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Differential effects of female sex hormones on cellular recruitment and tracheal reactivity after formaldehyde exposure

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HIGHLIGHTS

- Exposure to formaldehyde induces lung inflammation in rats.
- Female sex hormones mediates lung inflammation induced by exposure to formaldehyde.
- Tracheal reactivity in response to formaldehyde is mediated by sex hormones.
- Adhesion molecule expression in response to formaldehyde is mediated by sex hormones.
- Mast cell activation in response to formaldehyde is mediated by sex hormones.

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ABSTRACT

Female sex hormones (FSHs) exert profound regulatory effects on the course of lung inflammation due to allergic and non-allergic immune responses. As pollution is one of the pivotal factors to induce lung dysfunction, in this study we investigated the modulatory role of FSHs on lung inflammation after a formaldehyde (FA) exposure. For this purpose, lung and systemic inflammatory responses were evaluated in terms of leukocytes countings in bronchoalveolar lavage (BAL), peripheral blood and bone marrow lavage from 7-day ovariectomized (OVx) and Sham-OVx rats subjected to FA inhalation for 3 consecutive days. The hypothesized link between effects of FSHs on expression of adhesion molecules and mast cells degranulation was also studied. Once exposed to FA, Sham-OVx rats increased the number of total cells recovered in BAL and of leukocytes in peripheral blood, and decreased the counts in bone marrow. By contrast, in OVx rats upon FA exposure there was a reduction of the total cells counts in BAL and of blood leukocytes; lung expressions of ICAM-1 and Mac-1 were depressed, but the number of bone marrow cells did not vary. Estradiol treatment of OVx rats increased the total cells in BAL and decreased the number of blood leukocytes, whereas the number of bone marrow cell remained unaltered. Progesterone treatment, in turn increased the total cells in BAL and blood leukocytes, but decreased the number of bone marrow cells. OVx rats exposed to FA developed tracheal hyperresponsiveness to methacholine (MCh). A similarly altered response was found between the tracheal segments of Sham-OVx rats after FA exposure and that found in tracheae of naïve rats. Estradiol treatment prevented FA-induced tracheal hyperresponsiveness to MCh whereas progesterone was ineffective in this regard. In addition, OVx rats upon FA exposure significantly increased both, the ability of mast cell degranulation and serum corticosterone levels. In conclusion, it was found that FSHs act by distinct control mechanisms on FA-induced lung inflammation and tracheal hyperresponsiveness, since at low circulating levels of FSHs (such as those after OVx) there is some resistance to the development of a lung inflammatory response, but the cholinergic tracheal responsiveness is exacerbated. Our data also help to understand the involvement of FSHs on mast cells activity after pollutants exposure and add information regarding the role of FSHs on the mechanisms related to endothelium-leukocyte interactions.

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1. Introduction

Formaldehyde (FA) is a common environmental contaminant found in building materials, furniture carpets, wallpaper, plywood, floor coverings and sterilizing agents (Fló-Neyret et al., 2001; Krakowiak et al., 1998). FA is also emitted by tobacco smoke, urea-FA foam insulation, cosmetics, solvents and disinfectants (Ohtoshi et al., 1998). Despite the convincing evidence that correlates FA exposure with the development of lung inflammation (Green-Mckenzie and Hudes, 2005), it is that a contradictory array of lung responses arises after FA inhalation. More specifically, a reduced capacity of the bronchial smooth muscle to contract in response to a cholinergic stimulus coexisting with an intense neutrophil recruitment (Lino-dos-Santos-Franco et al., 2006).

The scenario is further complicated by the observation that, although mast cells have been reported to be the target of the FA action in the lung (Fujimak et al., 1992; Stenback et al., 1996; Lino-dos-Santos-Franco et al., 2006, 2009, 2010a) the exposure to this pollutant causes a downregulation of the allergic lung inflammation (Lino-dos-Santos-Franco et al., 2009). In parallel studies we have also demonstrated that FA exposure reduced expression of PECAM-1 and ICAM-1 in lung tissue of allergic rats (Lino-dos-Santos-Franco et al., 2009, 2010b). The subject is still controversial, since in a different model of allergic asthma it has been shown that FA exposure induces a worsening of the lung inflammation (Kita et al., 2003).

Clinical and experimental evidence indicate a role for female sex hormones (FSHs) have definite control influences on lung inflammation (Stanford et al., 2006; Haggerty et al., 2003; Skobellof et al., 1996). In this context, Cuzzocrea et al. (2000) demonstrated that estradiol protects carrageenan-induced pleurisy in rats. Moreover, Speyer et al. (2005) showed that FSHs exert a protective role in LPS-induced lung inflammation. On the other hand, our group have demonstrated that ovariectomy reduces allergic lung inflammation in rodents (Ligeiro de Oliveira et al., 2004). We have shown that FSHs inhibit mast cells degranulation (De Oliveira et al., 2007). Women are more susceptible for development of lung disease such as asthma when compared to men (Eliasson and Scherzer, 1986). In addition, postmenopausal women suffer less asthma symptoms episodes. Since pollutants are putative inducers of asthma deterioration, then we hypothesized that FSH could also interfere with lung inflammatory disease induced by FA exposure. At present there is little doubt that FSHs exert subtle, though definite, modulatory influences on both allergic and non-allergic lung inflammation. By taking into account that on the one hand pollutants like FA can also exert mast cell-mediated effects on lung inflammation and on the other hand, FSHs recognizedly influence these actions, we investigated the participation of FSHs after being called into play during a FA-induced lung inflammation. For this purpose, in this study we investigated the lung and systemic repercussions of FA exposure in ovariectomized (OVx) rats.

2. Materials and methods

2.1. Animals

Female Wistar rats (180–200 g) from Institute of Biomedical Sciences, University of São Paulo were housed in groups (5 rats per cage) in a light and temperature controlled room (12/12-h light-dark cycle, $21 \pm 2^\circ\text{C}$) with free access to food and water. The experiments were approved by the Institutional Animal Care Committee.

