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NEW ACCESS OF PULMONARY FIBROSIS INDUCED BY BLEOMYCIN IN RATS

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ABSTRACT

Background: Pulmonary fibrosis (PF) is a disabling and life-threatening disease, involved in inflammatory processes which proceed in fibroblast proliferation and consequent increase of connective tissue in lung parenchyma, leading to deterioration of function. Objective: to determinate the effectiveness of a new method of inducing pulmonary fibrosis with bleomycin (BLM) injected into the lumen of the trachea aided by palpation in rat. Materials and methods: The sample was consisted of 18 rats subjected to induced pulmonary fibrosis and their controls. After 3 days the animals were euthanized and lungs removed, which were processed for morphological analysis of the inflammatory infiltrate and check total collagen content of the lung hydroxyproline count in tissue. The GraphPad Prism 1.6 software was used for statistical analysis with ANOVA ONE WAY test with Tukey post-test, p values <0.05 were considered significant. Results: It was found that the inflammatory process induced by BLM behaved with infiltration of inflammatory cells reflecting inflammation and increased hydroxyproline with a significant difference. Conclusion: The new method proved to be a simple and efficient method for PF induction.

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INTRODUCTION

The PF is a chronic and progressive disease that affects the lung parenchyma irreversibly. The progression of the disease leads to the replacement of healthy lung tissue by fibrous connective tissue. It affects the disease lung compliance and gas exchange causing severe respiratory failure and death. The pathology develops secondarily to other pulmonary aggression, resulting from chronic inflammatory diseases, viral, radiotherapy, chemotherapy, among others. However, the origin of pulmonary fibrosis is still unknown in most of cases, so-called idiopathic pulmonary fibrosis (Kim *et al.*, 2006).

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The most used experimental model of PF in use today is induced by the BLM in mice (Grabarz, 2014). Several authors have reported the induction of pulmonary fibrosis in mice Wistar intratracheal medium, exposing the trachea, where, through a dissection is instilled BLM sulfate single dose of 100mg / kg into the trachea (Rossari, 2004). Other methods used to induce pulmonary fibrosis is through oral and nasal instillation in which, after the animals were anesthetized BLM is administered using intravenous catheters, such as small intratracheal probes (François *et al.*, 2014). Nevertheless, the most widely used model is the single intratracheal administration with only one application in low wall model accessing the trachea surgically, requiring the animals to an open procedure and risk aggravating (Chen *et al.,* 2015). Thus, this study aimed to verify the effectiveness of a new method of inducing pulmonary fibrosis with BLM injected into the lumen of the trachea aided by palpation in murine.

MATERIALS AND METHODS

Animals

Adult Wistar rats (*Rattus norvegicus albinus*, 200–300 g) were randomly assigned into one of 3 groups (n = 18, 6 eat group). Rats housed were in controlled conditions at 22 ± 2 °C under a 12-h light/dark cycle, with access to food and water ad libitum. The experiments reported in this study performed were in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals. The Ethics Committees Animal Use (CEUA) of the Tiradentes University, Sergipe, Brazil approved study (#070514).

Groups

The groups were made as follows: Sound group without any injuries or procedures; Vehicle group only subjected to instillation of saline solution injected by an insulin syringe in the lumen of the trachea with the aid of palpation; and Lesion group, animals FP induced by instillation of BLM injected by an insulin syringe in the lumen of the trachea with the aid of palpation.

Experimental design

The suggested method modifies the [3] method. Induction of PF was by intratracheal injections surgical procedure BLM sulfate, at doses of 10 U / kg in animals anesthetized with ketamine at 10% (95 mg / kg) and xylazine 2% (12 mg / kg). The instillation of BLM was performed using 100U needle used to inject insulin into the trachea with palpation guidance to locate the trachea. To get the tracheal lumen access, the needle was inclined at 10 degrees and 0.7cm above the line drawn on the upper limit of the shoulders of the mice (Figure 1). After 3 days, the induction of pulmonary fibrosis was started. It was removed the biological material to verify in situ FP (right and left lung). The material was processed for hitomorfológica analyze inflammatory infiltration and the quantitative measurement of hydroxyproline.

