

FULL ARTICLE

The chemokines secretion and the oxidative stress are targets of low-level laser therapy in allergic lung inflammation

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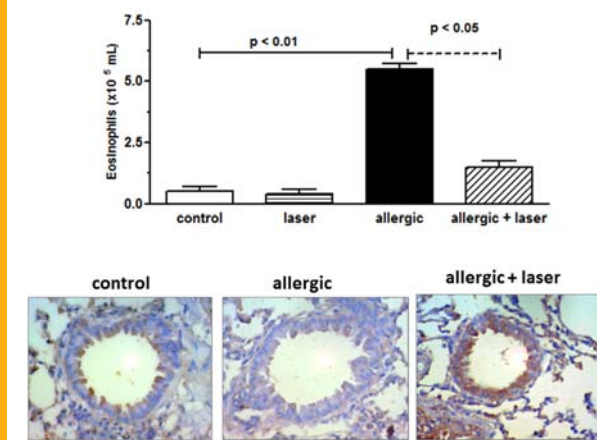
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Recent studies show that low-level laser therapy (LLLT) has an important anti-inflammatory action in acute lung inflammation. The present work explored if laser therapy is able to antagonize eosinophils and allergic inflammation induced by oxidative stress in Balb/c mice. Forty-eight hours after challenge, the leukocyte counting, ROS and nitrite/nitrate level, RANTES, CCL3, CCL8 as well as eotaxins were measured in the bronchoalveolar lavage fluid (BALF) of laser-treated mice or not. Into the lung, some chemokines receptors, the iNOS activity and mRNA expression, and the activities of superoxide dismutase (SOD), catalase, glutathione, NADPH oxidase activities and thiobarbituric acid reactive species (T-Bars) were measured. Laser-treated allergic mice presented reduction of both the ICAM-1 and eosinophil in the lungs. RANTES, CCL8, CCL3 and eotaxins were reduced in BALF of laser-treated allergic mice. In allergic mice lung LLLT decreased the CCR1 and CCR3 and restored the oxidative stress balance as well. Laser decreased the lipidic peroxidation in allergic mice lung as much as increased SOD, GPx and GR. It shows that LLLT on allergic lung inflammation involves leukocyte-attractant chemokines and endogenous antioxidant. Based on results, LLLT may ultimately become a non-invasive option in allergic lung disease treatment.

Graphical Abstract



The top figure illustrates the laser decreasing the eosinophils migration into BALF and the bottom figure shows the laser upregulating the expression of heme-oxygenase (anti-oxidant enzyme) in lung tissue anti-oxidant.

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1. Introduction

Asthma is one of the most common diseases, characterized by airway obstruction, airway inflammation and airway hyperresponsiveness to various stimuli [1]. Cytokines have an important action in the chronic inflammation response in asthma. Some inflammatory cells, as for example eosinophils, trachea and bronchi epithelial cells and smooth muscle cells produce and secrete cytokines that have important role in asthma, such as the cytokines released by the Th2 response lymphocytes [2, 3]. The exacerbated inflammatory response provoked by great amounts of these mediators is also responsible for features as inflammatory infiltrate and structural alterations in airway and lung [4].

In both animal and humans with allergic lung inflammation, eosinophils and lymphocytes are the main inflammatory cells found in the bronchoalveolar lavage or sputum, respectively, thus making these cells useful markers for evaluation of asthma severity [5–7]. Whereas the peak of eosinophils in the lung that occurs 48 hours after the antigenic challenge, studies concerned with LLLT efficiency during this critical period are important for understanding the effectiveness of the laser light [8]. Thus, we decided to investigate the laser effect on cell migration response at this time-point.

The allergic airway inflammation involves the action of diverse cell types (T-cells, eosinophils, mast cells, and dendritic cells) [5]. Besides interleukins secreted by Th2 lymphocytes, the cellular migration and these inflammatory cells interactions are also synchronized by chemical mediators called chemokines [9]. Chemokines are important for the events of migration and activation of inflammatory cells such as leukocytes, epithelial cells and alveolar macrophages at the allergic response through their receptors, which play an important role in the recruitment, leukocytes cell activation and resident cells in the allergic inflammatory process [7]. The inflammatory cells migration through specific chemokines receptors activation can guarantee a specific cellular response and thus initiate and perpetuate the allergic inflammation of airway and lung [10, 11].

Chemokines are recognized by receptors coupled to G-protein [12]. The most of chemokines receptors (CCR) identify more than one chemokine, and many chemokines can be bound to various receptors. This condition permits a great ability on the chemokines action [13]. Among the CCR, both the CCR1 and the CCR3 have an important role in allergic inflammatory diseases. Both can bind with other ligands, including RANTES and CCL3 [14, 15].

Besides the pro and the anti-inflammatory CD4⁺ lymphocytes mediators releasing in the airway and lung, the oxidative stress can also interfere with phospholipids, proteins, and nucleic acids. It is

mediated by reactive oxygen species (ROS) secretion, such as hydrogen peroxide, superoxide anion, singlet oxygen, hydroxyl radical, as well as nitric oxide (NO), species peroxyxynitrite anion and nitrogen dioxide [16]. These molecules participate in both the cellular dysfunction and the inflammation in asthmatic individuals and other species of mammals [17]. ROS species and the products of their metabolism are found in asthmatic with airway and lung diseases, such as chronic obstructive pulmonary disease (COPD) and asthma [18]. In the same sense, the defensive mechanisms against ROS is fundamental to alleviate the injury secondary to imbalance between the secretion of ROS and the antioxidant enzymes activity, including superoxide dismutase (SOD), glutathione peroxidase (GP) and catalase. These enzymes aid to the chronicity of the inflammatory response seen in allergic asthma [19].

Despite the recognized anti-inflammatory effect of laser therapy on wound healing situations [20] and diseases that affect the skeletal muscle [21], there is a growing number of reports showing the efficiency of LLLT in alleviating the late and early symptoms of pulmonary disease of different etiologies [22–25]. Nevertheless, the cellular signaling responsible for the beneficial effect of LLLT in allergic asthma has not been elucidated yet.

The anti-inflammatory effect of laser therapy in experimental models of lung inflammation is present in the reduction of inflammatory cells infiltrate as well as in the decrease of airway obstruction (bronchoconstriction). Probably the magnitude of these effects is due to the fact that LLLT interferes in both the secretion of inflammatory mediators and the expression of transcription factors which are responsible for lung inflammation. Regarding the laser effect on allergic lung inflammation, we recently reported that a diode laser with a wavelength of 660 nm reduces the bronchial hyperreactivity through a mechanism that seems to be modulated via RhoA. In the same study, we also revealed that LLLT lessened allergic lung inflammation, via STAT6 which can be one of the main targets of LLLT. We demonstrated that LLLT together with N-acetylcysteine reduce both the macrophage inflammatory protein-2 (MIP-2) expression and the generation of intracellular ROS of alveolar macrophages [26]. Some authors have demonstrated in experimental studies that laser treatment controls lung inflammation equilibrating the effect between pro-oxidant and anti-oxidant mediators in lung inflammation induced by intestinal ischemia and reperfusion [27]. Recently, we have demonstrated that laser therapy suppresses the oxidative stress-induced glucocorticoids resistance in alveolar macrophages by a cellular mechanism that involves the reduction of cytokine secretion and the increase of histone deacetylase activity [28]. In a model of acute lung inflammation, LLLT reduced

both the neutrophil recruitment and lung edema through mechanisms that involve downregulation of pro-inflammatory mediators, such as TNF and IL-1 β , as well as upregulation of anti-inflammatory mediators, such as IL-10 [29]. It has been reported that the laser effect on balance between pro- and anti-inflammatory mediators is the result of its action on transcription factors such as nuclear transcription factor- κ B (NF- κ B) and peroxisome proliferator-activated receptor- γ (PPAR γ) [30]. Therefore, we explored if LLLT attenuates the secretion of chemokines responsible for the attraction of eosinophils as well as the unbalance between the pro- and antioxidants proteins in lung from allergic mice. In this context, the present work was designed to study if the laser therapy could control the lung oxidative stress in a model of allergic asthma in mice.

