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ORIGINAL ARTICLE

Photobiomodulation therapy improves both inflammatory and fibrotic parameters in experimental model of lung fibrosis in mice

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Abstract Lung fibrosis (LF) is a chronic and progressive lung disease characterized by pulmonary parenchyma progressive lesion, inflammatory infiltration, and interstitial fibrosis. It is developed by excessive collagen deposition and other cellular matrix components, resulting in severe changes in the alveolar architecture. Considering the absence of effective treatment, the aim of this study was to investigate the effect of photobiomodulation therapy (PBMT) on the development of PF. For this purpose, we used C57BL6 mice subjected to induction of LF by bleomycin administration (1.5 U/kg) by orotracheal route and, after 14 days of the induction, mice were treated with PBMT applied to the thorax 1×/day for 8 days (wavelength 660 ± 20 nm, power 100 mW, radiant exposure 5 J/cm^2, irradiance 33.3 mW/cm^2, spot size 2.8cm^2, total energy 15 J, time of irradiation: 150 s) and inflammatory and fibrotic parameters were evaluated with or without PBMT. Our results showed that PBMT significantly reduced the number of inflammatory cells in the alveolar space, collagen production, interstitial thickening, and static and dynamic pulmonary elastance. In addition, we observed reduced levels of IL-6 e CXCL1/KC released by pneumocytes in culture as well as reduced level of CXCL1/KC released by fibroblasts in culture. We can conclude that the PBMT improves both inflammatory and fibrotic parameters showing a promising therapy which is economical and has no side effects.

Keywords Photobiomodulation therapy (PBMT) · Lung fibrosis · Lung elastance · Cytokines · Collagen production · Mice

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Introduction

Lung fibrosis (LF) is a chronic and progressive lung disease characterized by inflammation and development of excessive extracellular matrix deposition culminating in an irreversible loss of lung function [1, 2]. Data obtained by lung fibrosis foundation estimate that lung fibrosis affects 1 out of 200 adults over the age of 65 in the USA. Approximately 50,000 new cases are diagnosed each year and as many as 40,000 Americans die from LF by year. Moore et al. [3] showed that inflammatory episodes in fibrotic patients were related with the disease's progression, while other studies attribute the development of disease to capacity of epithelial cells origin to myofibroblasts, thus inducing proliferation and activation of fibroblasts [4]. Moreover, activated fibroblasts generate many cytokines and collagen contributing to the lung remodeling [5]. During the lung repair process, there is a production of TGF-beta, which is important to fibroblast differentiation in

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myofibroblast [6]. After that, there is an increased synthesis and secretion of collagen fibers followed by collagen deposition.

The biggest problem of LF is an ineffective treatment. Any treatment has demonstrated positive effects in the stabilization of disease or in the improvement of life quality. The first line of pharmacological therapy is based on the anti-inflammatories. Thus, steroidal anti-inflammatories, such as corticoids, are used due its wide action mechanisms. Oxygen therapy is recommended for patients who present hypoxemia. However, all of these therapies do have not good results on the disease control. So, new therapies are necessary. In this context, phototherapies emerge as promisor therapy.

Light-emitting diode (LED) is a photobiomodulation therapy (PBMT) characterized by the light emission non-coherent and non-collimated. Their photo stimulation exerts anti-inflammatory and anti-fibrotic effects by interfering with release of inflammatory mediators [5–7] and inhibiting fibroblast proliferation [8]. In previous studies, our group showed promisor effects of PBMT in experimental model of asthma [9]. Our results showed that PBMT in asthmatic mice reduced the lung cell recruitment, mucus production, oedema, and the tracheal's contractile response. Such effects were modulated by IL-10, IFN-gamma, and by mast cells.

Experimentally, the LF can be developed by bleomycin administration. It is an antineoplastic agent that can cause the lung toxicity. The lung toxicity is related to several inflammatory mediators by macrophages including TGF-beta, TNF-alpha, chemokines among others, which cause severe lung fibrosis as side effect.

Considering that the pathogenesis of LF is associated with exacerbated lung inflammation and elevated production and deposition of collagen in the lung parenchyma and, the PBMT showed important anti-inflammatory effects in addition to the modulation of fibroblast activity, our objective was to investigate the effects of PBMT on the development of LF induced by bleomycin.

Material and methods

Animals and groups of study

Male mice (C57BL/6) were obtained from the University Nove de Julho and maintained 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 Animal Care Committee University Nove de Julho (CoEP-UNINOVE, AN0038/2014).