2.2. Ovariectomy (OVx)

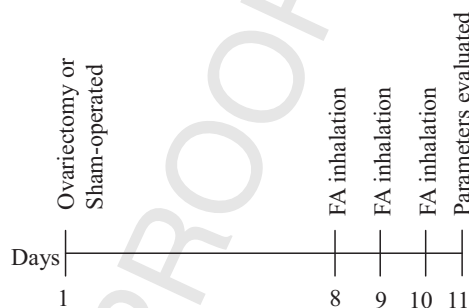
Rats were anesthetized with ketamine-xylazine by intraperitoneal route (100 mg/kg and 20 mg/kg, respectively). Upon laparotomy the ovaries were taken and removed free from adherent tissue. The surgical wound was sutured and animals received a single dose of Pentantibiotic® (570 mg/kg, im). Vaginal smears and quantification of the uterine weight were used in order to assess the effectiveness of OVx. Similarly operated rats, except for ovaries 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.

2.3. Exposure to formaldehyde (FA)

A standard glass chamber (20 L) coupled to an ultrasonic nebulizer device (Icel®, Brazil) was used to generate a constant airstream from an aqueous solution of formalin diluted to 1 wt% FA, as previously described (Lino-dos-Santos-Franco et al., 2006). The final concentration of methanol (present in the parent medium to prevent FA polymerization) in the nebulized solution was 0.32%. Rats (5/chamber) were subjected to daily 90-min sessions of FA inhalation for 3 consecutive days. The chosen concentration and period of FA exposure were found to induce a neutrophilic lung inflammation (Lino-dos-Santos-Franco et al., 2006).

2.4. Experimental design



Upon ketamine-xylazine anesthesia, the rats were OVx or not (Sham-operated) on day 1. After 7 days, the rats were subjected to 90-min sessions of FA inhalation (at days 8, 9 and 10). The rats were subjected to euthanasia (choral hydrate, >400 mg/kg ip followed by exsanguination of abdominal aorta) at day 11. The experimental design was selected considering our previous studies showing that after 7 days of ovaries removal, the serum levels of estradiol and progesterone and the uterus weight were significantly reduced (Ligeiro de Oliveira et al., 2004; De Oliveira et al., 2007).

2.5. Bronchoalveolar lavage (BAL)

According to De Lima et al. (1992) BAL fluid was taken from euthanized animals (choral hydrate, >400 mg/kg ip followed by exsanguination of abdominal aorta). A polyethylene tubing (1 mm inner diameter) was inserted in the trachea and the lung washed by flushing with RPMI-1640 culture medium (20 ml). The recovered BAL fluid was centrifuged ($170 \times g$ for 10 min at 20°C) and the resulting cell pellet was then resuspended in 1 ml of RPMI-1640. Cell suspensions (90 μl) were stained with 10 μl of 0.2% crystal violet and the total cell number were determined in Neubauer chambers.

2.6. Blood leukocytes and bone marrow cell counts

Peripheral blood leukocytes and bone marrow cells were quantified in samples obtained from the abdominal aorta and from aliquots of femoral lavage (FL), respectively. Blood aliquots were diluted (1:20) in Türk fluid (3% acetic acid), while the cells in FL were resuspended in PBS (1 ml) and stained with crystal violet (0.2%). The quantitative analyses of cells were performed in blood smears and FL samples stained with Rosenfeld's dye.

2.7. Flow cytometry

Total BAL cells were resuspended in Dulbecco-modified Eagle's medium (DMEM) containing 2% FCS. In order to prevent non-specific binding via the Fc receptor, the cells were blocked with Fc blocking at 4°C for 20 min and then stained for 30 min at 4°C with FITC-(ICAM-1) (FL1 detector), PE (Mac-1) antibodies (FL2 detector), according to the manufacturer's specifications. After being washed twice with cold HBSS, the cells were resuspended in paraformaldehyde (1%) and analyzed using a FACSCalibur flow cytometer (BD Bioscience, San Diego, CA). Data were analyzed using FlowJo software.

2.8. Quantification of serum levels of corticosterone

Blood samples were collected from rats 24 h after the last FA inhalation session. All the samples were collected in a standard time (between 10 h am and 11 h am). Aliquots of blood were centrifuged ($170 \times g$, 10 min), and the resulting sera were stored at -80°C . Corticosterone levels were determined using radioimmunoassay Kits (Coat-A-Count, Genesee). The detection limit for corticosterone was 25–1000 ng/ml.

Table 1

Wet uterine weights of rats, ovariectomized (OVx) or sham-operated (Sham-OVx), subjected to formaldehyde (FA) inhalation (1%, 90 min daily, 3 days). A naïve group of intact rats was used for control. Uteri were taken free of adherent tissue and weighted 24 h after the last FA inhalation.

Animal groups	Uterine weight (mg)
Naïve	514 ± 44
FA Sham-OVx	513 ± 42
FA OVx	128 ± 24*

Data are mean ± SEM from 6 animals in every group.

* $P < 0.05$ vs Sham-OVx.

2.9. Ex-vivo tracheal responsiveness to methacholine (MCh)

Isometric force development was quantified in tracheal rings mounted in a 15-ml organ bath by means of two steel hooks (De Lima and Da Silva, 1998). Force contraction was recorded using a force displacement transducer and a chart recorder (Powerlab®, Labchart, AD Instruments). Briefly, tracheal rings were suspended in organ bath filled with continuously aerated (95% O₂ and 5% CO₂) Krebs–Hanseleit (KH) solution at 37 °C. After a 40-min equilibrating period the tracheal tension was adjusted to 1 g. Tissue viability was assessed by replacing KH solution with 60 mM KCl buffer in the bath and comparing the contraction force generated with those obtained previously. Next, cumulative dose-response curves to MCh were constructed (Van Rossum, 1963).

2.10. Evaluation of mast cells degranulation

Rats lungs were removed and fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 24 h at 4 °C. Next, they were fragmented, washed, dehydrated in ethanol, clarified in xylene and embedded in histosec™ (MERK, USA). Sections were cut (3 µm; HYRAX M60, Zeiss, GR), mounted on slides, and stained with 0.25% toluidine blue and 0.25% borate sodium solution. Quantification of mast cell number and activation in tissue samples was performed with a high-power objective (×40) and measuring the area of analysis with the Software Axiovision (Zeiss, GR). Data were expressed as cells/mm² (analyzing at least 10 distinct sections per rat).