2. Material and methods

2.1 Ethical statement

This study was carried out in accordance to the Brazilian Guidelines for the Care and Use of Laboratory Animals Guide. The protocol was approved by the Federal University of São Paulo Committee on Ethics of Animal Experiments. Every surgery was performed under anaesthesia with pentobarbital (50 mg \cdot kg $^{-1}$), and all efforts were made to minimize suffering. For euthanasia, the mice received an excessive dose of chloral hydrate (>400 mg \cdot kg $^{-1}$, i.p.) under anesthesia.

2.2 Animals

The assays were accomplished on eight-week-old male BALB/c mice of the Federal University of São Paulo Development Center of Experimental Models for Medicine and Biology biotherium. They were kept in polypropylene cages (five per cage) under standard conditions of temperature (22–25 °C), relative humidity (40–60%) and 12 h light/dark cycle with access to food and water *ad libitum*.

2.3 Sensitization and antigen challenge

Mice were sensitized at days 1, 8 and 15 by subcutaneous (s.c) injection of 400 μ L of a suspension containing 100 mg grade V ovalbumin (OVA) bound to 4 mg of aluminum hydroxide in sterile phosphate buffered saline (PBS) solution. Seven days after the

second sensitization, the groups of animals were briefly anesthetized with halothane and intranasally challenged with OVA (10 μ g in 50 μ L of sterile saline solution), or received the same volume of sterile saline solution alone. These OVA exposures were performed twice a day, during two consecutive days; the untreated challenged animals received the same volume of saline sterile alone. All mice were euthanized 48 h after the first challenge by sectioning the abdominal aorta under deep chloral hydrate anesthesia (>400 mg \cdot kg $^{-1}$ i.p.), and then the measurements were carried out.

2.4 Low-Level Laser Therapy (LLLT)

Animals challenged with OVA were irradiated by using a continuous wave laser diode module (MM Optics, São Paulo, BRAZIL) with the following parameters: output power of 30 mW, 660 nm wavelength, spot size of 0.08 cm 2 , with exposure radiant of 112.5 J/cm 2 and irradiance of 0.375 W/cm 2 . The physical parameters of laser irradiation used herein are described in Table 1. The optical power was calibrated using a Newport 1835C multifunction optical power meter (Equipland, Oklahoma Road, Sao Jose, CA, USA). The mice were irradiated, via transcutaneous, on the main bronchus. Laser power was monitored during laser irradiation by collecting laser light with a partial reflection (4%) mirror. The laser dose was set at 9 J for 5 min after the sensitization period until the last OVA challenge, twice a day every 12 h. Elapsed forty-eight hours after the antigenic challenge the cellular migration as well as the oxidative stress in lung and BALF were analyzed.

2.5 Experimental groups

All four groups were studied as described in Table 2: 1) *control* group, consisted of sensitized non-manipulated mice; 2) *laser* group, non-allergic animals and treated with laser irradiation; 3) the *allergic* group, that consisted of mice subjected to antigen exposure,

Table 1 Laser Irradiation Parameters.

Laser Parameters	
Wavelength	660 nm
Potency	30 mW
Radiant exposure	112.5 J/cm 2
Irradiance	0.375 W/cm 2
Dose	9 J
Spot size	0.08 cm 2
Irradiation time	300 seconds
Contact	applied contactless

Table 2 Experimental Groups.

Group	Description
1) control	Non-sensitized and non-challenged mice
2) laser	Non-sensitized and non-challenged mice, treated with laser for 5 min every 12 h
3) allergic	Sensitized and challenged mice
4) allergic + laser	Allergic mice treated with laser for 5 min immediately after the sensitization period until the last OVA and to each 12 h

without treatment; 4) allergic + laser group: allergic animals, treated with laser.

2.6 Cellularity

BALF cells were collected from the airway lumen by flushing the airways with a $10 \text{ mg} \cdot \text{kg}^{-1}$ RPMI 1640 through the tracheal cannula. Total cell counts were obtained in BALF samples using an automated cell counter (Sellex, USA). The slides were fixed and stained using a Hema-tek 2000 (BioRad, USA) with Wright's-Giemsa stain. BALF cellularity data were expressed as $\text{cells} \cdot \text{mL}^{-1}$.

2.7 IgE

OVA-specific IgE levels in serum free were analyzed by ELISA. The results are shown as absorbance units at 492 nm.

2.8 ICAM-1

Paraffin sections of lung tissue were processed for standard immunohistological staining using the labeled streptavidin-biotin method and polyclonal rabbit anti-mice ICAM-1 antibody diluted at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The positive reaction was visualized as a yellowish brown stain following treatment with 3, 3'-diaminobenzidine. Immunohistochemical images were assessed using the Imaging Densitometer (AxioVision, Zeiss, USA) and a computer program (AxioVision).

2.9 ROS, RANTES, CCL8, CCL3 and Eotaxin in BALF and ICAM-1 in lung

The mediators in BALF were determined by Enzyme Linked Immuno Sorbent Assay (ELISA) using commercially available kits according to the manufacturer's instructions. The detection limit of these

assays was found to be in the range of $10 \text{ ng} \cdot \text{mL}^{-1}$ for ROS, $31.2 \text{ pg} \cdot \text{mL}^{-1}$ for RANTES, $2.34 \text{ pg} \cdot \text{mL}^{-1}$ for CCL8, $31.2 \text{ pg} \cdot \text{mL}^{-1}$ for CCL3, and $19.53 \text{ pg} \cdot \text{mL}^{-1}$ for eotaxin. For ICAM-1 in lung tissue, protein content was measured using the Lowry assay.

2.10 CCR1 and CCR3

The receptors were measured by ELISA using commercially available kits according to the manufacturer's instructions. The detection limit of these assays was found to be in the range of $10 \text{ pg} \cdot \text{mL}^{-1}$ for CCR1 and $12.5 \text{ pg} \cdot \text{mL}^{-1}$ for CCR3.

2.11 NOS activity

NOS activities in the lung homogenates were determined based on the $[^3\text{H}]\text{-L-arginine}$ to $[^3\text{H}]\text{-L-citrulline}$ conversion according to method described by [30].

2.12 Nitrite and nitrate

Total concentrations of nitrite and nitrate anions in BALF samples were determined by the Griess reaction as described by [31].

2.13 Antioxidant enzymes

SOD activity was measured using the hypoxanthine-xanthine system with absorbance of 550 nm as previously described by [32]. According to the method of [33] the catalase activity was evaluated by measuring the consumption of hydrogen peroxide with absorbance of 240 nm. Glutathione peroxidase (GP) analysis method was determined using Tert-butylhydroperoxide with absorbance of 340 nm as described by [34]. As previously described by [35] the glutathione reductase (GR) was measured through NADPH consumption with absorbance of 412 nm. The enzyme activity values were expressed as rela-

tive to the total protein content measured according to the method of [36].