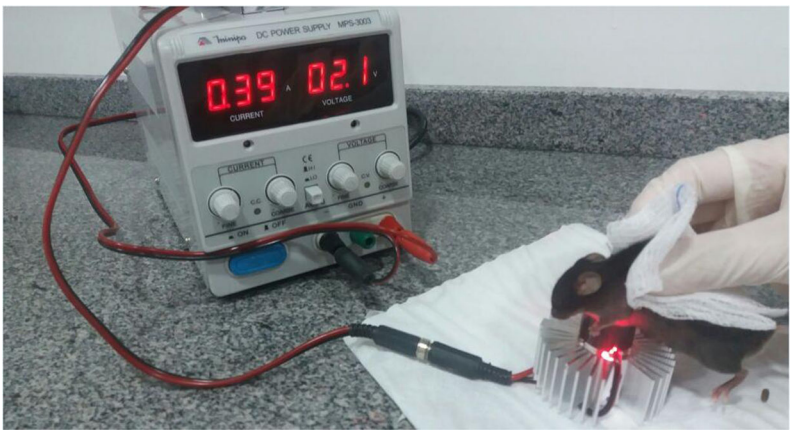
The animals were divided into four experimental groups: Basal, non-manipulated mice; F, mice subjected to bleomycin for LF induction; PBMT, mice treated with LED; and F+ PBMT, mice treated with LED 14 days after the induction of LF. Groups of mice were killed by sectioning the abdominal aorta under deep anesthesia with ketamine-xylazine by intraperitoneal route (100 and 20 mg/kg, respectively) at day 22.

Induction of lung fibrosis

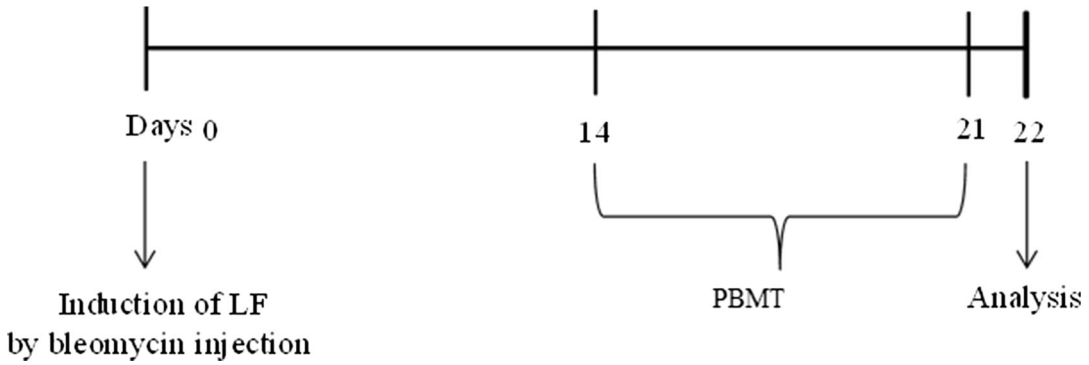
Lung fibrosis was induced by bleomycin administration by orotracheal route (1.5 U/Kg,) according to Moore et al. [3]. After light anesthesia, the administration of bleomycin was performed by mouth of mice through direct introduction into the trachea. For this administration, we used a micropipette.

Photobiomodulation therapy

The animals were irradiated in the respiratory tract (lungs and trachea) by direct contact with skin according to Cardoso Siqueira [9]. The PBMT was performed 14 days after the bleomycin injection. The animals were irradiated 1×/day for 8 days. The parameters of PBMT were based on previous studies [9] and the following: The peak of wavelength was 660 nm (full width half maximum 20 nm); power 100 mW, radiant exposure 5 J/cm^2, irradiance 33.3 mW/cm^2, spot size 2,8cm^2, total energy 15 J, time of irradiation150 s.



Experimental design



Evaluation of cells recovered in the bronchoalveolar lavage

In order to evaluated the lung inflammation, the tracheae of mice was cannulated and the alveolar space was flushed twice with phosphate-buffered saline (PBS, 1.5 ml total volume). The collected bronchoalveolar lavage (BAL) was centrifuged (1500 rpm for 15 min at 20 °C), and the resulting cell pellet was resuspended in 1 ml of PBS. The cell suspension was stained with crystal violet, and the total cell number was determined microscopically using a Neubauer chamber.

Measurement 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. Briefly, mice were anesthetized with a ketamine-xylazine mixture, 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. Lung static elastance (E_{st}) and lung dynamic elastance (E_{dyn}) were measured by means of end-inspiratory airway occlusions [10]. Closed chest represents the lung mechanical properties of respiratory tract, including the rib cage; and open chest represents the lung mechanical properties only of the lung. After an end-inspiratory occlusion, there was a fast initial drop in lung pressure ($\Delta \mathbf{P}1$) from the pre-occlusion value down to an inflection point (with pressure Pi), followed by a slow pressure decay ($\Delta \mathbf{P}2$) until a plateau pressure (P_{el}) corresponding to the elastic recoil pressure of the lung is reached. Whereas $\Delta \mathbf{P}1$ is associated with pressure dissipated against pulmonary resistance, $\Delta \mathbf{P}2$ reflects tissue viscoelastic properties or pendelluft. Taking into account the value of pre-inspiratory pressure (P_o), lung static elastance (E_{st}) was computed as the adjusted plateau pressure (P_{el}–P_o) recorded after 5 s of occlusion divided by the tidal volume. E_{dyn} was computed by dividing the adjusted inflection point pressure ($\mathbf{P}_i$–P_o) by the tidal volume. E_{st} and E_{dyn} were obtained as the means from five end-inspiratory occlusions, each one carried out after 1 min of normal mechanical ventilation.

