FULL ARTICLE

Light-Emitting Diode treatment ameliorates allergic lung inflammation in experimental model of asthma induced by ovalbumin

Vanessa Perosino Cardoso Siqueira,1 Marina Izadora Silveira Evangelista,1 Alana dos Santos,1 Rodrigo Labat Marcos,1 Ana Paula Ligeiro-de-Oliveira,1 Christiane Pavani,1 Amílcar Sabino Damazo,2 and Adriana Lino-dos-Santos-Franco*,1

1 Post Graduate Program in Biophotonics Applied to Health Sciences, University Nove de Julho (UNINOVE), São Paulo, Brazil
2 Department of Basic Science in Health, Faculty of Medical Sciences, Federal University of Cuiabá, Cuiabá, Brazil.

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Since asthma is a multifactorial disease where treatment sometimes is not effective, new therapies that improve the respiratory discomfort of patients are of great importance. Phototherapy as Light-emitting diode (LED) has emerged as a treatment that presents good results for diseases that are characterized by inflammation. Thus, our objective was to investigate the effects of LED on lung inflammation, by an evaluation of lung cell infiltration, mucus secretion, oedema, and the production of cytokines. Male Balb/c mice were or not sensitized and challenged with ovalbumin (OVA) and treated or not with LED therapy (1 h and 4 h after each OVA challenge). Twenty-four hours after the last OVA challenge, analyzes were performed. Our results showed that LED treatment in asthmatic mice reduced the lung cell infiltration, the mucus production, the oedema, and the tracheal’s contractile response. It also increased the IL-10 and the IFN-gamma levels. The effects of LED treatment on lung inflammation may be modulated by IL-10, IFN-gamma, and by mast cells. This study may provide important information about the effects of LED, and in addition, it may open the possibility of a new approach for the treatment of asthma.

* Corresponding author: e-mail: alsantosfranco@gmail.com
1. Introduction

Studies have demonstrated that allergic lung diseases have been increased significantly throughout the world, affecting the quality of life of many people [1]. Asthma is an allergic lung inflammatory disease that is characterized by inflammatory cells that are recruited into the lungs, including neutrophils, eosinophils, mast cells, lymphocytes, macrophages, an elevated mucus secretion, airway remodeling, and hyperresponsiveness [2].

Asthma can be divided into immediate and late phase. The immediate phase occurs between 10–20 min after the allergen exposure, and it does not last more than 4 hours. It is characterized by bronchoconstriction, increased mucus production and lung oedema. Such effects are a reflex of inflammatory mediators released by mast cells that are activated after the antigen challenge [3]. On the other hand, the late phase of asthma begin between 6–8 hours after antigen exposure and it has, among other characteristics, an intense lung cell infiltration that includes eosinophils, neutrophils, mast cells, and lymphocytes. The mediators released by these activated cells determine the epithelial injury, the activation of the sensorial nerves, the secretion of mucus, the bronchial hyperresponsiveness, and the remodeling [4].

Since asthma is a multifactorial disease, its treatment is sometimes not effective. In this context, new therapies that improve the respiratory discomfort of asthmatic patients are of great importance. Phototherapy has emerged as an alternative therapy, presenting good results for those diseases that are characterized by inflammation. Among these phototherapies, low level laser therapy (LLLT) has been extensively studied due its anti-inflammatory and antioxidant effects [5,6,7]. Another promising phototherapy is LED treatment, but there are not any studies related to the respiratory tract and LED therapy.

LED is a semiconductor diode that is characterized by a monochromatic light emission, non-coherent and non-collimated. Such characteristics are different of LASER, since its light emission to be coherent and collimated. However, LED therapy has been shown to be as efficient as LASER therapy, since 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 [8,9,10].

Considering that the pathogenesis of asthma is associated with an exacerbated lung inflammation and that LED therapy has shown important anti-inflammatory effects, our objective was to investigate the effects of LED treatment on lung inflammation. Our evaluation focused the lung cell infiltration, the mucus secretion, the oedema, the mast cell activation, the production of cytokines, and the tracheal responsiveness.