2.14 Lipid peroxidation

The lipid peroxidation was measured using the thiobarbituric acid-reactive substances (TBARS) method. TBARS levels were determined by absorbance at 535 nm and expressed as malondialdehyde equivalents (nmol/mg protein) as described by [37].

2.15 iNOS, HO-1 and Nrf2

Lung fragments were cut into pieces of 5 mm using a tissue chopper, flesh frozen in liquid nitrogen and stored at -80°C for Real Time-PCR (RT-PCR) analysis of genes expression. Primers used for iNOS mRNA quantification were forward 5'-GTGGTGCACAAGCACATTTG-3' and reverse 5'-GGCTGGACTTTTCACTGC-3'. For HO-1 mRNA quantification the primers used were forward 5'-AACTTTCAGAAGGGCCAGGT-3' and reverse 5'-CTGGGCTCTCCTTGTTC-3'. The primers used for Nrf2 mRNA quantification were forward 5' ACACGGTCCACAGCTCATC-3' and reverse 5'-TGTCATCAAATCCATGTCCTG-3'. The primers for β -actin were forward 5'-TTCAACGGCACAGTCAAGG-3' and reverse 5'-ACATACTCAGCACCAGCATCAC-3' were used as control. For the RT-PCR of NADPH oxidase subunit (gp91 phox), the Taqman inventoried assays-on-demand gene expression kits were purchased from Applied Biosystems. 18S rRNA was used as endogenous control.

2.16 HO-1

Inflation-fixed lungs were paraffin embedded and cut into 10 μm sections. The sections were blocked in a solution of 5% nonfat dry milk, 1% BSA, 5% goat serum in 0.01 M PBS, and 0.1% Triton X-100 before incubation overnight at 4°C with antibodies to ferritin or HO-1 in 1% BSA in 0.01 M PBS and 0.1% Triton X-100 at dilutions of 1:100 and 1:500, respectively. The signal was detected with peroxidase-conjugated avidin and diaminobenzidine. The slides were examined on an Olympus BX41 light microscope.

2.17 Nrf2 immunohistochemistry in lung

Paraffin embedded sections of lung were collected on positively charged glass slides, deparaffinized, re-

hydrated and subjected to antigen retrieval by incubation in Target Retrieval solution, pH 6.0 for 25 min at 90°C , followed by a 20 min cooling period at room temperature. The sections were then, treated with 0.3% hydrogen peroxide in water for 15 min to quench endogenous peroxidase activity. Following a rinse in Tris buffered saline with 1% Tween-20 (TTBS), the slides were subjected to two blocking steps: (i) 15 min incubation with 0.15 mM glycine in PBS, and (ii) 30 min incubation with 1% normal horse serum with a rinse in TTBS in between. The slides were then incubated with rabbit polyclonal antibody to human Nrf2, GCS (Santa Cruz Biotechnology, Santa Cruz, CA), MDA, NQO1 (Abcam, Cambridge, MA) or respective isotype IgG control at a 1:100 dilution in blocking buffer followed by several rinses TTBS. This was followed by a 30 min incubation with biotinylated goat-anti rabbit IgG (Vector Laboratories) and visualization of bound antibody by the Avidin-Biotin system (Vector Laboratories) and diaminobenzidine substrate. The sections were counter-stained with Meyer's hematoxylin (Scytek Laboratories) mounted with coverslips, and examined on an Olympus BX41 light microscope.

2.18 Data analysis and statistical procedures

All data are expressed as mean \pm S.E.M. and were analyzed by One-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons with exception for the iNOS expression experiments. Statistical significance was established at $p < 0.05$.

3. Results

3.1 Leukocyte lung infiltration

To determine the effect of LLLT on the leukocyte lung infiltration induced by antigen exposure, we assessed the total count of cells in BALF and performed histological analysis as illustrated in Figure 1. It can be observed that the total count of cells in BALF of antigen-challenged rats was significantly increased when compared with control group (1A) and that such increase was significantly reduced by LLLT. Diff-Quik staining of cells revealed that most of the new cells were eosinophils and that the eosinophils number in BALF from antigen-challenged mice was largely reduced by LLLT (1B). Histological examinations also revealed increased eosinophilic inflammation in airways wall in the antigen-challenged mice compared with control group (1C); on the contrary, laser treatment reduced eosinophilic in-

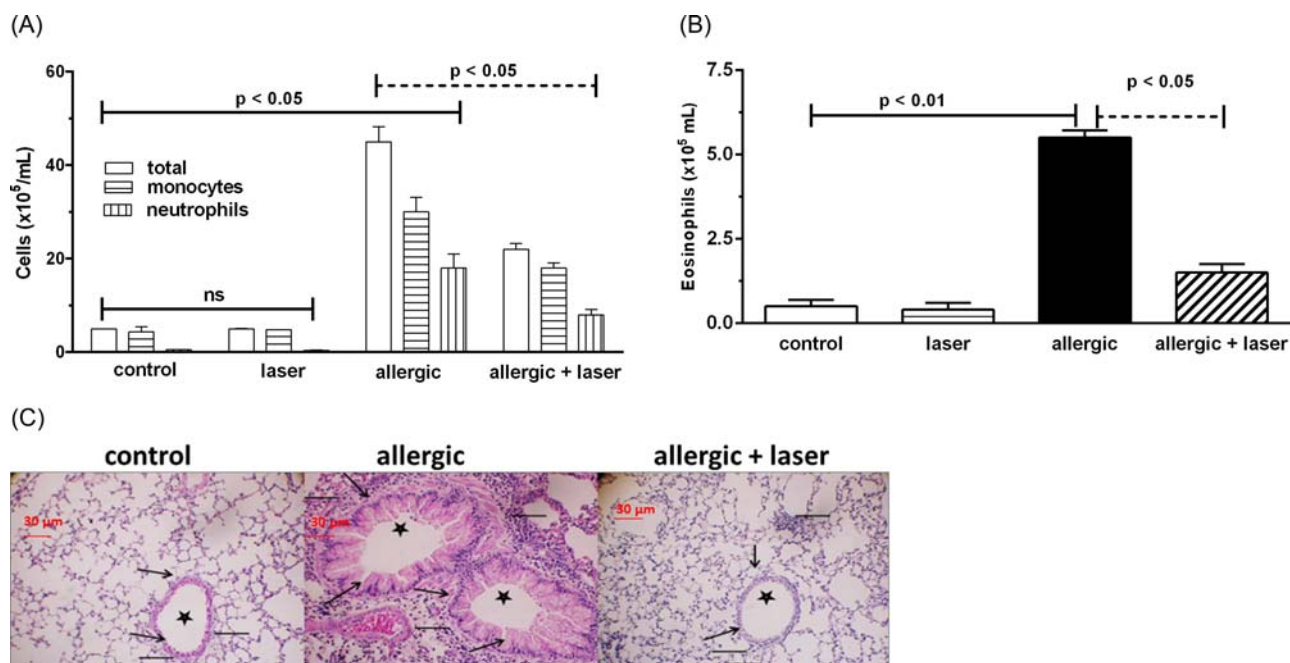


Figure 1 LLLT on cellular infiltrate in lung: The count of the total cells (A) as well as the eosinophils, lymphocytes, neutrophils and monocytes (B) were carried out in BALF obtained from the control, allergic and laser-treated allergic mice 48 h after the last OVA challenge. Sections of formalin-fixed lungs (C) were stained with hematoxylin and eosin before examination with light microscopy (original magnification, ×100). The laser irradiation dose was set at 9 J for 5 min after the sensitization period and to each 12 h during 48 h after the first OVA challenge g. Each bar represents the mean ± SE from 5 different animals. Results were considered significant when $p < 0.05$. — Peribronchial region; ★ bronchial contractility; → Inflammatory cells. The lung morphometry of all experimental groups is described in Table 3. Each bar represents the mean ±SE from 5 different animals. Results were considered significant when $p < 0.05$. — 100 μm.

flammation induced by antigenic challenge compared with antigen-challenged mice. The pulmonary morphometry is described in Table 3.