Evaluation of collagen production as well as interstitial tissue thickening

Histological analyses were performed according to Miranda da Silva et al. [11]. Lung tissues were stained with Picrossirius for collagen and interstitial tissue thickening analysis. The collagen analysis was performed by image using the Image Pro plus program, while the interstitial tissue thickening was

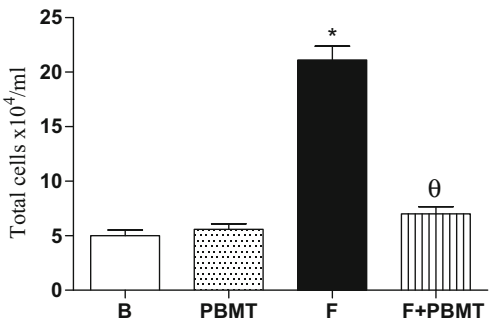


Fig. 1 *Effect of PBMT on the lung cell recruitment*. Groups of mice were subjected or not to lung fibrosis and after 14 days were treated or not with PBMT for eight consecutive days. After 24 h of the last PBMT, the total number of cells recovered in the bronchoalveolar lavage was determined. Data represent the mean ± SEM of eight animals. $^*P < 0.05$ in relation to B and PBMT groups; $^{\theta}P < 0.05$ in relation to F group

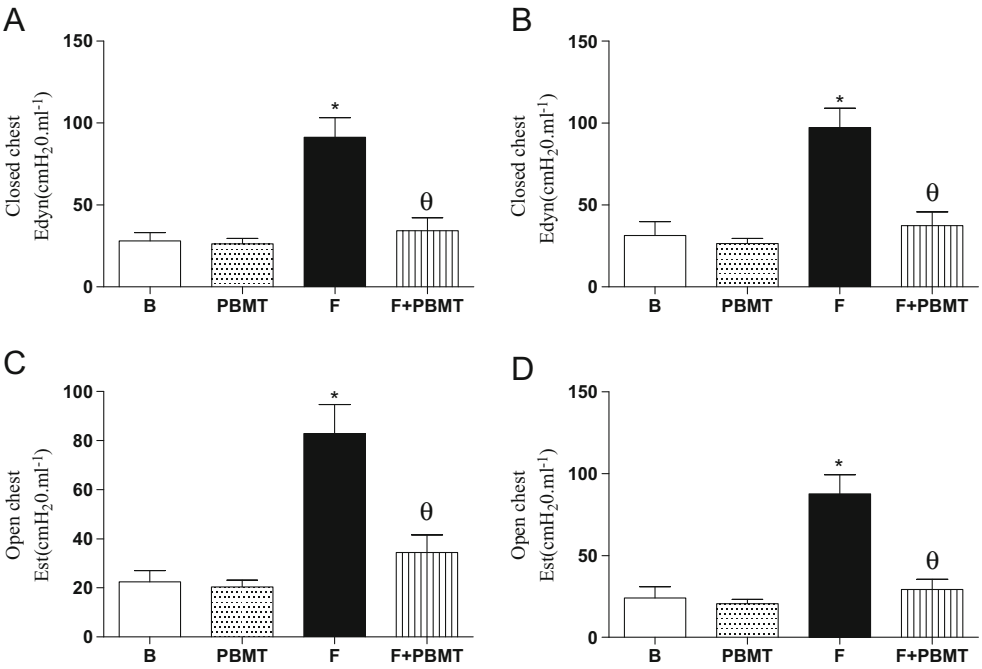


Fig. 2 *Effect of PBMT on the lung elastance*. Groups of mice were subjected or not to lung fibrosis and after 14 days were treated or not with PBMT for eight consecutive days. After 24 h of the last PBMT, the dynamic elastance (**a**, **b**) and the lung static elastance (**c**, **d**) were determined. Data represent the mean ± SEM of eight animals. $^{*}P < 0.05$ in relation to B and PBMT groups; $^{\theta}P < 0.05$ in relation to F group

determined by histomorphometric technique and below are described the scores used for the evaluations.

Interstitial tissue thickening: 0, absence; 1, increased cellularity in interstitial tissue; 2, increased cellularity in interstitial tissue and tissue thickness in up to one cell layer; 3, increased cellularity in interstitial tissue and tissue thickness in up to two cell layers; 4 increased cellularity in the interstitial tissue and tissue thickness with visible presence of collagen fibers.

Quantification of cytokines released in the bronchoalveolar lavage fluid and in the supernatant of fibroblasts and pneumocytes in culture

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 picogram of cytokine produced per milliliter. The assays were made in duplicate for every sample using standard curves for IL-1beta, TNF-alpha, IL-17A, IL-6, IL-10, IFN-gamma, and CXCL-1/KC.

Isolation and culture of lung fibroblasts and pneumocytes

The lungs were removed, fragmented, and immersed in sterile enzymatic digestion solution (2.5 mg dispase II/l and 50 μg/ml DNase I), penicillin (1%), and streptomycin (1%) at 37 °C for 60 min. After digestion, the material was filtered and the cells resuspended and cultured in specific media as each cell type.

Fibroblasts

Cells were suspended in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum + 4.5 g glucose/L and 1% gentamycin and transferred to Petri dishes and incubated at 37 °C, 5% CO_2 in a humidified greenhouse for 20 min. The non-adherent cells were removed and discarded. Adherent cells were collected and transferred to a cell culture bottle of 175 cm^2 and incubated under the same conditions as above until they reach at least 90% desired confluence (1×10^5 cells). After that, cells were collected using 1% trypsin solution and the cell viability was assessed by tripan blue exclusion. Cells were cultured with or without bleomycin and irradiated or not with LED. The parameters used to LED irradiation were similar to in vivo studies.