2. Materials and Methods

2.1 Animals

Male 2-month-old Balb/c mice were obtained from the University Nove de Julho, and maintained in a light and temperature-controlled room (12/12-hour 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, AN0005/2015).

2.2 Induction of experimental asthma

Male mice were sensitized (10 μg of ovalbumin plus 10 mg of alumen, OVA-alum) by subcutaneous route. One week later, mice received another injection with OVA-alum by same route (booster). After 3 days of booster, the mice were submitted to an inhaled antigen challenge (aerosolized 1% OVA in PBS, 15 minutes) for three consecutive days. We used an ultrasonic nebulizer device (Icel®, SP, Brazil) coupled to a plastic inhalation chamber (20 L). The analyses were performed 24 hours after the last challenge with OVA. The mice were killed by sectioning the abdominal aorta under deep anesthesia with ketamine-xylazine (100 mg/kg and 20 mg/kg, respectively, ip) [11,12].

2.3 LED treatment

LED treatment was performed 1 and 4 h after each challenge with OVA. The application of LED was performed in the respiratory tract by direct contact with skin according earlier studies [5]. The period of treatment was based on the development of immediate phase of asthma (between 10 min and 4 hours after the antigen challenge). Thus, we could interfere in the initial phase, in order to prevent the development of late phase.

The parameters of LED are described below:
- Wavelength: 660 nm (Full Width Half Maximum 20 nm); Potency: 100 mW, Radiant exposure: 5 J/cm², Irradiance: 33,3 mW/cm², area: 2,8 cm², Total energy: 15 J, time of irradiation: 150 s.
2.4 Groups of study

Mice were divided into 3 experimental groups: OVA, identified as asthmatic mice; LED, identified as non-asthmatic mice treated with LED, and OVA + LED, identified as asthmatic mice treated with LED. In a parallel set of experiments, we also evaluated non-manipulated mice in order to determine basal parameters. However, the results obtained demonstrated that there are not differences between basal and LED groups. So, we showed LED group as control. Moreover, we used 6 animals per group.

2.5 Experimental design

![Experimental design diagram]

2.6 Evaluation of inflammatory response

In order to determine the number of cells recruited into the lung, we cannulated the tracheae of mice and the lungs were 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.

The differential cell count was performed by flow cytometry. Therefore, BAL cells were incubated with monoclonal antibodies anti-CD3 FITC surface, anti-CD11b and anti-Ly6G PerCP (Becton Dickinson - BD®, East Rutherford, NJ, USA) to phenotypic characterization of lymphocytes, macrophages, and granulocytes (neutrophils and eosinophils) respectively. Samples were acquired in BD Accuri flow cytometer analyzed in CSampler software (Becton Dickinson - BD®, East Rutherford, NJ, USA).

2.7 Evaluation of mobilization cell process

In order to understand the cell mobilization process, we quantified blood cells using an automated hematology analyzer Mindray BC 2800 Vet. Bone marrow cells were obtained by lavage of the femoral bone (5 ml). The fluid was centrifuged (1500 rpm for 15 min at 20 °C), and the pellet was resuspended in 1 ml of PBS. The cells were stained with crystal violet (0.2 %) and quantified in Neubauer chamber.

2.8 Lung histological analysis

Lung fragments were fixed in paraformaldehyde (4 %) for 24 h and after that the fragments were dehydrate in alcohol. Followed by alcoholic dehydration and diaphanization by xylene, the fragments were embedded in paraffin, deparaffinized, and sectioned at 5 μm (microtome HYRAX M60, Zeiss, GR). Moreover, the tissues were stained with hematoxylin and eosin for analysis of cell infiltration; toluidine blue for analysis of mast cells and; PAS (Pericid acid Shiff) for mucus and oedema analysis.

The data of score follow above.

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

**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 presence of plasma and red blood cells in the interstitial space.

**Perivascular infiltration:** 0, absent; 1, the discontinuous presence of leukocytes around the blood vessel; 2, the presence of continuous monolayer of leukocytes around the blood vessel; 3, the presence of leukocytes continuous layer containing two or three cells thick around the blood vessel; 4, presence of leukocytes continuous layer containing more than four cells thick around the blood vessel.