2.11. Pharmacological treatments

Four hours before every FA inhalation session, groups of OVx rats were subjected to subcutaneous injection of 17β-estradiol (280 µg/Kg) or progesterone (200 µg/Kg). Controls consisted of OVx rats subjected to injection with the corresponding volumes of the hormone vehicles (distilled water for progesterone and 17β-estradiol). The doses of estradiol and progesterone utilized were selected considering our previous studies showing that an increased systemic levels of estradiol and progesterone in comparison to OVx non-treated rats (De Oliveira et al., 2007).

2.12. Statistical analysis

Data are expressed as mean ± SEM and comparisons among the experimental groups were analyzed by one-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons using the GraphPad prism software V.5. Statistically significant differences were considered for P values less than 0.05.

3. Results

3.1. Effect of female sex hormones on lung inflammation induced by formaldehyde inhalation

Firstly, we assessed the effectiveness of ovariectomy. As can be observed in Table 1, ovariectomy resulted in a significant loss of uterine weight and as previously described (Ligeiro de Oliveira et al., 2004) caused morphological changes in vaginal cells compatible with the diestrus phase (data not shown).

A significant increase in the number of leukocytes was observed in the BAL fluid of Sham-OVx rats exposed to FA (21.8 ± 2.8 × 10⁵ cells; $n=6$) when compared to the naïve group (10.3 ± 0.8 × 10⁵ cells; $n=6$). However, ovariectomy caused a significant decrease (11.8 ± 0.7 × 10⁵ cells; $n=6$) in the number of leukocytes recovered in the BAL of OVx rats exposed to FA when compared to the Sham-OVx group and did not differ from the naïve group. The treatments of OVx animals with estradiol or progesterone caused an increase in the num-

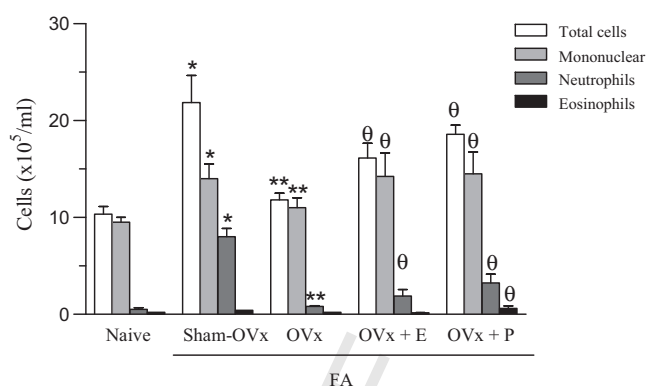


Fig. 1. Effect of female sex hormones on pulmonary cellular recruitment after formaldehyde inhalation. Groups of rats were subjected to ovariectomy at day 1 (OVx) and after 7 days the rats were subjected to FA inhalation (3 consecutive days, 1%, 90 min/day). Control group consisted of rats submitted to similar manipulations excepting the ovaries removal (Sham-OVx). In a parallel set of experiments, group of OVx rats were treated with estradiol or progesterone during 3 days before each FA inhalation. Lung inflammation was assessed by quantification of the recruited cells present in bronchoalveolar lavage (BAL) 24 h after FA exposure. The basal parameters were obtained of non-manipulated rats (Naïve). Data are mean ± SEM of 6 animals per group. * $P < 0.05$ relative to the naïve group; ** $P < 0.05$ relative to the FA Sham-OVx group; ^θ $P < 0.05$ relative to the FA OVx group.

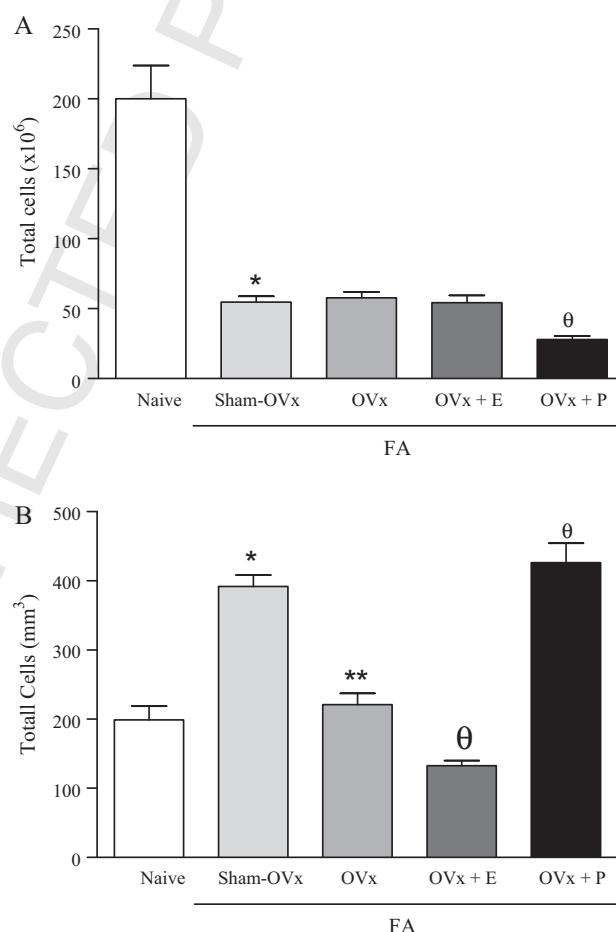


Fig. 2. Effects of female sex hormones on bone marrow cells and on peripheral blood cells in rats exposed to formaldehyde. Groups of rats were subjected to OVx and after 7 days were subjected to FA inhalation (3 consecutive days; 1%, 90 min/day). The femoral lavage (A) and the blood leukocytes counts (B) were performed 24 h after FA inhalation. The parameters were also analyzed after the treatment of OVx rats with estradiol or progesterone. Data are mean ± SEM of 6 animals per group. * $P < 0.05$ relative to the naïve group; ** $P < 0.05$ relative to the FA Sham-OVx group; ^θ $P < 0.05$ relative to the FA OVx group.