Pneumocytes

The cells were suspended in RPMI 1640 medium plus 1% gentamycin, and they were placed in Petri dishes and incubated at 37 °C, 5% CO_2 in a humidified greenhouse for 20 min. The unbound cells were carefully collected and transferred to a cell culture bottle in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% gentamycin, and then incubated

at 37 °C, 5% CO_2 in a humidified greenhouse until they reached at least 90% confluence. After reaching the desired confluence, the cells were collected using 1% trypsin, and the number and purity of the pneumocytes was assessed by Papanicolau's staining. Cells were cultured with or without bleomycin and irradiated or not with LED.

Statistical analyses

Data were expressed as the means ± SEM, and comparisons among the experimental groups were analyzed by one-way ANOVA followed by the Student's Newman-Keuls test for multiple comparisons using the GraphPad software V.5. *P* values less than 0.05 were considered statistically significant.

Results

LED treatment reduces cell influx in the BAL of fibrotic mice

Figure 1 showed that F+PBMT group had a decreased number of total cells recovered in the BAL (striped bar) when compared to non-treated group (black bar). In addition, no

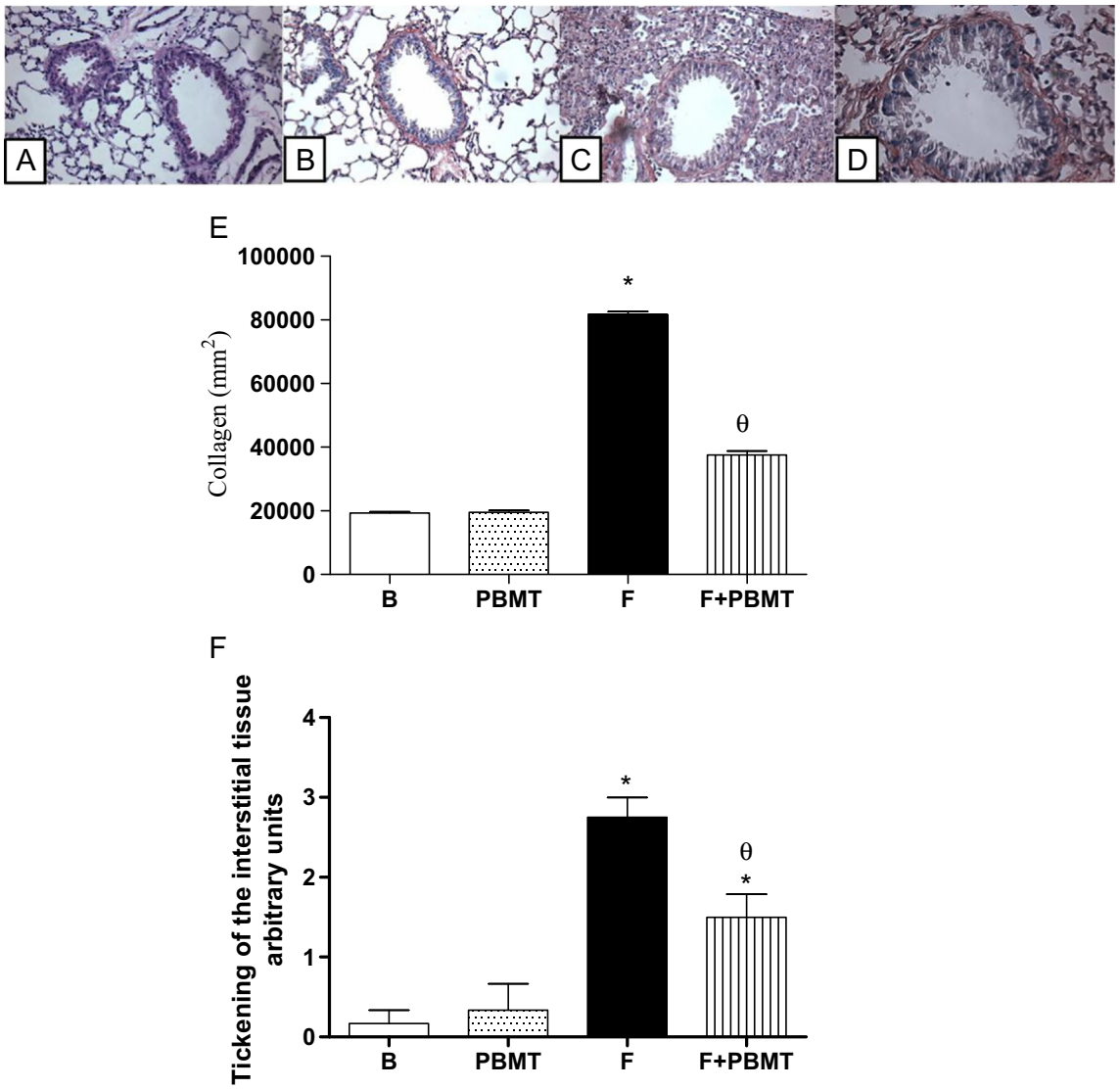


Fig. 3 *Effect of PBMT on the collagen production* (**e**) *and on the tickening of the interstitial tissue* (**f**). Groups of mice were subjected or not to lung fibrosis and after 14 days were treated or not with PBMT for eight consecutive days. After 24 h of the last PBMT, the lungs were removed and submitted to histomorphometric analysis. A = basal; B = PBMT; C = F; D = F+PBMT. Data represent the mean ± SEM of eight animals. *$P < 0.05$ in relation to B and PBMT groups; $^{\theta}P < 0.05$ in relation to F group

differences were observed among the B (white bar), PBMT (dots bar) and F+PBMT (striped bar) groups.

