesterone and estradiol. *Am J Physiol Cell Physiol.* 2007; 293(3):120-8.
- Stanford KI, Mickleborough TD, Ray S, Lindley MR, Koceja DM, Stager JM. Influence of menstrual cycle phase on pulmonary function in asthmatic athletes. *Eur J Appl Physiol.* 2006;96(6):703-10.
- Ligeiro de Oliveira AP, Oliveira-Filho RM, Da Silva ZL, Borelli P, Tavares De Lima W. Regulation of allergic lung inflammation in rats: interaction





- between estradiol and corticosterone. *Neuroimmunomodulation*. 2004;11(1):20-7, <http://dx.doi.org/10.1159/000072965>.
8. Haggerty CL, Ness RB, Kelsey S, Waterer GW. The impact of estrogen and progesterone on asthma. *Ann Allergy Asthma Immunol*. 2003;90(3):284-91, [http://dx.doi.org/10.1016/S1081-1206\(10\)61794-2](http://dx.doi.org/10.1016/S1081-1206(10)61794-2).
  9. Skobellof EM, Spivey WH, Silverman R, Eskin BA, Harchelroad F, Alessi TV. The effect of the menstrual cycle on asthma presentations in the emergency department. *Arch Intern Med*. 1996;156(16):1837-40.
  10. Hellings PW, Vandekerckhove P, Claeys R, Billen J, Kasran A, Ceuppens JL. Progesterone increases airway eosinophilia and hyper-responsiveness in a murine model of allergic asthma. *Clin Exp Allergy*. 2003;33(10):1457-63, <http://dx.doi.org/10.1046/j.1365-2222.2003.01743.x>.
  11. Miyaura H and Iwata M. Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. *J Immunol*. 2002; 168(3):1087-94.
  12. De Oliveira AP, Peron JP, Damazo AS, Franco AL, Domingos HV, Oliani SM, et al. Female sex hormones mediate the allergic lung reaction by regulating the release of inflammatory mediators and the expression of lung E-selectin in rats. *Respir Res*. 2010;24:11-115.
  13. Speyer CL, Rancilio NJ, McClintock SD, Crawford JD, Gao H, Sarma JV, et al. Regulatory effects of estrogen on acute lung inflammation in mice. *Am J Physiol Cell Physiol*. 2005;288(4):881-90.
  14. Lino-dos-Santos-Franco A, Domingos HV, Damazo AS, Breithaupt-Faloppa A, Ligeiro de Oliveira AP, Costa SKP, et al. Reduced allergic lung inflammation in rats following formaldehyde exposure: long-term effects on multiple effector systems. *Toxicology*. 2009;256(3):157-63, <http://dx.doi.org/10.1016/j.tox.2008.11.011>.
  15. De Lima WT, Sirois P, Jancar S. Immune-complex alveolitis in the rat: evidence for platelet activating factor and leukotrienes as mediators of the vascular lesions. *Eur J Pharmacol*. 1992;213(1):63-70.
  16. de Lima WT, da Silva ZL. Contractile responses of proximal and distal trachea segments isolated from rats subjected to immunological stimulation: role of connective tissue mast cells. *Gen Pharmacol*. 1998;30(5):689-95, [http://dx.doi.org/10.1016/S0306-3623\(97\)00377-7](http://dx.doi.org/10.1016/S0306-3623(97)00377-7).
  17. Proust B, Nahori MA, Ruffie C, Lefort J, Vargaftig BB. Persistence of bronchopulmonary hyper-reactivity and eosinophilic lung inflammation after anti-IL-5 or -IL-13 treatment in allergic BALB/c and IL-4Ralpha knockout mice. *Clin Exp Allergy*. 2003;33(1):119-31, <http://dx.doi.org/10.1046/j.1365-2222.2003.01560.x>.
  18. Fu CL, Ye YL, Lee Y, Chiang BL. Effects of overexpression of IL-10, IL-12, TGF-beta and IL-4 on allergen induced change in bronchial responsiveness. *Respir Res*. 2006;7(1):72, <http://dx.doi.org/10.1186/1465-9921-7-72>.
  19. Kelly M, Hwang JM, Kubes P. Modulating leukocyte recruitment in inflammation. *J Allergy Clin Immunol*. 2007;120(1):3-10, <http://dx.doi.org/10.1016/j.jaci.2007.05.017>.
  20. Mitchell VL, Gershwin LJ. Progesterone and environmental tobacco smoke act synergistically to exacerbate the development of allergic asthma in a mouse model. *Clin Exp Allergy*. 2007;37(2):276-86, <http://dx.doi.org/10.1111/j.1365-2222.2007.02658.x>.
  21. Tan K, McFarlane L, Lipworth B. Modulation of airway reactivity and peak flow variability in asthmatics receiving the oral contraceptive pill. *Am J Respir Crit Care Med*. 1997;155(4):1273-7, <http://dx.doi.org/10.1164/ajrccm.155.4.9105066>.
  22. Mitchell VL, Van Winkle LS, Gershwin LJ. Environmental tobacco smoke and progesterone alter lung inflammation and mucous metaplasia in a mouse model of allergic airway disease. *Clin Rev Allergy Immunol*. 2012;43(1-2):57-68, <http://dx.doi.org/10.1007/s12016-011-8280-0>.
  23. Hogan SP, Mould A, Kikutani H, Ramsay AJ, Foster PS. Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyper-reactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. *J Clin Invest*. 1997;99(6):1329-39, <http://dx.doi.org/10.1172/JCI119292>.
  24. Balzar S, Strand M, Rhodes D, Wenzel SE. IgE expression pattern in lung: relation to systemic IgE and asthma phenotypes. *J Allergy Clin Immunol*. 2007;119(4):855-62, <http://dx.doi.org/10.1016/j.jaci.2006.12.642>.
  25. Hamano N, Terada N, Maesako K, Numata T, Konno A. Effect of sex hormones on eosinophilic inflammation in nasal mucosa. *Allergy Asthma*. 1998;19(5):263-9, <http://dx.doi.org/10.2500/108854198778557773>.