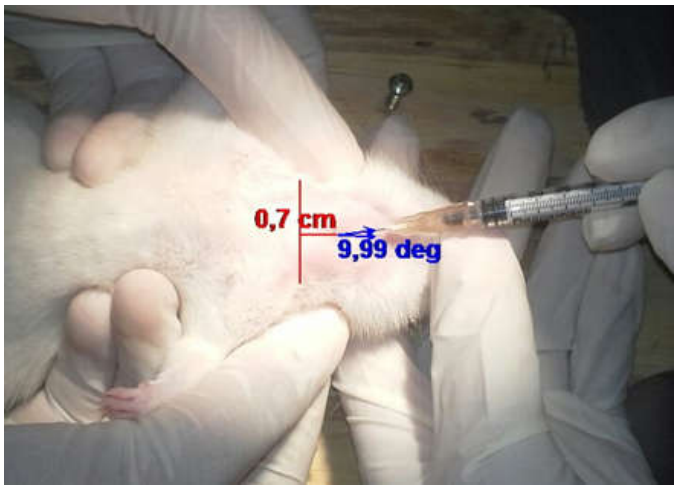

Figure 1. Demonstration of Pulmonary Fibrosis induced by injecting bleomycin directly into trachea without surgery

Histological analysis of the inflammatory infiltrate

The histological sections stained were with hematoxylin–eosin (HE) according to the procedure described by Albuquerque-Júnior *et al.* (2009). The infiltration type and the inflammatory process were classified as acute when polymorphonuclear cells predominated; or chronic when mononuclear cells predominated. The fixer used in this study was 70% buffered formalin. Lung tissue with approximately 3 mm thickness was fixed in formalin for 48 hours.

Inclusion and Court

In the dehydration process, successive washings of the lung tissues were performed in increasing concentrations of alcohol (70%, 95%, 95%, 100%, 100%).

Staining with hematoxylin-eosin

The staining technique was as follows: after the hydration process followed by washing in running water and distilled water the slides, they were bathed in hematoxylin for a period of 3 minutes followed by rinsing with water for removal of excess dye. Then it proceeded with eosin staining for 30 seconds. After this, the blades have undergone a new dehydration process by washing with increasing concentrations of alcohol (80%, 95%, 100%). Next, the slides were placed in xylene (diaphanization), where they remained for 10 minutes. In this staining, the following inflammatory cells were analyzed: lymphocytes, macrophages, neutrophils, and plasma cells. The slides were photographed with microscope (Olympus BX51, Tokyo, Japan) equipped with a digital camera (Olympus DP71, Tokyo, Japan). Images were acquired with a resolution of 2040 x 1536 pixels and saved in file in TIF format.

Morphological Analysis of Inflammatory infiltrates

To evaluate the histological and histopathological features associated with inflammatory lung process, it was analyzed the histological sections stained with hematoxylin-eosin (HE). The predominance of cells involved in the inflammatory process it was by recognition and counting of leukocytes with identification of polymorphonuclear and mononuclear cells. The slides were photomicrographed in four histological fields (magnification of 100x) and were digitized using Olympus 200® image capture software. The images were overlapped by 100 test reticle containing a total of 500 points counting on each blade preventing the counting of the cells. The average of the cells was obtained through summation ratio structures, and the quantitative analysis fields (Albuquerque-Júnior *et al.*, 2009).

Measurement of hydroxyproline of the pulmonary tissue

Each right and left lung was weighed, homogenized and divided into microtubes equally. One of the microtubes were used for measurement of lung hydroxyproline content. Content of Total lung collagen was determined by the dye binding method MAK008-1KT Hydroxyproline Assay Kit (Sigma-Aldrich, representing north / Brazil). The left lung tissues were cut and then added 10 mg Ehrlich solution (1 M dimethylaminobenzaldehyde (DMBA) in 70% propanol and 20% perchloric acid). After cooling to room temperature, the absorbance is measured at 560 nm in a microplate reader (Thermo Plate reader tp) and the amount of hydroxyproline was determined relative to a standard curve prepared using known concentrations of hydroxyproline. Trans-4-hydroxy-L-

proline (10 g / mL) was used as a standard solution (Li *et al.,* 2015).