LED treatment reduces dynamic and static elastances of fibrotic mice

As can be observed in Fig. 2, F+PBMT group (striped bar) showed reduced dynamic and static elastances (panels A–D) when compared to the F group (black bar). In addition, we noted that this reduction occurred not only in open chest (A and C), but also in closed chest (B and D). Moreover, we showed elevated dynamic and static elastances in F groups (striped bar) independently of their conditions (open or closed chest) when compared to the control groups B (white bar) and PBMT (dots bar). No differences were found between B and PBMT groups.

LED treatment reduces collagen production and interstitial tissue thickening of fibrotic mice

Figure 3 showed reduced collagen production in F+PBMT group (panel D) when compared to PBMT and F groups, which are represented respectively by panels C and E. No differences were observed between B and PBMT (panels A and B, respectively).

In panel E, we showed that F+PBMT group (striped bar) had reduced collagen production when compared to F group (black bar). Moreover, elevated collagen production was found in F group in relation to B (white bar) and PBMT (dots bar) groups.

In panel F, we observed that F+PBMT group (striped bar) had reduced interstitial tissue thickening when compared to F group (black bar). Moreover, elevated interstitial tissue thickening was observed in F+PBMT group in relation to B (white bar) and PBMT (dots bar) groups.

LED treatment increases IFN-gamma in the BAL fluid of fibrotic mice

In order to understand the effects of PBMT on the lung fibrosis, we evaluated inflammatory and anti-inflammatory cytokines released in the BAL fluid. In Fig. 4, we showed reduced levels of TNF-alpha, IL-17A, and IL-6 (panels B, C, and D, respectively) in F+PBMT group (striped bar) when compared to the F group (black bar). No differences were noted in the levels of IL-1beta and IL-10 among groups of study (panels A and E). On the other hand, the F+PBMT group showed increased IFN-gamma levels (striped bar) when compared to F group (black bar) as well as B (white bar) and PBMT (dots bar) groups (panel F).

LED treatment reduces CXCL1/KC released by fibroblasts in culture of fibrotic mice

Considering that fibroblasts are important cells involved in the development of lung fibrosis, we investigated the effects of PBMT on the functional state of these cells. We can observe in Fig. 5 that no differences were found in the levels of IL-6, IL-1beta, IL-10, and TNF-alpha (panels A, B, C, and D) of F+PBMT group (striped bar) in relation to F group (black bar).

In panel E, no differences were noted in the levels of IFN-gamma among groups of study. However, F+PBMT group (striped bar) decreased CXCL1/KC when compared to F group (black bar) as well as B (white bar) and PBMT (dots bar) groups (panel F).

LED treatment reduces IL-6 and CXCL1/KC released by pneumocytes in culture of fibrotic mice

We can observe in Fig. 6 panel A, F+PBMT group (striped bar) presented reduced levels of IL-6 released by pneumocytes in culture when compared to F group (black bar). Moreover, we showed that the levels of IL-1beta, IL-10, TNF-alpha, and IFN-gamma of F+PBMT group did not differ from F group (panels B, C, D, and E). However, F+PBMT group decreased CXCL1/KC when compared to F group as well as B (white bar) and PBMT groups (dots bar) (panel F).

Discussion

The aim of this study was to evaluate a new therapy for the treatment of LF, considering that it is a chronic and progressive lung disease whose treatment is not effective. In this context, light treatment has shown significant positive effects on the lung diseases; however, its mechanisms need to be explored.

We investigated the effects of PBMT on the development of LF using an experimental model induced by bleomycin. This model is well established in the literature that is characterized by lung inflammation, activation of epithelial cells, fibroblasts, and increased deposition of collagen fibers in the lung parenchyma [3].

As we could expect, bleomycin administration evoked a marked influx of leukocytes in the BAL, increased pulmonary elastance, and collagen production. The augmented number of leukocytes in the BAL was reversed by PBMT.