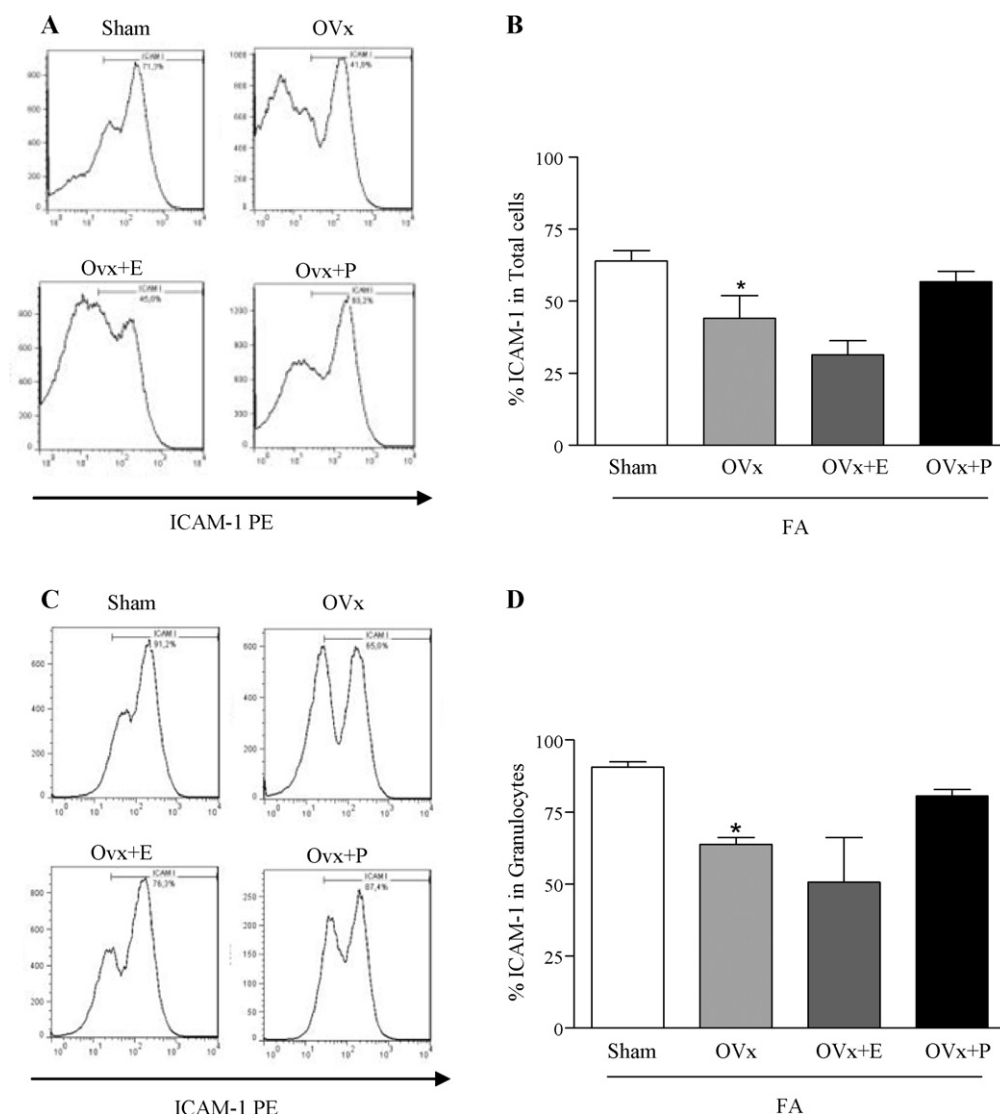


Fig. 3. Expression of ICAM-1 in total leukocytes (Panels A and B) and granulocytes (Panels C and D) collected in BAL of ovariectomized rats exposed to formaldehyde. Groups of rats were subjected to OVx and after 7 days were subjected to FA inhalation (see Section 2). Data are mean $\pm$ SEM from 5 animals. * $P < 0.05$ relative to the FA Sham-OVx group.

ber of leukocytes recovered in the BAL (OVx+E = $16.1 \pm 1.5 \times 10^5$ cells; OVx+P = $18.5 \pm 0.9 \times 10^5$ cells; $n = 6$) when compared to the untreated OVx rats ($11.8 \pm 0.7 \times 10^5$ cells) (Fig. 1).

Differential cell analyses indicated that after FA inhalation BAL of Sham-OVx rats had an increased number of recruited mononuclear and neutrophils, without significant eosinophils migration. It was noteworthy that FA inhalation of OVx rats evoked a BAL cell profile similar to that found in the naïve group.

The treatment with estradiol caused a significant increase in the number of mononuclear and neutrophils recovered in BAL of OVx rats upon FA inhalation when compared to the OVx untreated group. There was an increase of the countings of all recovered BAL cell types in the OVx rats upon progesterone treatment (OVx+P) when compared to OVx untreated group.

3.2. Effects of female sex hormones on bone marrow cells and on peripheral blood leukocytes in rats exposed to formaldehyde

Since that OVx rats presented reduced lung inflammation after FA exposure, we evaluated whether FSHs could affect the mobilization of leukocytes from the blood and bone marrow to lung compartment. Fig. 2 (Panel A) shows that Sham-OVx rats exposed

to FA inhalation exhibited a significant decrease in the bone marrow cells count ($54.7 \pm 4.2 \times 10^6$ cells; $n = 5$) when compared to the naïve group ($200.0 \pm 23.8 \times 10^6$ cells; $n = 5$). On the other hand, OVx failed to change the number of bone marrow cells ($57.8 \pm 4.11 \times 10^6$ cells; $n = 5$) in relation to found in Sham-OVx rats ($54.7 \pm 4.2 \times 10^6$ cells; $n = 5$).