**Peribronchial infiltration:** 0, absent; 1, the discontinuous presence of leukocytes around the bronchioles; 2, the presence of continuous monolayer of leukocytes around the bronchioles; 3, the presence of leukocytes continuous layer containing two or three cells thick around the bronchioles; 4, presence of leukocytes continuous layer containing more than four cells thick around the bronchioles.

**Degranulation of mast cells:** 0, the presence of intact mast cells; 1, the presence of 25 % of degranulated mast cells; 2, the presence of 50 % of degranulated mast cells; 3, the presence of 75 % of degranulated mast cells; 4, the presence of 100 % of degranulated mast cells.
2.9 Quantification of cytokines in bronchoalveolar lavage fluid

Cytokines levels were determined in BAL supernatant samples. The results were expressed as pg of cytokine produced per ml. IL-4, TNF-alpha, IL-1beta, IL-17 A, IL-10, and IFN-gamma were quantified using ELISA kits purchased from Biolegend (San Diego, USA). Determinations were made in duplicate for every sample using standard curves according to the manufacturer’s specifications.

2.10 Maximum contractile response of tracheal tissue

In order to investigate the effects of LED treatment on smooth muscle contraction, isometric force development was quantified in tracheal rings mounted in a 15-ml organ bath by means of two steel hooks. Force contraction was recorded using a force displacement transducer and a chart recorder (Powerlab®, Labchart, AD Instruments). Tracheal rings were suspended in organ bath filled with continuously aerated (95% O2 and 5% CO2) Krebs-Hanseleit (KH) solution at 37 °C. After 40-min, the tracheal tension was adjusted to 0.5 g and was added methacholine (MCh, 10⁻⁵M) in order to obtain maximum contractile response.

2.11 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.

3. Results

3.1 LED treatment reduced the cell infiltration by decreased number of lymphocytes, macrophages and granulocytes in asthmatic mice

Figure 1 (Panel A) showed elevated number of total cells recruited in BAL of OVA group in relation to LED group. On the other hand, LED treatment in asthmatic mice reversed the increased cell influx in the BAL when compared to the OVA group. No differences were observed between OVA + LED and LED groups.

Panels B, C and D showed elevated number of macrophages, lymphocytes, and granulocytes recruited in the BAL of asthmatic mice in relation to LED group. We also observed reduced all types of cells in the BAL (macrophages, lymphocytes, and granulocytes) of OVA + LED group when compared to OVA group and did not differ from LED group.

The dot plot of differential cells is presented in Figure 2. We can observe in panels A, D and G the percentage of lymphocytes of LED, OVA and OVA + LED groups respectively. In panels B, E and H we showed the percentage of macrophages of LED, OVA and OVA + LED groups. Finally, in panels C, F and I we showed the percentage of granulocytes of LED, OVA and OVA + LED groups.

3.2 LED treatment modified the cell mobilization in the blood and in the bone marrow in asthmatic mice

Figure 3 (Panel A) we can observe increased number of neutrophils in the blood in OVA group in relation to LED group. We did not observe differences in monocytes, basophils, lymphocytes, and eosinophils in the blood of OVA group when compared to the LED group. On the other hand, LED treatment in asthmatic mice reduced neutrophils in the blood when compared to OVA group.

In panel C we showed decreased number of cells in the bone marrow in OVA group in relation to LED group. Moreover, increased number of cells in the bone marrow of OVA + LED group was observed in relation to OVA group.

3.3 LED treatment increased IL-1 beta, IL-10 and IFN-gamma levels in the BAL fluid of asthmatic mice

Figure 4 (Panel A) showed increased IL-4 levels in OVA group in relation to LED group and the LED treatment in asthmatic mice (OVA + LED group) did not reversed this response.

Panels B and F showed that no differences in IL-1beta and IFN-gamma levels in BAL fluid were found between LED and OVA groups. However, mice of OVA + LED group enhanced IL-1beta as well as IFN-gamma levels in relation to LED group and did not differ from OVA group.

