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Effects of formaldehyde exposure on the development of pulmonary fibrosis induced by bleomycin in mice

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ABSTRACT

Environmental and Occupational pollution has been extensively studied because of its serious implications on the human health. Formaldehyde (FA) is a pollutant widely employed in several industries and also in anatomy, pathology and histology laboratories. Studies have shown the correlation between FA exposure and development or worsening of asthma. However, the effect of FA exposure on the pulmonary fibrosis (PF) is unknown. PF is a progressive and chronic lung disease with high incidence and considerable morbidity and mortality. Few studies have shown a worsening of PF after pollutants exposure such as ozone and nitrogen dioxide. Therefore, our objective was to assess the effects of FA on the PF. Male mice C57BL6 were treated or not with bleomycin (1,5 U/kg) and exposed or not to FA inhalation (0.92 mg/m^3, 1 h/day, 5 days/week during 2 weeks). Non-manipulated mice were used as control. Our data showed that FA exposure in fibrotic mice increased the number of granulocytes in the bronchoalveolar lavage followed by elevated levels of interleukin 1 beta and interleukin 17. In addition, FA exposure in fibrotic mice enhanced the gene expression of C-X-C motif chemokine ligand 1 (CXCL1) and tumor necrosis factor alpha (TNF-α) in the lung. We also showed an increase in the collagen production, while lung elastance was reduced. **No** differences were found in the mucus production, oedema and interstitial thickening in the lung tissue of fibrotic mice after FA exposure. In conclusion our study showed that FA exposure aggravates the lung neutrophils influx and collagen production, but did not alter the lung elastance, mucus production, oedema and interstitial tickening. This work contributes to understand the effects of pollution in the development of PF.

1. Introduction

Formaldehyde (FA) is a pollutant widely employed in several industries and also in anatomy, pathology and histology laboratories. Studies have shown the correlation between exposure to FA and development or worsening of asthma [1–6]. In addition, FA exposure during the pregnancy causes changes in the immune system of offspring, culminating to impaired defenses against infectious or allergic stimulus by fetal programming mechanisms [7,8]. Thus, the effects of FA have been studied extensively; however, the link between FA and pulmonary fibrosis (PF) is not still established.

PF is a progressive and chronic condition, whose etiology is unknown and the incidence is high [9]. It is characterized by lung inflammation and loss of the original architecture of the lung due to excessive and disorganized expression and deposition of collagen and extracellular matrix leading to lung remodeling and respiratory failure [10,11]. The Pulmonary fibrosis can be developed experimentally by bleomycin administration. Bleomycin is an antineoplastic agent that can cause lung toxicity. This toxicity is related to several inflammatory mediators including transforming growth factor-beta (TGF-beta) and tumor necrosis factor-alpha (TNF-alpha), chemokines among others, which cause severe lung fibrosis as side effect.

The accumulation of vascular exudate and inflammatory cells within the alveolar space causes epithelial injury. These exudates increase the proliferation of resident fibroblasts and their differentiation into myofibroblasts [12]. The balance between inflammatory and anti-inflammatory cytokines is very important for the control of the disease. In this context, transforming growth factor- beta (TGF-beta) is produced

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by inflammatory cells inducing the differentiation of fibroblasts into myofibroblasts. After that, the synthesis and secretion of collagen is substantially increased causing lung remodeling [13]. In addition, it is an important sign for induction of interleukin 17 (IL-17), which amplifies the development of lung fibrosis [14]. It is well established that interleukin 17 (IL-17) promotes the recruitment of neutrophils and might be responsible by the mortality in patients with PF [15]. Similarly, CXC chemokine superfamily, such as CXCL1 is also produced by alveolar macrophages and it plays a central role in the PF regulating the neutrophil influx [16]. It has been shown that the level of CXCL1 into the lung correlates with neutrophil influx [17]. Interleukin 6 (IL-6) is an inflammatory cytokine produced by many cells and its inhibition reduces the lung fibrosis in murine model [18]. Anti-inflammatory cytokines including interleukin 10 (IL-10) and interferon gamma (IFN-γ) are increased in patients with PF and may be used as a biomarker or a therapeutic target because its anti-fibrotic effects [19].

There are few studies in the literature evaluating the effects of pollutants on the worsening of PF. Epidemiological study correlated interstitial lung diseases with inhaled agents including cigarette smoke, organic antigens and wood dusts [20]. Moreover, Johannson et al. [21] showed that exposure to ozone and nitrogen dioxide induced deleterious effects on the PF. Such studies suggest that the pollution may contribute to the worsening of PF. Thus, additional studies are needed. In this context, we evaluated the effects of FA exposure, since it is an important occupational and environmental pollutant. Our study prioritized the effects of FA on the cell recruitment, release and gene expression of cytokines, collagen and mucus production, oedema, interstitial thickening and lung elastance.