Treatment with progesterone caused a decrease in the number of bone marrow cells in OVx rats ($28.1 \pm 2.45 \times 10^6$ cells; $n = 5$) and whereas estradiol did not modify the bone marrow cellularity when compared to OVx untreated group ($57.8 \pm 4.11 \times 10^6$ cells; $n = 5$).

In panel B, we observed that FA inhalation determined a significant increase in the total blood leukocytes count of Sham-OVx rats (392.0 ± 16.5 cells/mm³; $n = 5$) when compared to naïve rats (199.0 ± 20.0 cells/mm³; $n = 5$). In contrast, OVx rats upon FA inhalation reduced the number of blood leukocytes (221.0 ± 16.3 cells/mm³; $n = 5$) regarding the Sham-OVx rats (392.0 ± 16.5 cells/mm³; $n = 5$). Estradiol treatment caused a decrease in the number of blood leukocytes in OVx rats (133.0 ± 7.5 cells/mm³; $n = 5$), whereas the treatment with progesterone increased the blood leukocytes (426.6 ± 27.8 cells/mm³; $n = 5$) when compared to the OVx untreated rats (221.0 ± 16.3 cells/mm³; $n = 5$).

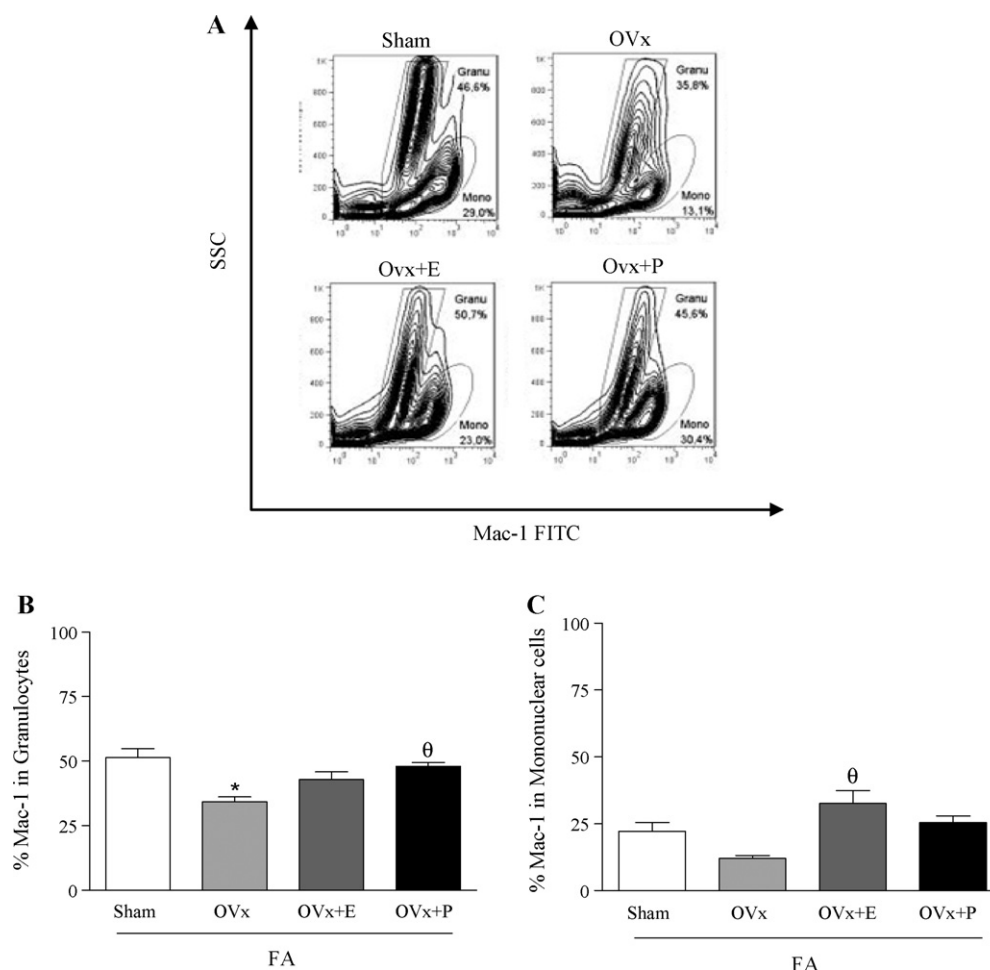


Fig. 4. Effects of FA exposure on expression of Mac-1 in total leukocytes (Panels A and C) and granulocytes (Panels A and B) collected in BAL of ovariectomized rats. Groups of rats were subjected to OVx and FA inhalation (see Section 2). Data are mean $\pm$ SEM from 5 animals. * P < 0.05 relative to the FA Sham-OVx group; $^{\theta}P$ < 0.05 relative to the FA OVx group.

3.3. Determination of Mac-1 and ICAM-1 expression in cells of BAL after formaldehyde exposure in ovariectomized rats

In order to understanding the reduced lung inflammation after FA exposure in OVx rats, we investigated the role of FSHs on expression of adhesion molecules. As shown in Fig. 3, OVx caused a decrease of ICAM-1 expression (CD54) in both cellular types (total cells, panels A and B) and granulocytes (panels C and D) by BAL cells in relation to data obtained from animals with intact ovaries (Sham-OVx group). On the other hand, the treatments with estradiol or progesterone did not modify ICAM-1 expression of OVx rats.

In Fig. 4, we can observe a decreased Mac-1 expression (CD11b/CD18) in granulocytes (Panels A and B) and mononuclear cells (Panels A and C) by BAL cells of OVx rats upon FA inhalation when compared to observed in Sham-OVx rats. In panel B we noted that only progesterone of OVx rats upon FA inhalation treatment increased Mac-1 expression when compared to OVx untreated rats. On the other hand, in panel C we observed that only estradiol treatment increased of Mac-1 expression when compared to OVx group.