We can observe in panel C that the TNF-alpha levels in BAL fluid in all groups of study did not differ. We also observed that IL-17 A levels were increased in OVA group in relation to LED group. No differences were observed in OVA + LED group (Panel D).
Panel E showed elevated level of IL-10 in both groups (OVA and OVA + LED) in relation to LED group. Moreover, OVA + LED group showed an additional increase in relation to OVA group.

3.4 LED treatment reduced mast cell degranulation and mucus production in asthmatic mice

Figure 5 Panels A, B and C showed the mast cell degranulation of LED, OVA and OVA + LED groups respectively. The representative graph of figures A, B and C can be observed in panel G. It showed elevated degranulation of mast cell in OVA group when compared to LED group. On the other hand, we can also observe decrease mast cell degranulation in OVA + LED group in relation to OVA group. No differences were observed between LED and OVA + LED groups.

Panels D, E and F showed the mucus production of LED, OVA and OVA + LED groups respectively by the presence of goblet cell metaplasia in the bronchial epithelium and a thick mucus layer on the epithelial surface. The representative graph of figures D, E and F can be observed in panel H. It
showed elevated mucus production in OVA group when compared to LED group. On the other hand, we can also observe decrease mucus production in OVA + LED group in relation to OVA group. No differences were observed between LED and OVA + LED groups.

3.5 LED treatment reduced oedema and vascular infiltration in asthmatic mice

Figure 6, Panels A, B and C showed oedema and vascular infiltration of LED, OVA and OVA + LED groups respectively. Table 1 represents the result of Figure 6.

Figure 6 and Table 1 showed that OVA group had an increased oedema when compared to LED group. On the other hand, the OVA + LED group
reduced the oedema in relation to OVA group and did not differ from LED group.

We also observed elevated bronchiolar and vascular infiltrate in OVA group when compared to LED group. However, OVA+LED group reduced vascular infiltrate in relation to OVA group. No differences were observed in relation to bronchiolar infiltrate between OVA and OVA+LED groups.

Table 1  Oedema and cell infiltration in allergic mice after LED treatment.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>ODEMA</th>
<th>BRONCHIOLAR INFILTRATE</th>
<th>VASCULAR INFILTRATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LED</td>
<td>0.2 ± 0.4</td>
<td>0.2 ± 0.4</td>
<td>0.3 ± 0.5</td>
</tr>
<tr>
<td>OVA</td>
<td>3.3 ± 0.5*</td>
<td>1.8 ± 0.7*</td>
<td>3.8 ± 0.4*</td>
</tr>
<tr>
<td>OVA+LED</td>
<td>1.7 ± 0.5*</td>
<td>1.5 ± 0.5*</td>
<td>1.8 ± 0.7*</td>
</tr>
</tbody>
</table>

LED treatment reduces oedema and cell infiltration in asthmatic mice. Group of mice were sensitized (days 1 and 7, OVA, sc) and after 12 days the animals were submitted to an inhaled antigen challenge (aerosolized 1 % OVA, 15 min) for three consecutive days. In addition, group of mice were treated with LED 1 and 4 h after each OVA challenge or not. The oedema, bronchiolar and vascular infiltration was determined 24 h after the last OVA challenge by histological analysis. Data mean ± SEM of 6 animals per group and 5 different fields per animal. *P < 0.05 in relation to LED group; **P < 0.05 in relation to OVA group.

Figure 3  LED treatment modulates the cell mobilization in asthmatic mice. Group of mice were sensitized (days 1 and 7, OVA, sc) and after 12 days the animals were submitted to an inhaled antigen challenge (aerosolized 1 % OVA, 15 min) for three consecutive days. In addition, group of mice were treated with LED 1 and 4 h after each OVA challenge or not. The analysis of cells in the blood (A) and in the bone marrow (B) was performed 24 h after the last OVA challenge. Data mean ± SEM of 6 animals per group. *P < 0.05 in relation to LED group; **P < 0.05 in relation to OVA group.