3.2 IgE

Figure 2 represents the laser effect on levels of antibody IgE in allergic animal's serum. It shows that the levels of IgE in serum were measured by ELISA 48 h after the first OVA challenge. A marked increase in IgE concentration in allergic mice was observed.

Otherwise, LLLT profoundly decreased the levels of IgE in serum of asthmatic animals.

3.3 Immunohistochemistry for ICAM-1

LLLT effect on ICAM-1 protein concentration is shown in Figure 3. Pulmonary localization of ICAM-1 was marked with immunohistochemical staining. ICAM-1 protein in lung was significantly higher in the allergic group compared with the control group (3A). There was a significant decrease on ICAM-1

Table 3 Description of pulmonary morphometry.

control	allergic	allergic + laser
— Peribronchialregion without edema	— Peribronchiolaredema	— Reduction of peribronchiolar edema after LLLT
★ Absence of bronchoconstriction: not have alteration of bronchial contractility	★ Bronchoconstriction: increase of bronchiolar contractility with hyperplasia of smooth muscle	★ Reduction of bronchoconstriction after LLLT
→ Absence of inflammatory cells in peribronchial regions	→ Increasing of cellular migration	→ Reduction of cellular migration after LLLT

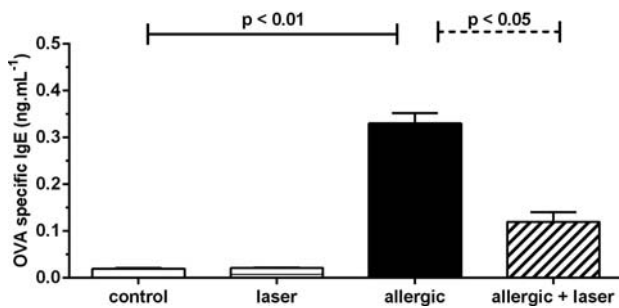


Figure 2 LLLT on total immunoglobulin E (IgE) in serum: The total immunoglobulin (IgE) in serum of all experimental groups is represented herein. Serum obtained from control and allergic mice were assayed for IgE by enzyme-linked immunosorbent assay (ELISA). The IgE level in serum was measured 48 h after the last antigenic challenge. The laser irradiation dose was set at 9 J for 5 min after the sensitization period to each 12 h during 48 h after the first OVA challenge. Each bar represents the mean \pm SE from 5 different animals. Results were considered significant when $p < 0.05$.

protein in lungs harvested from animals treated with laser (3A). The presence of ICAM-1 in allergic animals lungs is confirmed by immunohistochemical staining (yellowish brown) (3B) and it is also evidenced by LLLT effect directly on ICAM-1. In the photomicrography, the arrow (\rightarrow) indicates the presence of ICAM-1 in peribronchial region, and the star (\star) evidences the presence of ICAM-1 in the lung parenchyma. LLLT abolished the ICAM-1 secretion in lung parenchyma, but there is still a little expression of this adhesion molecule in peribronchial region. As expected, the control group did not present immunohistochemical stain for ICAM-1. Laser irradiation in the control group animals showed no effect on ICAM-1 expression.

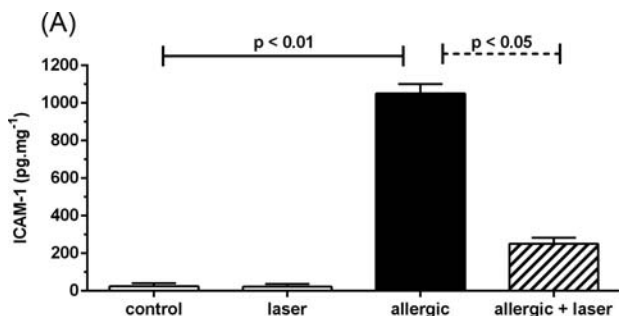


Figure 3 LLLT on ICAM-1 in lung: The changes on ICAM-1 protein in mice lung among the groups (control, allergic and laser-treated allergic) were determined 48 h after the last challenge and they are illustrated herein. The ICAM-1 concentration in lung tissue was assessed by ELISA technique (A). For immunohistochemical localization of ICAM-1 in lung, the positive reaction was visualized as a yellowish brown stain (B). Both assays were performed 48 hours after the last challenge. The laser irradiation dose was set at 9 J for 5 min after the sensitization period and to each 12 h during 48 h after the first OVA challenge. The arrow indicates the localization of ICAM-1 in the peribronchial region, and the star indicates the ICAM-1 localization in the lung parenchyma. Each bar represents the mean \pm SE from 5 different animals. Results were considered significant when $p < 0.05$.

3.4 ROS, RANTES, CCL8, CCL3 and eotaxin in BALF

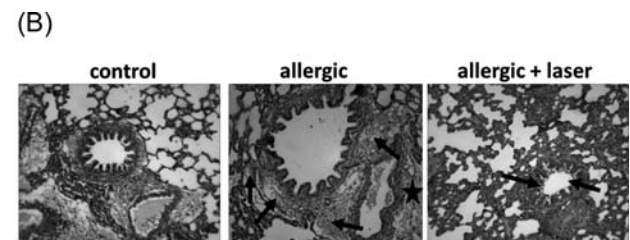
As it is seen in Figure 4 the levels of ROS (4A), RANTES (4B), CCL8 (4C), eotaxin (4D), and CCL3 (4E) in BALF of allergic mice were significantly increased compared with those in the control group. On the contrary, the levels of ROS, RANTES, CCL8, CCL3 and eotaxin in allergic mice BALF were reduced after laser therapy when compared with allergic mice. There is no significant difference between the control and laser groups.

3.5 CCR1 and CCR3 in lung

As it can be observed in Figure 5, both the CCR1 and the CCR3 receptors for chemokines RANTES and CCL8, respectively, are augmented in allergic mice when compared with control group. It was revealed that LLLT reduced the CCR1 (5A) and CCR3 (5B) concentration in allergic mice lung tissue when compared to allergic mice not treated with laser. The CCR1 and CCR3 receptors concentration in lung tissue was not different among the control and laser groups.