3.4. Quantification of corticosterone levels after FA exposure in ovariectomized rats

Since we observed a reduced expression of adhesion molecules in OVx rats, we investigated a possible correlation with corticosterone levels. It is well establish the modulation of corticosterone

levels on expression of adhesion molecules. Fig. 5 shows that there was an increase of serum corticosterone levels in OVx rats upon FA inhalation relative to Sham-OVx rats. Moreover, Sham OVx rats subjected to FA inhalation significantly increased the levels of serum corticosterone relative to naïve rats.

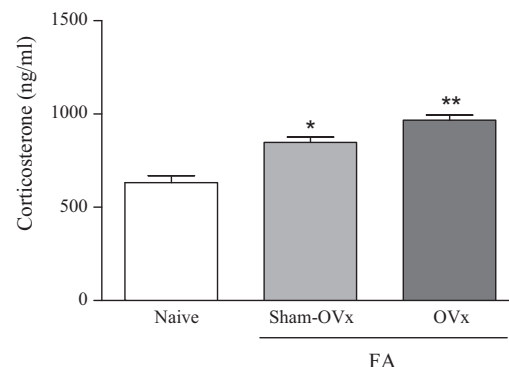


Fig. 5. Effects of OVx on levels of corticosterone of rats exposed to FA inhalation. Groups of rats were subjected to OVx and after 7 days were subjected to FA inhalation for 3 consecutive days (1%, 90 min/day). The quantification of corticosterone was determined in sample of serum after 24 h the last FA inhalation. Data are mean $\pm$ SEM from 5 animals. * P < 0.05 relative to the naïve group; ** P < 0.05 relative to FA Sham-OVx group.

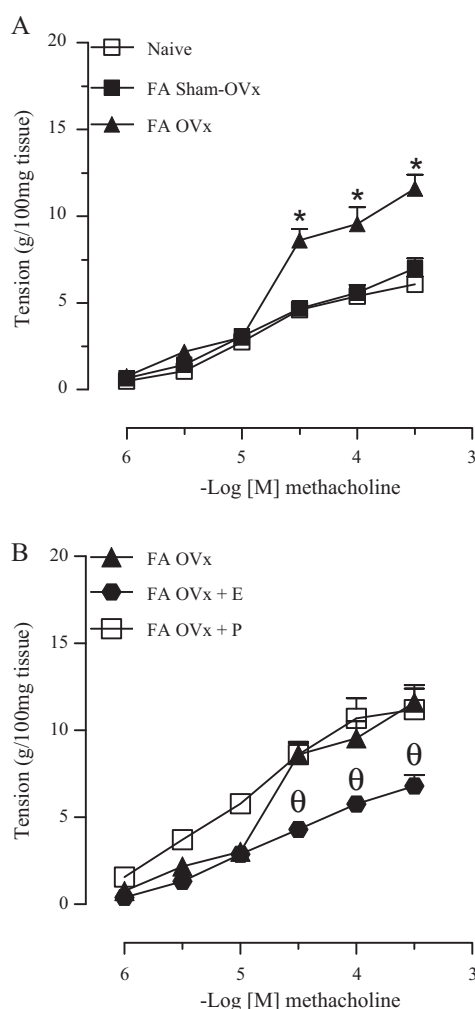


Fig. 6. Involvement of female sex hormones on airways reactivity after formaldehyde inhalation. Groups of rats were subjected to OVx and FA inhalation (see Section 2). After 24 h last FA exposure the concentration-effect curve for methacholine (MCh) were constructed using an organ bath system. Data are expressed as mean $\pm$ SEM of 6 animals. * $P < 0.05$ relative to the FA Sham-OVx group; $\theta P < 0.05$ relative to the FA OVx group.

3.5. Role of female sex hormones on tracheal reactivity to methacholine after formaldehyde inhalation

As shown in Fig. 6 (Panel A), OVx in rats exposed to FA displayed tracheal hyperresponsiveness to MCh when compared to the Sham-OVx group ($E_{\max_{OVx}} = 11.6 \pm 0.8$ g vs $E_{\max_{Sham-OVx}} = 7.0 \pm 0.58$ g). No differences were found between the FA-exposed Sham-OVx and naïve groups ($E_{\max_{naïve}} = 6.0 \pm 0.46$ g).

In panel B it is seen that the tracheal hyperresponsiveness to MCh observed in the OVx group was reduced by treatment with estradiol ($E_{\max_{OVx+E}} = 6.8 \pm 0.6$ g vs $E_{\max_{OVx}}$ untreated = 11.6 ± 0.7 g). No differences were found when rats were treated with progesterone ($E_{\max_{OVx+P}} = 11.2 \pm 1.24$ g) and OVx untreated groups.

3.6. Mast cells degranulation analysis

Lung mast cells of naïve rats did not show significant degranulation (Fig. 7A and Table 2). Conversely, lung fragments of the Sham-OVx rats upon FA inhalation showed significant degranulation. Degranulation was also evidenced by the presence of granules in the surrounding extracellular space (Fig. 7B, Table 2). On the other hand, a more intense mast cell degranulation was found in

Table 2

Mast cell degranulation in samples of lungs of rats, ovariectomized (OVx) or sham-operated (Sham-OVx), subjected to formaldehyde (FA) inhalation (1%, 90 min daily, 3 days). A naïve group of intact rats was used for control. Parallel groups of FA OVx rats were treated with 17 β -estradiol (E) or with progesterone (P). Data are mean $\pm$ SEM from 5 rats per group.

Groups	Mast cells number/mm ²		
	Intact	Degranulated	%
Naive	17.6 $\pm$ 0.5	0.4 $\pm$ 0.1	0.0
FA Sham-OVx	13.3 $\pm$ 1.2*	7.8 $\pm$ 1.2*	36.9
FA OVx	6.0 $\pm$ 3.4§	12.1 $\pm$ 2.4§	66.8
FA OVx + E	13.3 $\pm$ 1.2¶	4.8 $\pm$ 1.2¶	26.5
FA OVx + P	6.5 $\pm$ 0.5	11.6 $\pm$ 0.5	64.0

* $P < 0.05$ vs Naïve group.

§ $P < 0.05$ vs FA Sham-OVx group.