Figure 4  LED treatment increased IL-1 beta, IL 10 and IFN-gamma levels in the BAL fluid of asthmatic mice. Group of mice were sensitized (days 1 and 7, OVA, sc) and after 12 days the animals were submitted to an inhaled antigen challenge (aerosolized 1 % OVA, 15 min) for three consecutive days. In addition, group of mice were treated with LED 1 and 4 h after each OVA challenge or not. The quantification of cytokines was performed 24 h after the last OVA challenge by ELISA. Data mean ± SEM of 6 animals per group. *P < 0.05 in relation to LED group; **P < 0.05 in relation to OVA group.
Moreover, OVA + LED group showed elevated bronchiolar and vascular infiltrate in relation to LED group.

3.6 LED treatment reduced the maximum tracheal contractile response in asthmatic mice

Table 2 showed that mice of OVA group presented elevated tracheal contractile response in relation to LED group. We also observed that mice of OVA + LED group reduced the tracheal responsiveness in relation to OVA group.

4. Discussion

We investigated the effects of LED treatment on the local inflammatory parameters using an experimental model of asthma induced by ovalbumin (OVA). This model is well-established in the literature that is characterized by an increased number of cells in the BAL, elevated levels of cytokines, increased mucus production, oedema, and bronchial hyperresponsiveness [12, 13, 14]. The objective of our study was to evaluate a new approach for the treatment of lung diseases that was based on the photobiomodulation therapy. The photobiomodulation therapy principle was introduced more than 30 years ago, being applied primarily in dermatology, in order to accelerate the healing process of labial herpes, paresthesias, dental hypersensitivity, and postoperative procedures [15]. For the first time, we are showing here, the beneficial effects of LED therapy on experimental model of asthma.

Light therapy has been investigated for the treatment of lung inflammatory diseases in both animal [5, 6, 16, 17] and human [18, 19] models. These promising results encourage new studies.

LED therapy has been shown to be as efficient as LASER therapy. It is an effective alternative to the LASER, since it can be produced through multiple arrangements. LED light stimulation seems to exert anti-inflammatory effects in some inflammatory processes, including the regeneration of Achilles tendon [20, 21].

Table 2 Tracheal contractile maximum response in allergic mice after LED treatment.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>CONTRACTILE RESPONSE (g/100 mg tissue)</th>
</tr>
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<tbody>
<tr>
<td>LED</td>
<td>6.24</td>
</tr>
<tr>
<td>OVA</td>
<td>18.40*</td>
</tr>
<tr>
<td>OVA + LED</td>
<td>7.66q</td>
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</tbody>
</table>

LED treatment reduces maximum tracheal responsiveness in asthmatic mice. Group of mice were sensitized (days 1 and 7, OVA, sc) and after 12 days the animals were submitted to an inhaled antigen challenge (aerosolized 1% OVA, 15 min) for three consecutive days. In addition, group of mice were treated with LED 1 and 4 h after each OVA challenge or not. The determination of tracheal maximum contractile response to methacholine (MCh) was performed 24 h after the last OVA challenge. Data mean ± SEM of 6 animals per group. *P < 0.05 in relation to LED group; qP < 0.05 in relation to OVA group.
Considering that asthma is a chronic inflammatory lung disease, we decided to investigate whether a drug therapy could be replaced by LED treatment. The LED parameters were based on LASER irradiation techniques that have already been used in different models of lung diseases [16,17,22]. Despite of the LASER characteristics are different of LED in terms of light emission, we admit based on the literature that the effects of both are similar. LED therapy has been shown to be as efficient as LASER therapy, since 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 [8,9,10]. Besides that, some aspects of LED such as lower cost as well as larger area of coverage are vantages when compared to LASER. In this context, we did not find studies evaluating the role of LED on the lung diseases, but we found some studies with LLLT therapy and we based on them. Wang et al. [23] showed that LLLT decreased the number of total cells and eosinophils recovered in BAL in experimental model of asthma. These authors also observed that LLLT reestablished the Th1/Th2 balance. Another study showed that LLLT inhibits bronchoconstriction, Th2 inflammation and airway remodeling in asthma model [24]. Thus, based on these studies, we supported that LED therapy could have beneficial effects on lung inflammation.