The participation of inflammatory cells in the development and progression of LF is controversial. Many authors attribute the progression of disease to an excessive and disorganized deposit of collagen and other extracellular matrix components, resulting in severe changes in

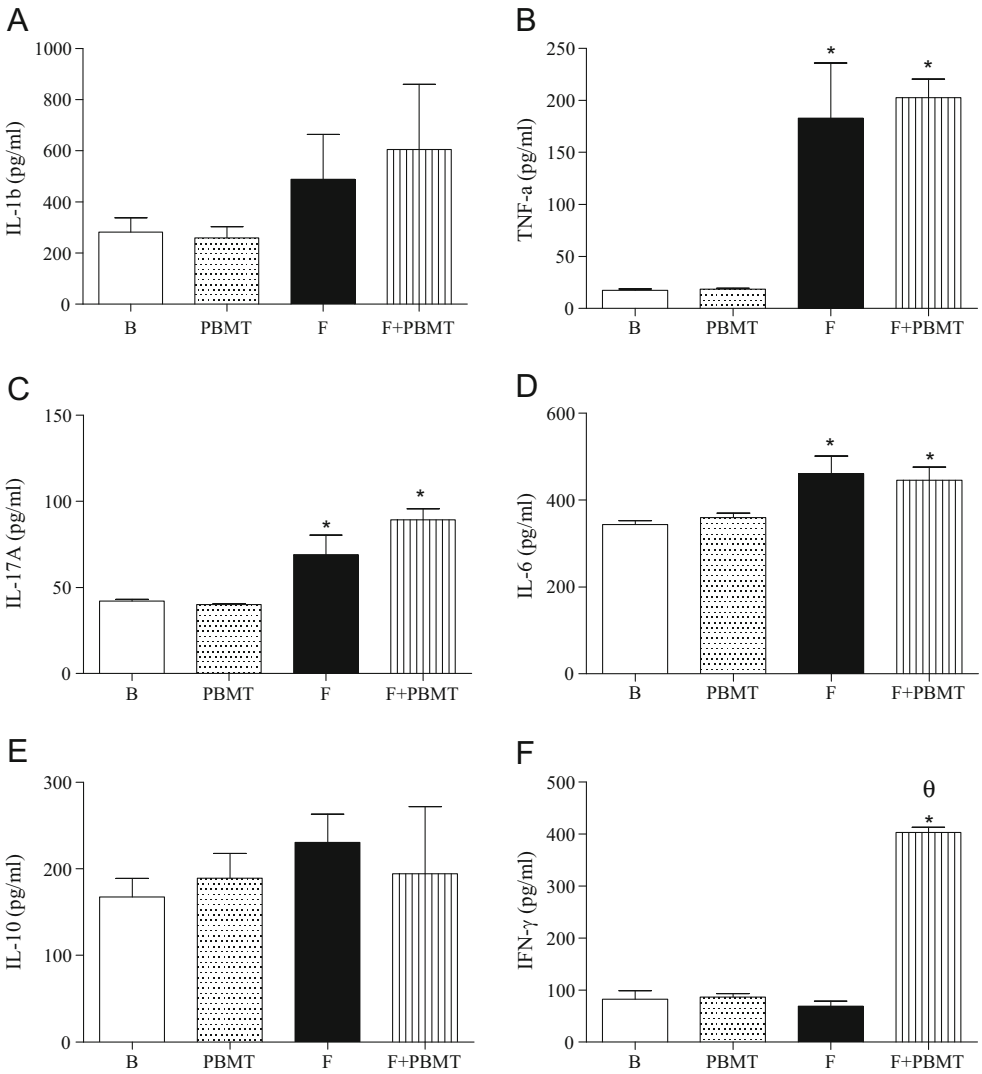


Fig. 4 *Effect of PBMT on the cytokines release in the bronchoalveolar lavage fluid.* Groups of mice were subjected or not to lung fibrosis and after 14 days were treated or not with PBMT for eight consecutive days. After 24 h of the last PBMT, the cytokines were quantified. Data represent the mean ± SEM of eight animals. $^{*}P < 0.05$ in relation to B and PBMT groups; $^{\theta}P < 0.05$ in relation to F group

alveolar wall architecture [12]. On the other hand, evidences suggest important role of inflammatory cells in the development of LF. In this context, we showed that PBMT exerts beneficial effects reducing lung cell infiltration. Still, the exudate accumulation of vascular and inflammatory cells within the alveolar space causes epithelial injury increasing the proliferation of resident fibroblasts and differentiation into myofibroblasts [13].

Our data showed that the anti-inflammatory effects of PBMT are in agreement with other data of the literature, which were utilized photobiomodulation in different models of lung disease [9, 14–16]. Moreover, we can note that almost of the studies were performed with laser therapy and, independent on the differences between laser and LED, both therapies, exert important effects ameliorating the development of lung inflammation. The main difference between laser and LED is the light emission. Laser is characterized by coherence while LED is non-coherent. However, the coherence of laser light is not responsible for the effects of therapy, because this property is lost in the first layers of biological tissues [5–7]. Thus, despite of the light emission of laser to be different from LED, we admit based on the literature that the effects of both are similar.

Laser therapy has been studied for the treatment of inflammatory lung diseases in animal models [11, 14–17], as well as in humans [18, 19]. On the other hand, there were few studies evaluating the biological effects of LED on the lung diseases, but they seem to be as effective as laser. Recently, our group showed beneficial effects of LED treatment on asthma development [9]. We showed that LED treatment reduced the lung cell infiltration in the lung, mucus production, and oedema. These effects might be modulated by the IL-10, IFN-gamma, and mast cells.