¶ $P < 0.05$ vs FA OVx group.

lung of OVx rats after FA inhalation group (Fig. 7C and Table 2) as compared with Sham-OVx rats exposed to FA inhalation. Estradiol treatment prevented the lung mast cells degranulation when compared to the untreated OVx rats upon FA inhalation (Fig. 7D and Table 2). However, the treatment of rats with progesterone did not change the degranulation of lung mast cells (Fig. 7E and Table 2).

4. Discussion

In this study, we have investigated the role of FSHs on the acute lung inflammation caused by FA inhalation. As FA is an indoor and outdoor pollutant (Fló-Neyret et al., 2001; Krakowiak et al., 1998), there is an increasing concern regarding its deleterious effects notably in the lung (Sherriff et al., 2006). Our studies have been performed in rodents using a model of short term exposure to FA. Our findings suggest that ovariectomy downregulates the cellular migration into the lungs after FA exposure and increases the ability of mast cells to degranulate, thus explaining the tracheal hyper-reactivity. Our data suggest that estradiol, but not progesterone prevents the tracheal hyperreactivity. Similarly, estradiol and progesterone mediate positively the lung inflammation induced by FA exposure.

Female sex hormones exert profound effects on the immune system and inflammatory response (Stanford et al., 2006; Yang et al., 2005; Haggerty et al., 2003; Skobeloff et al., 1996). In this context, a number of clinical and experimental evidence show that estradiol and progesterone exert pro and anti-inflammatory actions depending on the nature (allergic or non-allergic) of the inflammatory agent (De Oliveira et al., 2010, 2007; Speyer et al., 2005; Ligeiro de Oliveira et al., 2004).

We have found exposure of male rats to FA lead to a lung inflammatory response where mast cells-derived mediators play an important role (Lino-dos-Santos-Franco et al., 2006, 2009, 2010a). By the other hand, FA exposure also blunted allergic lung inflammation by mechanism involving mast cells piecemeal degranulation (Lino-dos-Santos-Franco et al., 2009). Keeping in mind that fluctuations of FSHs levels are related to airway dysfunctions and that postmenopausal women might reduce/develop asthma symptoms (Stanford et al., 2006; Cohen and Holbrook, 2004; Haggerty et al., 2003; Tan, 2001), we decided to evaluate whether FSHs mediate the lung inflammatory response caused by FA inhalation as well as the involvement of mast cells activation in this process.

In comparison to Sham-OVx group, OVx rats developed a less intense cell infiltration into the lung after FA exposure and decreased number of circulating blood leukocytes, but did not modify the number of cells in the bone marrow. These data show that FA exerts systemic effects and the reduced lung inflammation in OVx rats did not result from a deficiency in the mobilization of leukocytes from the blood as well the bone marrow to the lung.