Our results showed that LED treatment was effective, since it reduced lung inflammation after OVA challenged, which is characterized by an increased number of lymphocytes, macrophages, and granulocytes in the BAL. In addition, no increase in neutrophils in the blood was observed. This effect might be attributed to cell retention in the bone marrow, because we found an elevated number of cells in this compartment after the LED treatment. In addition, the OVA group showed reduced number of cells in the bone marrow due to mobilization process, considering that we found elevated number of cells in the blood as well as in the BAL. It is reasonable to admit that LED treatment ameliorated the lung inflammation, and consequently, the stimulus for the exit of bone marrow cells was drastically reduced, resulting in an increased number of cells in the bone marrow. Thus, we do not infer that LED caused direct systemic effects, but that they were indirect. Similarly, Wang et al. [23] showed that LLLT decreased the number of total cells and eosinophils recovered in BAL in experimental model of asthma, showing that both LLLT as well as LED seem to have good results on the lung influx. Moreover, these authors also showed that LLLT reestablished the Th1/Th2 balance as we observed in our studies.

It well recognized that not only the number of cells, but also their functional status is very important for the development and amplification of inflammatory response. Thus, we investigated the effects of LED treatment on the cytokines released in the BAL fluid supernatant. Elevated levels of anti-inflammatory cytokines, including IL-10 and IFN-gamma, were observed after the LED treatment. On the other hand, we observed that LED treatment did not reverse the elevated level of IL-4, and also increased IL-1beta. No differences were observed in TNF-alpha and IL-17 among the groups. Thus, we believe that LED treatment promotes its anti-inflammation response.
flammatory effects by the reestablishment of homeostasis, increasing the defense mechanisms. Moreover, we must consider that the asthmatic response is complex and other inflammatory mediators are involved, such as leukotriene, IL-13, IL-5 among others, which can be eventually modulated by LED. IL-10 and IFN-gamma are important anti-inflammatory cytokines that regulate the cellular influx. In general, the gene expression of the cytokines was not affected by LED treatment (data not shown). Eventually, we did find differences among the groups, due to the moment that the experiments were performed. Maybe, if we had analyzed later, we could have found some differences.

Other parameters were also reduced by LED treatment, including mucus production, lung edema, and tracheal contractile response. After the OVA challenge, an inflammatory cascade was triggered by the mast cell activation. Mast cells are activated by OVA and these cells constitute an important immunological sentinel. When mast cells are activated, they release a wide spectrum of inflammatory mediators, which modulate the cellular migration, the mucus production, edema, and the airways hyperresponsiveness [3]. Following this line of thought, we investigated the role of LED treatment on the mast cell degranulation. Our data showed that LED treatment significantly reduced the mast cell degranulation and this effect may be responsible, at least in part, for the reduced cell migration, mucus production, edema, and tracheal responsiveness. Comparing our data with a study using LLLT, we can note that both lights, inhibited bronchoconstriction in asthma model [24]. In addition, in earlier studies of our group using LLLT in lung inflammation model induced by pollutant, we showed similar effects of LED on the mast cell degranulation [5]. Thus, we admit that LED and LLLT had beneficial effects on the lung inflammation due their actions in the same points, including cell influx, mast cells degranulation and airways hyper-responsiveness. In this context, lights therapies are an important alternative to treatment of allergic diseases such as asthma.

5. Conclusion

In conclusion, our results showed that LED treatment reduces the lung cell infiltration in the lung, mucus production, and edema. These effects might be modulated by the IL-10, IFN-gamma and mast cells. This study may provide important information about the effects of LED treatment. In addition, it may open the possibility of a new approach for the asthma treatment.

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Competing Interests The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Asthma is a multifactorial disease where treatment sometimes is not effective and, in this context new therapies are of great importance. Phototherapy as Light-emitting diode (LED) has emerged as a promisor treatment presenting good results for diseases which are characterized by inflammation. This study open the possibility of a new approach for the asthma treatment showing that drug therapy chould be replaced by LED treatment.