Statistical analysis: The program GraphPad Prism 6.01 was used for all statistical analyses. Results are expressed as the mean±SD. Statistical significance (p<0,05) was determined using a ONE-WAY ANOVA, and Tukey's test of quantitative measurement. Each time point was analyzed separately.

RESULTS

It was realized the standardizing of the pulmonary fibrosis induction by instillation of BLM was carried directly into the trachea the animal in vivo and without the need for surgery. Thus lower risk of death in invasive procedure and difficult to perform. HE staining was performed to observe the pathological change with pulmonay fibrosis.

Histological examination of lung samples showed that BLM induced classical FP through the new method of induction in injury group (Figure 2). The mean count of Lymphocytes (p <0.001) and macrophages (p <0.001) in the injury control group were different in relation to the healthy group. And in relation to the vehicle, lymphocytes (p <0.001) and macrophages (p <0.05) (Figure 3).

Figure 4 shows the average quantity of collagen deposition in rats with induced by BLM in mice. This analysis was performed with hydroxyproline, demonstrating that the lesion group showed a denser deposition of this compound demonstrated the arrangement for collagen formation in this tissue significantly manner towards healthy groups (p <0.01) and vehicle (p <0.05). The average between the groups was in the healthy group (0.07 ± 0.0053); in the vehicle group (0.08 ± 0.028); and the lesion group (0.11 ± 0.037).

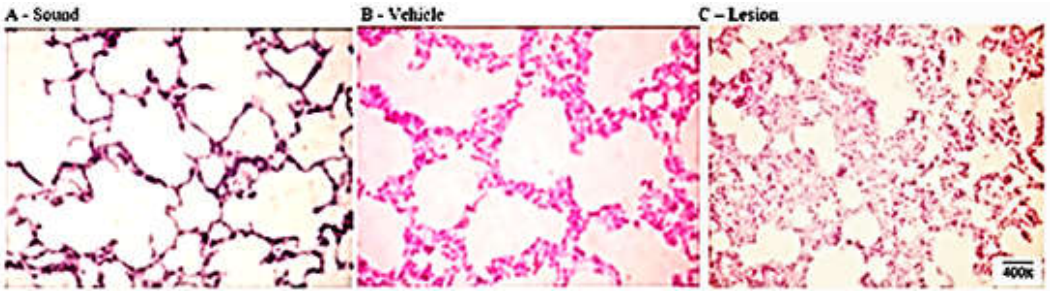

Figure 2. Histopathological blades of rats with moderate inflammatory cell in the control group (HE , 400x magnification)

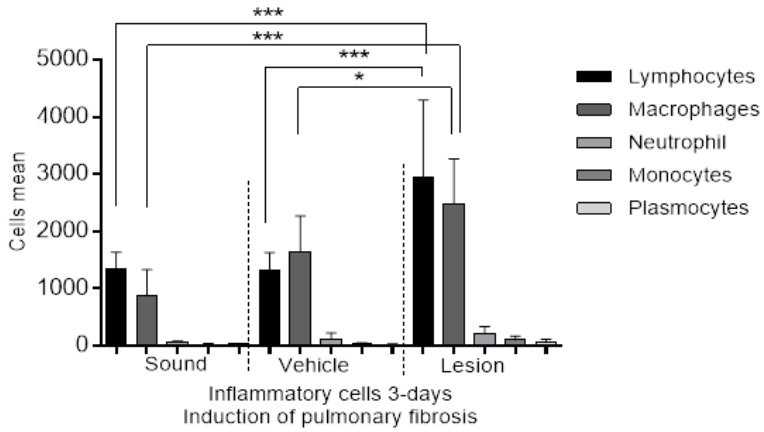

Figure 3. Mean types on Inflammatory cells polymorphonuclear and mononuclear in 3 days in the induction of pulmonary fibrosis for bleomycin. ANOVA ONE-WAY and Tukey pos-test; *p<0,05, **p<0,01, and ***p<0,001