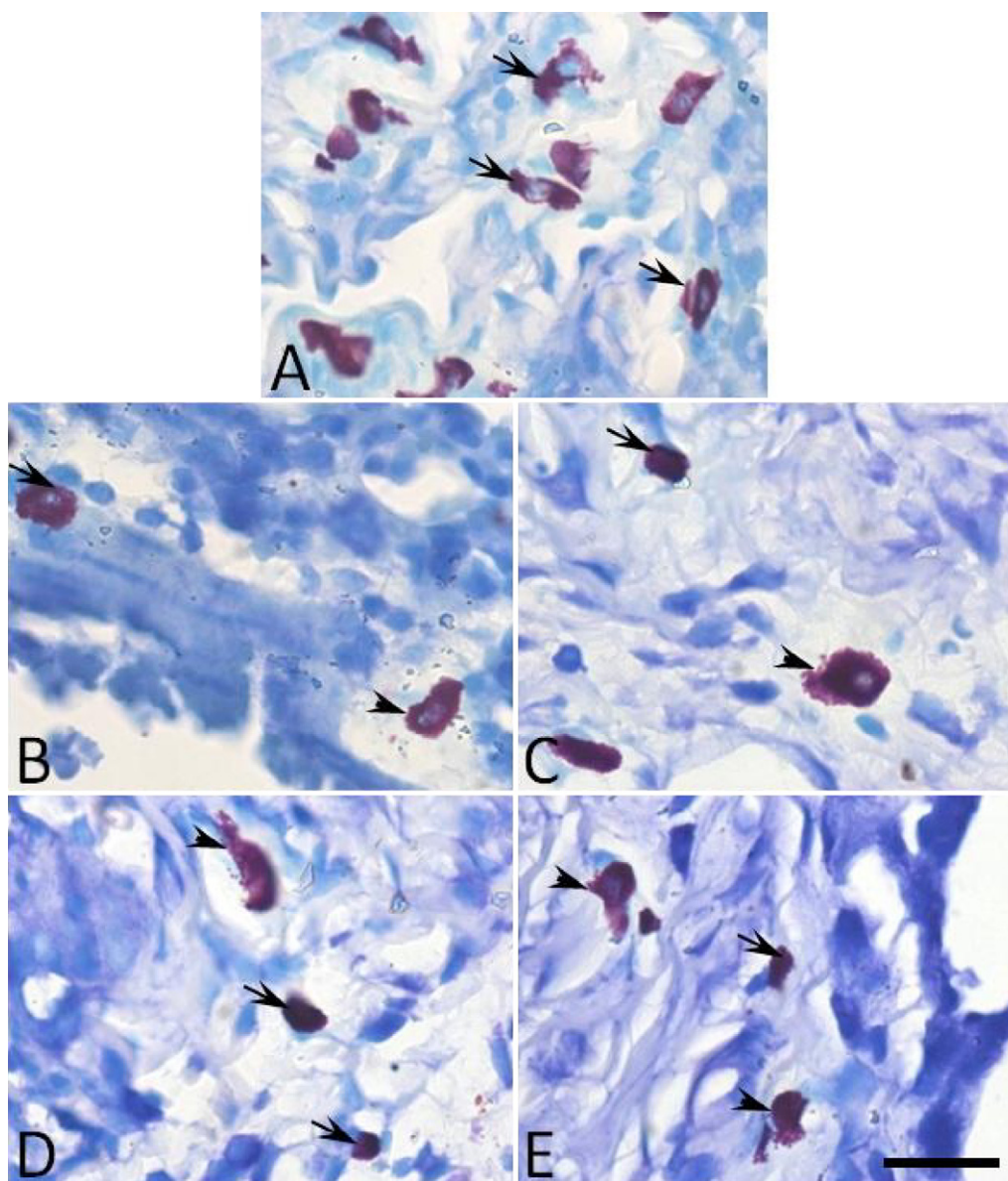


Fig. 7. Mast cells degranulation in ovariectomized rats exposed to formaldehyde inhalation. Panel A mast cells from naïve rats were intact and metachromatic (arrows), localized near the bronchus. Panel B: lung sections from Sham-OVx rats showing mast cell degranulation (arrowhead). Panel C: OVx induced activation of mast cells (arrowhead). Panels D and E: Treatment with estrogen and progesterone in rats previously OVx caused some intact (arrow) and degranulated (arrowhead) mast cells in the lung ($n = 3$). Toluidine blue stained. Quantification of mast cell number and activation in tissue samples was performed with a high-power objective ($\times 40$) and measuring the area of analysis with the Software Axiovision (Zeiss, GR). Data were expressed as cells/mm² (analyzing at least 10 distinct sections per rat). Scale bars = 10 μ m.

Conversely, FA exposure of OVx rats caused a tracheal hyperreactivity to MCh. Overall, our studies revealed that airways reactivity and cellular recruitment into the lung after FA exposure are controlled differentially by FSHs.

Being so, since our data indicated that FA inhalation not only affected the lung but also exerted systemic effects, we have considered the possibility that a previous FA inhalation might modify the mechanisms involving the induction of immune response. Similarly, FSHs might modulate the course of immune response.

Considering that leukocytes migration into inflamed tissues involves complex interactions of these cells with the endothelium through regulated expression of surface adhesion molecules, we investigated the role of FSHs in this scenario. We observed that ovariectomy induced a reduced expression of Mac-1 and ICAM-1, and this could contribute to decreased cell migration into the lung after FA exposure. Interestingly, the expression of Mac-1, but not of ICAM-1, was positively mediated by estradiol and progesterone.

ICAM-1 is well established as a molecule involved in neutrophil, macrophage and lymphocyte vascular transmigration (Kelly et al., 2007), whereas MAC-1 is expressed on the surface of monocytes, granulocytes, macrophages and natural killer (Solovjov et al., 2005).

Corticosterone exerts potent anti-inflammatory and immunosuppressive effects, including the reduction of both functional activity of phagocytes and its expression of adhesion molecules (Cammeron and Hamid, 2001). Besides, inhibition of corticosterone synthesis caused an increase of cell recruitment into lung of OVx rats upon allergic challenge (Ligeiro de Oliveira et al., 2004). After our observation that OVx reduced the expression of adhesion molecules in BAL cells, and keeping in mind that corticosterone exerts strong anti-inflammatory effects, an investigation on the levels of corticosterone in OVx rats after FA exposure was in order.

In this study we found that FA exposure *per se* induced an increase of serum levels of corticosterone in both Sham-OVx and OVx group. As our studies were carried out 10 days after the surgi-

cal procedures, these data allowed us to suggest that the increase of corticosterone levels might not be attributed to the surgical stress. We showed that FA exposure in OVx rats increased the corticosterone levels relative to Sham-OVx rats.

FA exposure had definite effects on bone marrow, as viewed by the significant reductions of cell counts in both Sham-OVx and OVx groups. Though on the one hand it is conceivable that FA-induced corticosterone release can suppress bone marrow germinative activity, on the other hand such suppression does not appear to be linked to the reduced lung inflammation observed in the OVx group. Overall, taking into account the OVx-induced increase of corticosterone levels, the reduced lung inflammation observed in OVx rats exposed to FA might be, at least in part, due to the interaction of FSHs with corticosterone. Thus, we propose that this association could be involved in the mechanisms underlying leukocyte-endothelial cells interaction, notably in the adhesion cell molecules.

Albeit OVx rats did not develop an exuberant lung inflammation after FA exposure, in the present study we found a high rate of lung mast cells degranulation. Interestingly, our data showed that estradiol prevents whereas progesterone exacerbates mast cells degranulation caused by FA exposure. However, we observed that estradiol and progesterone treatments of OVx rats increased the cellular recruitment in BAL. Thus, despite the fact that ovariectomy increase mast cells degranulation, such event did not seem to correlate with the increment of cell recruitment into the lung observed after FA exposure.

The presence of inflammatory cells in the lung was found not to be required for the induction of bronchial hyperreactivity in lung inflammation models (Lino-dos-Santos-Franco et al., 2006; Tournoy et al., 2000). Here we demonstrated that FA exposure caused tracheal hyperreactivity that coexisted with a reduced lung inflammatory response. As our data indicated that estradiol decreases and progesterone increases the mast cell degranulation, we can infer that in this model, estradiol exerts protective effects on tracheal reactivity, likely by mechanisms involving estradiol receptors on mast cells (Zaitzu et al., 2007). Interestingly as we previously reported that tracheal reactivity of male rats was not affected by FA exposure (Lino-dos-Santos-Franco et al., 2006), then our data reinforce a role of FSHs on lung repercussions of FA exposure. Male (Lino-dos-Santos-Franco et al., 2006) and female Sham-OVx rats subjected to FA inhalation develop similar lung inflammatory response. Taken together, these results indicate that male rat mast cells regulate both cellular recruitment and airways reactivity, whereas mast cells from female rats are involved only in airways reactivity.

Our study demonstrates that at low levels of circulating FSHs, female are more resistant to develop a lung inflammatory response to FA, but present exacerbated upper airway reactivity. These data strongly suggest that lung inflammation and upper airway reactivity due to FA exposure are under distinct control of FSHs. In conclusion, our data contribute to the understanding of the putative involvement of gender differences on mast cells after pollutants exposure and add information regarding the dual role of FSHs on the control of lung inflammation.

Conflict of interest statement

None.

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