3.6 NOS in BALF

Figure 6 represents the laser effect on NOS in BALF of mice of all experimental groups. After 48 hours the antigenic challenge did not affect NOS activity dependent on Ca^{+2} , but significantly increased the activity of NOS independent of Ca^{+2}



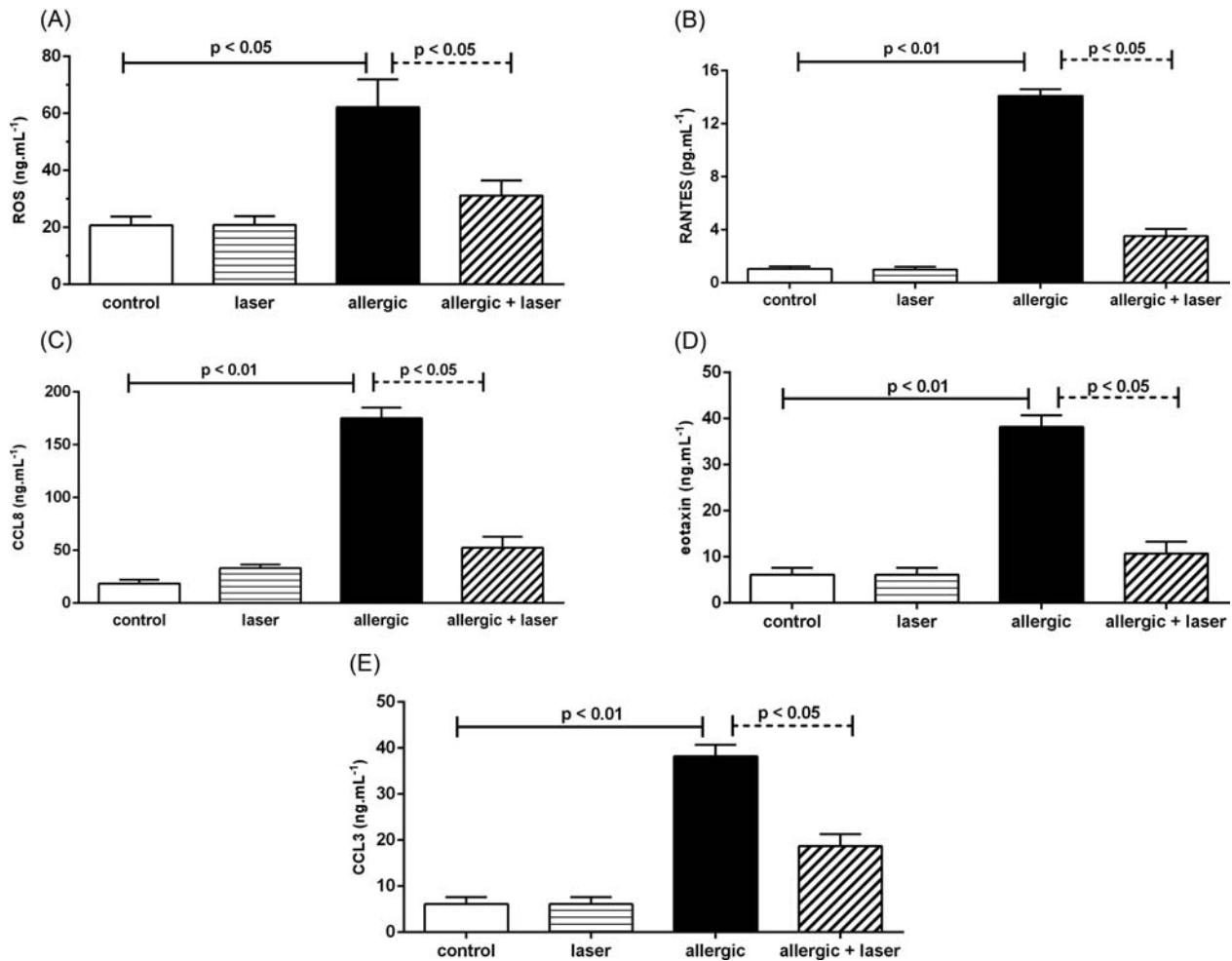


Figure 4 LLLT on chemokines in BALF: ROS and chemokines concentration in BALF of the control, allergic and laser-treated allergic group is illustrated herein. BALF fluids obtained from control and antigen-challenged mice were assayed for ROS (A), RANTES (B), CCL8 (C), CCL3 (D) and eotaxin (E) by enzyme-linked immunosorbent assay (ELISA) 48 hours after the last antigenic challenge. The laser irradiation dose was set at 9 J for 5 min after the sensitization period and to each 12 h during 48 h after the first OVA challenge. The cytokines in BALF were assayed 48 h after the last antigen challenge. Each bar represents the mean \pm SE from 5 different animals. Results were considered significant when $p < 0.05$.

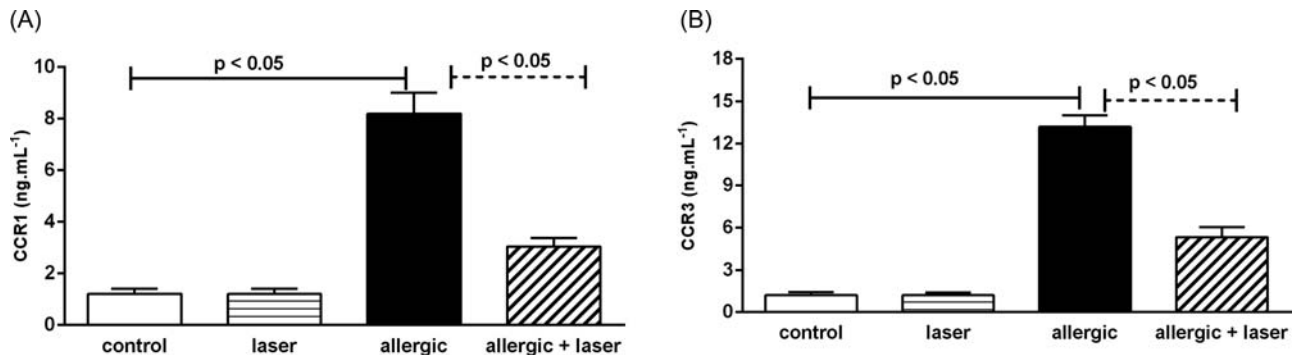


Figure 5 LLLT on CCR1 and CCR3 in lung: It illustrates the CCR1 (A) and CCR3 (B) concentration in lung tissue of animals in the control, allergic and laser-treated allergic group that was determined 48 h after the last challenge by ELISA kit in which the intensity of this colored product analyzed by spectrophotometer (450 nm) is directly proportional to the protein concentration of both the CCR1 and the CCR3 present in the lung tissue. The laser dose was set at 9 J for 5 min after the sensitization period and to each 12 h during 48 h after the first OVA challenge. Each bar represents the mean \pm SE from 5 different animals. Results were considered significant when $p < 0.05$.

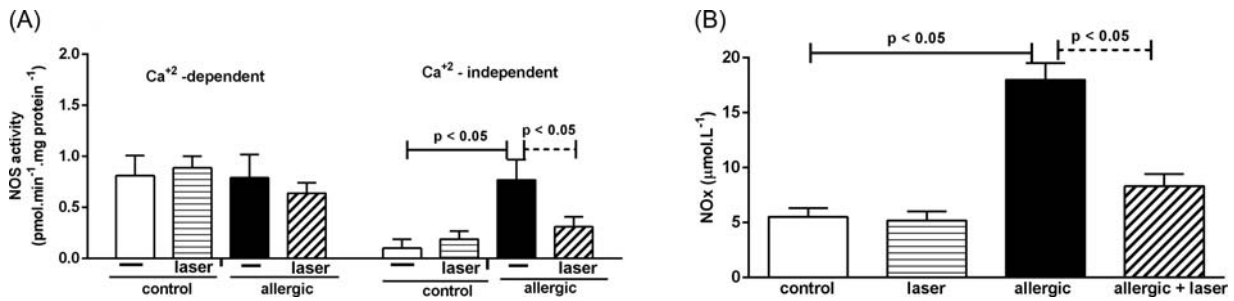


Figure 6 LLLT on NO in BALF: The effect of NOS activity and NO concentration in BALF of the control or allergic mice, treated or not with laser is illustrated herein. NOS activity (A) and NO concentration (B) were assessed by L-arginine conversion to citrulline and Griess reaction, respectively. The laser irradiation dose was set at 9 J for 5 min after the sensitization period and to each 12 h during 48 h after the first OVA challenge. Each bar represents the mean \pm SE from 5 different animals. Results were considered significant when $p < 0.05$.