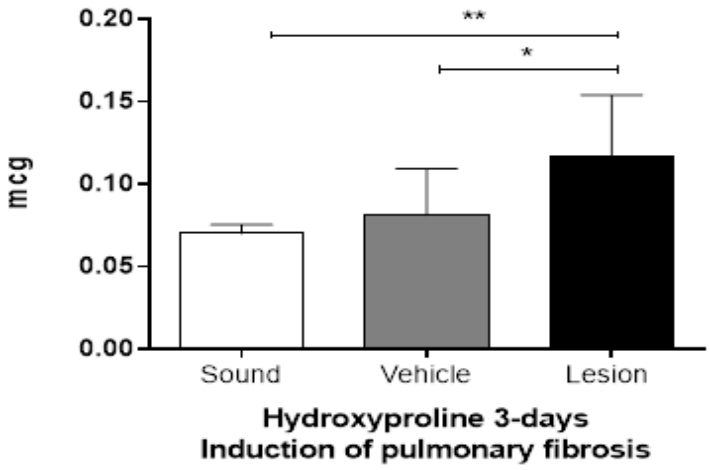

Figure 4. Hidroxyproline analysis in the 3-days at pulmonar fibrosis for bleomycin in rats. Induction intratracheal injections surgical procedure bleomycin sulfate, at 10 U / kg doses. ANOVA ONE-WAY and Tukey pos-test; *p<0,05, **p<0,01, and *

DISCUSSION

In order to investigate how is the development of PF a widely used method is to experimental lung injury induced by BLM (Asker *et al.,* 2015). The PF induction method has been optimized by applying various doses of intraperitoneal application (Asker *et al.,* 2015), intravenous (Kurokawa *et al.,* 2010), subcutaneously (Della Latta *et al*., 2015) and nasal instillation (François *et al.,* 2014), however, the most widely used model is the single intratracheal administration with only one application in low wall model accessing the trachea surgically, requiring the animals to an open procedure and risk aggravating (Chen *et al.,* 2015). In this study, we used a new method standardizing the induction of PF by instillation of BLM directly into the living animal trachea, without surgery, only using an insulin syringe. With this procedure it was possible to verify the possibility of PF induction without the need for surgical access to the trachea followed by instillation of BLM. As the group subjected to induction, group injury, inflammatory cells presented the type lymphocytes and macrophages in the lung tissue predominant form. In this study, after the inflammatory cell count, the most frequent cells were polymorph nucleate inflammatory cells (neutrophils) and mononuclear (macrophages and lymphocytes) in relation to other types of polymorph nucleate cells (neutrophils, monocytes and plasma cells (Jin *et al*., 2012). Histological analisis was performed in three days, after the first event involving the first pro-inflammatory cells. Ou *et al.* (2008) Report that in FP is common to find lymphocytes and macrophages. This statement is strengthened by Todd *et al.* (2013), Bargagli *et al.* (2011) and Subbian *et al*. (2011) when reporting substantial contribution to these cells in BLM-induced lung inflammation. As for Rossari (2004), the cells most often found during the acute phase of pulmonary fibrosis are macrophages.

According to Das and Roy (2015) in acute lung inflammation, inflammatory cells responds injuries, repairing or removing damaged tissues caused by physical, chemical or biological events. The process involves endothelial cells, epithelial cells and alveolar macrophages; and chemokines, adhesion molecules and secreting tissue factor (Papakonstantnou and Karakiulakis 2009). Monocytes are directed to the site followed by macrophages. Macrophages in turn, are responsible for the high concentrations of reactive oxygen species and nitrogen (Papakonstantnou and Karakiulakis 2009); (Smith *et al.,* 2010). It was observed in this study, the increase in hydroxyproline in the lung tissue injury in group also induced by BLM in relation to Healthy and vehicle groups. This finding is explained by the involvement of more events of inflammation that leads to pulmonary fibrosis. Hydroxyproline is an amino acid present in the composition of collagen induced pro-fibrotic proteins synthesized by transdifferentiation of quiescent fibroblasts into myoblasts by Hunninghake and Schwarz (2007), Smith *et al.* (2010), Cheresh *et al*. (2013), Luo *et al.* (2014), Shi et al. (2014), Kim *et al.* (2015) and hence increase of collagen expression by Li *et al.* (2015).

Conclusion

It is concluded that the induction proposed by this study, by injection instilled into the trachea without surgical access was satisfactory, producing inflammatory cells and increase in hydroxyproline production resulting from PF. The induction by direct instillation of BLM into the trachea without surgical access and aided by palpation is a quick and economical method; eliminates the complications of surgical procedures and reduces the risk of death of the animals.

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