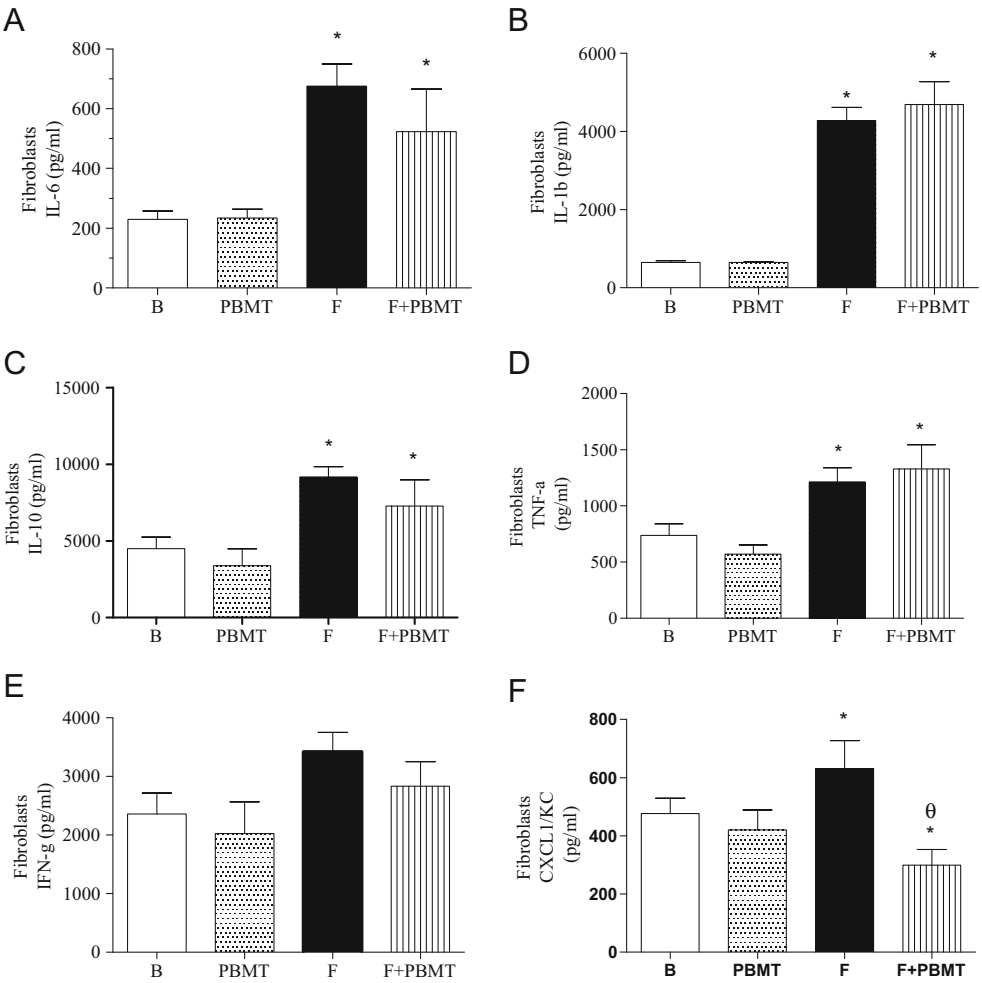


Fig. 5 *Effect of PBMT on the cytokines release in the fibroblasts in culture*. Lungs from non-manipulated mice were removed and the fibroblasts were isolated and cultured with or without bleomycin. In parallel, cultures of fibroblasts were treated or not with PBMT directly on the culture plate. After 24 h, the supernatants were collected and the cytokines were evaluated. Data represent the mean ± SEM of eight animals. $^{*}P < 0.05$ in relation to B and PBMT groups; $^{\theta}P < 0.05$ in relation to F group

Subsequently, we analyzed the dynamic and static lung elastance, important parameters related to mechanic proprieties of lung, which they are altered in PF. We showed elevated dynamic and static elastance after bleomycin administration, and PBMT reversed these responses. Our study was performed with closed and open chest. This approach allows the assessment of all components involved in breathing (chest wall and lung) as well as assessing only the lung tissue response. Thus, closed chest represents the lung mechanical properties of chest wall and lung, while open chest represents the mechanical of only the lung tissue. Our data showed that PBMT reduced elastance in both situations.

Considering the central role of collagen in the development/progression of LF, we evaluated the collagen production. Our data showed reduced collagen deposition after the PBMT. These results might be responsible, at least in part, by reduced lung elastance as well as interstitial tissue thickening. Besides that, they are very promising considering that nowadays any treatment is effective to reverse collagen deposition in the lung in patients with fibrosis. Recently, clinical data indicate that LED may improve the skin fibrosis. The authors showed that the cellular features of skin fibrosis were modulated by decreased of cellular proliferation, collagen production, and migration speed of human skin fibroblasts [20].

The irradiation was performed locally directly on the respiratory tract on the skin, and thus its effects were circumscribed to the site of the injury. In general, we have seen that our data are in accordance with the literature data regarding the effects of light on lung cell recruitment. Despite of the reduced cell recruitment, the PBMT did not modify the inflammatory cytokines released. On the other hand, we also showed an increase in the levels of IFN-gamma that in fibrosis model can regulate the transcription of TGF-beta gene [21].