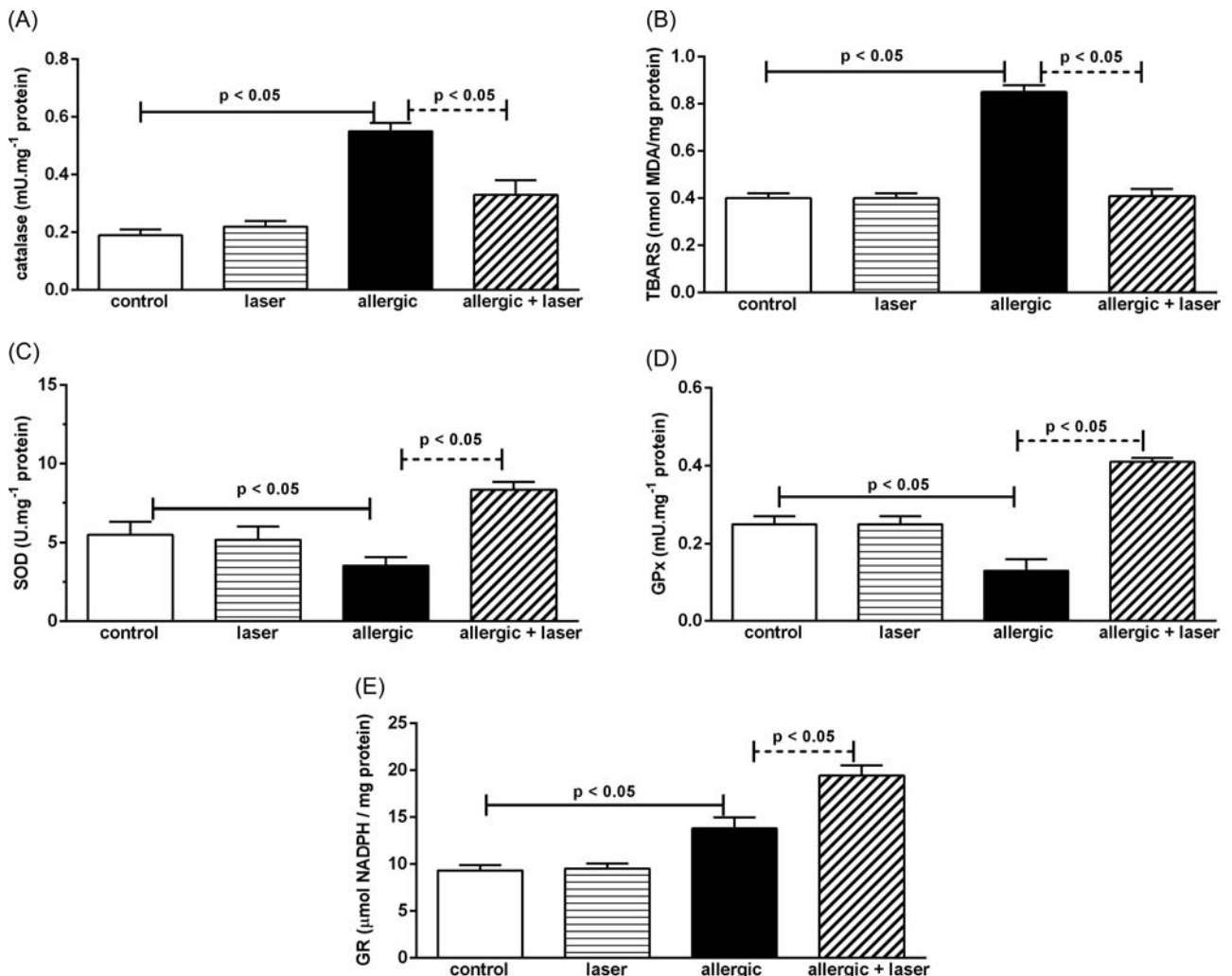


Figure 7 LLLT on oxidant enzymes and lipid peroxidation in lung: Catalase activity and thiobarbituric acid reactive species content as well as the activity of both the SOD and the glutathione peroxidase/reductase in lung of allergic or control mice and treated or not with laser are represented herein. Lung fragments were equally homogenized and the activity of each enzyme was assessed according to description on the “Material and Methods” section. The laser irradiation dose was set at 9 J for 5 min after the sensitization period and to each 12 h during 48 h after the first OVA challenge. Each bar represents the mean \pm SE from 5 different animals. Results were considered significant when $p < 0.05$.

(6A), which was avoided by LLLT. In addition, the nitrite and nitrate concentration in allergic animals BALF (6B) were significantly augmented in relation to the control group; on the contrary, laser treatment reduced the nitrite and nitrate concentration in allergic mice lung. LLLT did not provoke effect on both the NOS and the NOx concentration in the control group mice.

3.7 Catalase, TBARS, SOD, GPx and GR in lung

As shown in Figure 7, the lung catalase activity as well as the TBARS content was increased in the allergic group when compared to the control mice, and LLLT reversed this increase (7A and 7B). The activity of GR as well as GPx and SOD was reduced in the allergic group. Otherwise, LLLT upregulated GR, GPx and SOD activities (7C–E). LLLT did not provoke any effect on these enzymes in control mice.

3.8 iNOS, NADPH oxidase, HO-1 and Nrf2 mRNA expression

Figure 8 represents the protein markers mRNA expression of the oxidative stress in lung of allergic, control and laser-treated allergic mice. As it can be observed, the allergic mice presented a concentration of iNOS and NADPH oxidase higher than the control mice. On the contrary, the HO-1 as well as the Nrf2 expression was downregulated in allergic mice when compared to the control group. LLLT effect was different to each protein once the laser therapy downregulated both the mRNA expression of iNOS (8A) and NADPH oxidase (8B) when compared to allergic animals not irradiated. Oppositely, the laser upregulated the HO-1 (8C) and Nrf2 (8D) expression in comparison to allergic mice. There is no significant difference among the control and laser groups.