2. Material and methods

2.1. Animals

The animals were obtained from the University Nove de Julho and the experiments were approved by the Animal Care Committee University Nove de Julho (CoEP-UNINOVE, AN0038/2014). Male C57BL/6 mice were maintained in light and temperature-controlled room (12/12-hour light-dark cycle, 21 ± 2 °C), with free access to food and water.

2.2. Experimental model of pulmonary fibrosis (PF)

The animals were subjected to a single bleomycin injection by orotracheal route (1.5 U/kg) under soft anesthesia (ketamine-xylazine) [22,23].

2.3. Exposure to formaldehyde inhalatio

The animals were exposed or not to FA inhalation (0.92 mg/m^3, 1 h/day, 5 days/week during 2 weeks) seven days after the PF induction. The dose as well as the time of FA exposition was based on previous studies [7,8].

2.4. Groups of study and experimental design

The animals were divided into 4 groups (n = 6 animals) and they were killed by sectioning the abdominal aorta under deep anesthesia with ketamine-xylazine by intraperitoneal route (100 mg/kg and 20 mg/kg, respectively) 24 h after the last FA inhalation. Below are listed the experimental groups:

Fibrosis + FA vehicle inhalation (F group): Animals submitted to bleomycin injection and exposed to FA vehicle.

Formaldehyde inhalation (FA group): Animals submitted to bleomycin vehicle injection and exposed to FA inhalation.

Fibrosis + Formaldehyde inhalation (F + FA group): Animals submitted to bleomycin injection and exposed to FA inhalation.

Basal (B group): Non-manipulated animals.

2.5. Evaluation of lung cell influx

The number of cells migrated to the bronchoalveolar lavage (BAL) was determined according to Maiellaro et al. [7]. The total cells as well as the differential cells were determined by microscopy.

2.6. Quantification of cytokines generated by BAL cells

Cytokines levels were determined in the BAL supernatant samples according to the manufacturer's specifications using ELISA kits purchased from Biolegend (San Diego, USA). Results were expressed as pg of cytokine produced per ml.

2.7. Quantification of gene expression of cytokines

Gene expression of cytokines was determined according to Silva Macedo et al. [24]. RT-PCR was performed using Taqman real-time PCR assay (Applied Biosystem, USA) for the following molecules: TNF-α: (Mm00443258_m1), IL-1β (Mm00434228_m1), IL-17(Mm01189488_m1), CXCL1 (Mm04207460_m1) and, TGF-β (Mm01178820_m1). Sequence Detection Software 1.9 (SDS) was used for the analysis and mRNA expression was normalized to HPRT expression.

2.8. Evaluation of collagen production in the lung tissue

Histological analyses were performed according to Miranda da Silva et al. [1] and it was evaluated by 1 blinded observer. Lung tissues were stained with Picrossirius and the analyses were performed by image using the Image Pro plus program.

2.9. Determination of lung elastance

Lung elastance is a measure of the tendency of a hollow organ to recoil toward its original dimensions upon removal of a distending or compressing force and it was determined on anesthetized mice using a volumetric ventilator (MV215, Montevideo, UY) in open as well as closed chest [22]. Briefly, mice were anesthetized with a ketamine and xylazine, tracheostomized, and subjected to conventional ventilation with a quasi-sinusoidal flow pattern with a tidal volume of 10 ml/kg of mouse body weight, a frequency of 100 breaths/min, and a positive end expiratory pressure of 2 cmH_2O. Flow and pressure signals from the transducers were analogically lowpass filtered, sampled and stored for subsequent analysis.

2.10. Evaluation of mucus production, oedema and interstitial tissue thickening

Histological analyses were determined by histomorphometric technique by 1 blinded observer. Below are described the scores used for the evaluations.

2.10.1. Mucus accumulation

0, absent; 1, discontinuous presence on the epithelial surface; 2, presence of goblet cell metaplasia in the bronchial epithelium and continuous epithelial surface; 3 presence of goblet cell metaplasia in the bronchial epithelium and a layer of thick mucus on the epithelial surface; 4, a partial obstruction of the bronchiolar lumen by a layer of mucus.

2.10.2. Oedema

0, absent; 1, minimal presence of plasma in the interstitial tissue; 2, presence of large amounts of plasma in the interstitial tissue; 3, presence of plasma and red blood cells in the interstitial space; 4, abundant

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