In order to explain the mechanisms involved in the PBMT, we evaluated the effects of irradiation on fibroblasts and type II pneumocytes cultured in vitro. Our data showed that PBMT in vitro reduced the release of

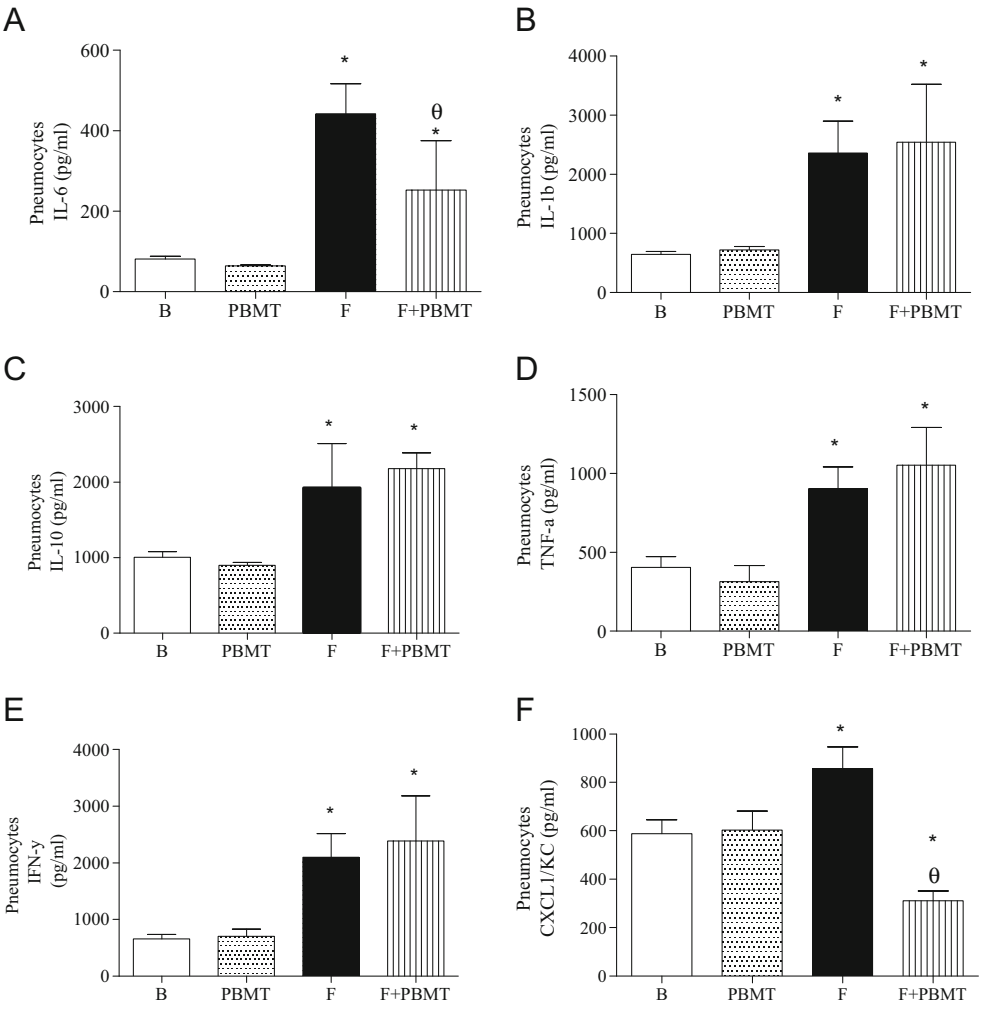


Fig. 6 *Effect of PBMT on the cytokines release in the pneumocytes type II in culture*. Lungs from non-manipulated mice were removed and the pneumocytes were isolated and cultured with or without bleomycin. In parallel, cultures of pneumocytes were treated or not with PBMT directly on the culture plate. After 24 h, the supernatants were collected and the cytokines were evaluated. Data represent the mean ± SEM of eight animals. $^{*}P < 0.05$ in relation to B and PBMT groups; $^{\theta}P < 0.05$ in relation to F group

CXCL1/KC by fibroblasts and pneumocytes in culture as well as reduced the levels of IL-6 by pneumocytes in culture. Thus, we can suppose that the anti-fibrotic effect of LED was due to its modulation on the fibroblast activity. In fact, other studies showed that the LED treatment modulated the skin fibrosis thought reducing cellular fibroblast proliferation and migration speed, inhibiting profibrotic cytokines and decreasing synthesis and deposition of collagen [22, 23].

Considering that these cytokines modulate pulmonary cell recruitment, especially neutrophils, we believe that PBMT might modulate the release of these cytokines and thereby reduced the influx of cells into the lung. IL-6 is an inflammatory cytokine produced by several cell types, and a study shows that its inhibition decreases lung fibrosis in a murine model [24]. Furthermore, it has been suggested that activation of the CXCR2 chemokine receptor by CXCL1/KC (chemokine derived from keratinocytes) and CXCL2/MIP-2 (inflammatory protein of macrophages-2) may play a relevant role in angiogenesis [25, 26], in the neutrophil recruitment [27], and in hypernociception [28].

We can conclude that PBMT reduced cell migration, lung elastance, and collagen production, which are important for the development and progression of PF. Thus, our data point to the promising effects of PBMT to treat PF, constituting an additional tool, which might reflect an improvement in patients' quality of life.

Acknowledgments We thank Alessandro Melo De Ana by LED device.

Compliance with ethical standards

Competing interests The authors declare that they have no conflicts of interest.

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