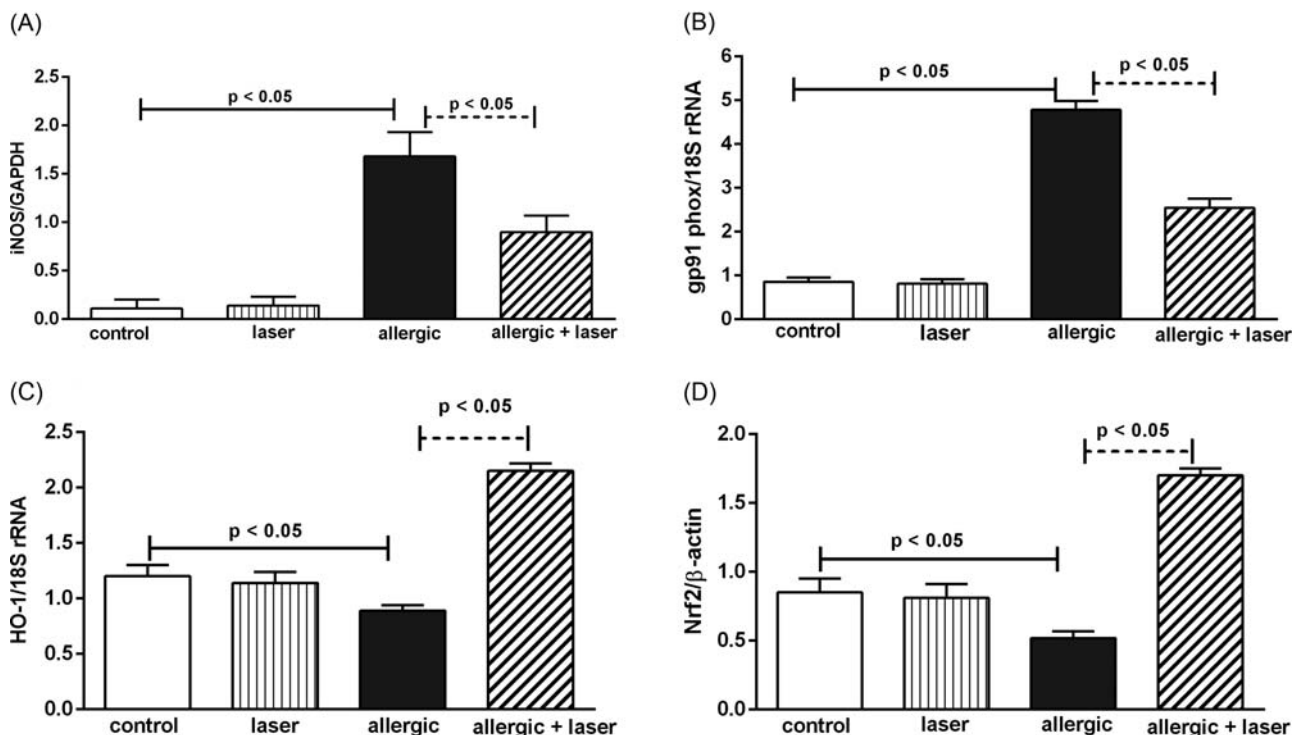


Figure 8 LLLT on iNOS, NADPH oxidase, HO-1, Nrf2: The mRNA expression of the enzymes iNOS, NADPH oxidase, HO-1, Keap-1 as well as the transcription factors Nrf2 in lung of the control, allergic and laser-treated allergic group is illustrated herein. The mRNA expression for iNOS (A), NADPH oxidase (B), HO-1 (C) and Nrf2 (D) in lung tissue were evaluated through Real Time-PCR 48 h after the last OVA challenge. The laser irradiation dose was set at 9 J for 5 min after the sensitization period and to each 12 h during 48 h after the first challenge. Each bar represents the mean \pm SE from 5 different animals. Results were considered significant when $p < 0.05$.

3.9 Immunohistochemical localization for HO-1 and Nrf2

Figure 9 illustrates the lung localization of HO-1 and Nrf2 in mice of the control, allergic, and laser-treated allergic groups. Lung localization of both HO-1 and Nrf2 marked with immunohistochemical staining are represented in (9A) and (9B), respectively. The presence of HO-1 and Nrf2 in allergic mice lungs was downregulated in allergic mice when compared to the control group; however, in laser-treated allergic mice the immunohistochemical stain for HO-1 and Nrf2 was markedly higher when compared to the group control. LLLT in the control group animals showed no effect on HO-1 and Nrf2 expression in lung tissue.

4. Discussion

In the present manuscript we observed that the laser dose at the level of 9 J produced *in vivo*, effect on the eosinophils migration and the levels of leukocytes-attractant chemokine's, as well as the oxidative stress balance. In order to avoid that accumulated laser doses in a short time between the irradiations would cause some deleterious effect on LLLT or even eliminate its beneficial effect, herein the mice were irradiated every 12 h during the sensitization period until the last antigenic challenge.

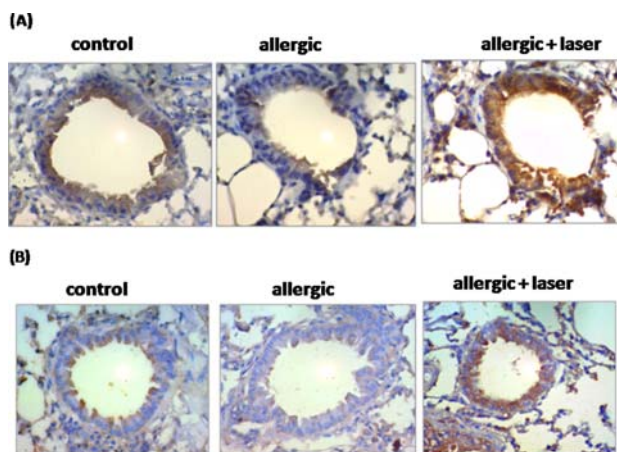


Figure 9 LLLT on HO-1 and Nrf2 in lung: The changes in HO-1 as well as in Nrf2 proteins among the groups (control, allergic and laser-treated allergic) were assessed 48 h after the last OVA challenge and they are illustrated herein. The localization of HO-1 (A) and Nrf2 (B) in lung was determined by immunohistochemical technique in which the positive reaction for both the HO-1 and Nrf2 was visualized as a brown stain. The laser irradiation dose was set at 9 J for 5 min after the sensitization period and to each 12 h during 48 h after the first OVA challenge.

There is currently no therapy able to heal the symptoms of allergic asthma. Current therapies have the ability to control the inflammatory process characteristic of asthma, but the onset of immune activation against the antigen and the predisposition of individuals cannot be controlled [31]. The use of corticoids is the main therapy for asthmatic patients. However, although corticosteroids are efficient if used in high doses or for long periods of time they present many side effects that increase the treatment cost. Besides it, some patients have resistance to the glucocorticoids therapeutic effects; therefore these subjects need high doses of corticoids, resulting in an increased risk of systemic side effects [32]. For patients with these problems there is an urgent need to develop new anti-inflammatory therapies with immunomodulatory activity to provide alternative ways for treating asthma. LLLT is a promissory candidate because it has some important characteristics such as low cost and noninvasive irradiation for instance, which permits the patient to be treated in the ambulatory without sedation; and mainly the absence of side effects, even when used for a long period.

Regarding the leukocyte migration in allergic mice, our results demonstrated that LLLT reduces the number of mononuclear cells and neutrophils of these animals, but the marked effect was seen when the laser decreased the eosinophils number in BALF. Recently, we demonstrated a beneficial effect of the laser treatment on leukocytes migration in allergic mice lung [33]. However, in this occasion, we focused on bronchial hyperreactivity and the transcription factor STAT6 at a period of 24 hours after the last challenge. Herein we focused on the participation of eosinophils and eosinophils attractant chemokines as well as the oxidative stress. Our results indicate that the laser effect on eosinophil migration is, at least, partially mediated by the decrease of the lung concentration of ICAM-1 in allergic mice lung. In addition, LLLT drastically reduced the levels of eosinophils-attractants chemokines such as eotaxin, RANTES, CCL8 and CCL3. We also demonstrated that the chemokines receptors, CCR1 and CCR3, in allergic mice lungs were also attenuated by LLLT. Taking the results presented into consideration, evidence show that LLLT interferes on different steps of the inflammatory cascade, and maybe it is very advantageous to use LLLT.

One of the most important results of this present study was the laser effect in reducing the levels of antibody IgE in serum of allergic animals. Some authors have also shown that LLLT reduces the IgE levels [34], but herein we demonstrated that LLLT applied since the beginning of the sensitization until the last antigenic challenge impairs the rise of IgE levels in serum at a period of 48 hours after challenge. Even if there is fluctuation in IgE levels during the time period before reaching the ends of

48 hours, the LLLT seems to provide an environment with a moderate response of both the immune system and the oxidative burst. It has been suggested that allergic individuals treated with laser for a long period can activate a milder immune response when re-exposed to the antigen [23]. Therefore, it is important to highlight that the idea of the IgE levels reduction after laser does not mean to reduce the antigenic ability of asthmatic individuals; on the contrary, the use of laser therapy is focused on controlling the antigenic response exacerbation. Naturally, LLLT efficiency on allergic asthma becomes higher if the laser maintains non-exacerbated levels of IgE in serum. Nevertheless, we cannot affirm with the experiments herein presented what the threshold dose capable of generating beneficial effect is. Thus, the laser optimal dose and the irradiation moment able to permit a significant allergic inflammation reduction for a long time must be investigated.

Some reports have evidenced the beneficial effects of LLLT on airway and lung diseases, in both acute and chronic inflammatory processes [36–38]. Besides that, the mechanism of laser therapy in lung allergic inflammation has not been fully elucidated. In the present study, we evidenced that laser treatment for a long period presented an anti-inflammatory effect related to the eosinophils attractant chemokines and ROS inhibition.

The present study proposes that the phototherapy action mechanism in allergic mice has driven not only the downregulation of leukocytes attractant chemokines, but also attenuated the ROS generation. In fact, herein we evidenced that LLLT beneficial effect on allergic lung inflammation can be controlled via activation of transcription factors responsible for the generation of anti-oxidant mediators with capability to restore the oxidative equilibrium in stress status. We revealed for the first time that laser treatment with one unique wavelength and the same dosimetry is able to produce a dual effect on oxidative stress in allergic lung, seen that LLLT downregulated oxidant agents but also up regulated antioxidant agents. Our results evidenced that the laser effect in counterbalancing the oxidative metabolism in allergic mice lung involves the participation of both the HO-1 and the transcription factor Nrf2.

Some authors have shown that the treatment with an iNOS inhibitor reduces eosinophil migration to the lungs of allergic animals [40]. We have previously demonstrated that LLLT reduced the neutrophils migration at the same time that diminished iNOS expression in lung of animals submitted to intestinal ischemia and reperfusion [41]. Our results herein presented showed that LLLT reduces the iNOS mRNA expression as well as the NOS activity. Thereupon, the NO production in allergic mice BALF was also decreased post laser treatment.

Thus, it is reasonable to suggest that in the present manuscript, the LLLT effect on NO can influence the eosinophils migration.

ROS also adds balance to the oxidative stress in allergic asthma. In the present study, we demonstrated that in laser-treated allergic mice lungs there is a significant upregulation of SOD, GP, GR and catalase activities. On the contrary, our results showed that lipid peroxidation is elevated in challenged mice lungs due to the fact that TBARS content is increased, and that LLLT attenuates it. Although some authors have highlighted the LLLT potential role in protection against oxidative stress [51] independently on the activities of intracellular enzymatic ROS scavengers, such as SOD, GP and catalase most studies indicate that the LLLT beneficial effect on oxidative stress status depends on its action on pro- and anti-oxidants agents [52–55]. Moreover, some authors showed LLLT protects the oxidative stress-activated animal cells through NADPH oxidase activity inhibition, thus showing another mechanism by which the laser therapy is able to reduce both the generation of OH⁻ and the lipid peroxidation [56].

In most cell systems, NADPH oxidase is an important source of ROS. NADPH oxidase activation is mediated by translocation of the cytosolic subunits, such as p47^{phox}, p67^{phox}, p40^{phox} and the GTPase to the plasma membrane subunits, as for example gp91^{phox} and gp22^{phox} [57]. NADPH oxidase activation in lung results in superoxide anions increase, which are toxic in allergic asthma [58]. Our results showed that LLLT reduces the activity of NADPH oxidase by the gp91^{phox} subunit decrease in allergic mice lung. These results indicate that LLLT can act directly on ROS secretion, but it also impairs the NADPH oxidase activity. In addition, this finding reinforces the idea that LLLT in pathological conditions acts controlling the exacerbated production of ROS, and it results in beneficial effect.

Enzymes such as catalase, glutathione and SOD are critical components of antioxidant response and, for the most part, are constitutively expressed. Among these inducible enzymes, HO-1 appears to be the most strongly induced and highly regulated [59]. Some authors have reported that a reduction of oxidant stress associated with HO-1 upregulation in animals challenged with OVA is closely related to a reduction of inflammatory cells including eosinophils, neutrophils, and lymphocytes in lung [60]. Our results in the present manuscript showed that both the dose and the time irradiation used in LLLT in OVA-challenged mice influenced the HO-1 activity resulting in increasing values of this enzyme in lung tissue, showing for the first time the cellular mechanism by which the laser treatment presents an anti-inflammatory action via antioxidant enzymes in the allergic lung inflammation.

Some authors have also reported that HO-1 can attenuate the cellular infiltration by interfering in cell rolling, adhesion, and migration, probably by downregulating the expression of adhesion molecules on the endothelium [61, 62]. Regarding the LLLT effect on adhesion molecules, our results showed that ICAM-1 expression in challenged mice lung was decreased after LLLT and at the same time the laser treatment increased the HO-1 activity. Despite it, we did not investigate whether the LLLT effect on ICAM-1 expression can be modulated by HO-1.

Among the transcription factors responsible for synthesizing antioxidant enzymes with the goal of compensating the ROS exacerbated secretion as well as the activation of oxidant enzymes, the nuclear factor E2-related factor (Nrf2) takes up a prominent position. It binds to common regulatory elements termed “antioxidant response elements” (ARE) in the regions of a wide range of antioxidant and detoxification genes triggering their activation, which include the HO-1, NADPH, glutathione, SOD, and catalase [65].

Our results demonstrated that the action mechanism responsible for the LLLT beneficial effect on oxidative stress in allergic lung inflammation has, as one of its target, the transcription factor Nrf2 because the allergic mice treated with laser presented a rise of Nrf2 expression in lung tissue. It means that the beneficial effect of LLLT is truly linked to antioxidant actions ranging from the reduction of prooxidant enzymes until the up regulation of transcription factor responsible for the generation of antioxidant mediators. Ultimately, our results demonstrated that the cellular signaling required by LLLT for attenuating both the eosinophil migration and the eosinophils-chemoattractant chemokines levels in allergic mice lung involves the upregulation of antioxidant enzymes HO-1 through Nrf2 activation.

Finally, the present study argues the proposed use of LLLT as a non-pharmacological therapeutic tool that can be applied non-invasively for diseases control that compromise the airway and lung. Therefore, our results suggest that a lung inflammatory condition attenuated by LLLT can reduce the frequency of asthmatic crisis, an event where the bronchial hyperreactivity (bronchoconstriction) is very significant becoming an emergency condition in which LLLT has not been effectively quick. For this reason, the idea is that phototherapy can work associated to conventional therapy always with the goal of finding a laser dosimetry able to minimize the steroids dose chronically used. Further studies are needed to investigate the effect of laser therapy associated with pharmacological therapy, especially bronchodilators and corticosteroids.

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