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Low Level Laser Therapy (LLLT) Decreases Pulmonary Microvascular Leakage, Neutrophil Influx and IL-1β Levels in Airway and Lung from Rat Subjected to LPS-Induced Inflammation

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Background and Objective. Low level laser therapy (LLLT) is a known anti-inflammatory therapy. Herein we studied the effect of LLLT on lung permeability and the IL-1β level in LPS-induced pulmonary inflammation.
Study Design/Methodology. Rats were divided into 12 groups (*n=7 for each group*). Lung permeability was measured by quantifying extravasated albumin concentration in lung homogenate, inflammatory cells influx was determined by myeloperoxidase activity, IL-1β in BAL was determined by ELISA and IL-1β mRNA expression in trachea was evaluated by RT-PCR. The rats were irradiated on the skin over the upper bronchus at the site of tracheotomy after LPS.
Results. LLLT attenuated lung permeability. In addition, there was reduced neutrophil influx, myeloperoxidase activity and both IL-1β in BAL and IL-1β mRNA expression in trachea obtained from animals subjected to LPS-induced inflammation.
Conclusion. LLLT reduced the lung permeability by a mechanism in which the IL-1β seems to have an important role.

KEY WORDS: laser therapy; inflammation; lung permeability; bronchoalveolar lavage; interleukin-1β (IL-1β); lipopolysaccharide (LPS).

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INTRODUCTION

The inflammation of the airway and lung induced by the Gram-negative bacterial lipopolysaccharide (LPS) is characterized by an increased contractile response of smooth muscle [1]. When infused into experimental animals, LPS induces airway and lung inflammation [2] caused by the accumulation of lymphocytes, mast cells, macrophages and neutrophils. It has been proposed that the release of mediators from these inflammatory cells contributes directly or indirectly to changes in airway structure and function [3].

Increased levels of cytokines are a hallmark of inflammatory diseases [4]. With respect to lung disorders, cytokines appear to play particularly prominent roles in the pathogenesis of adult respiratory distress syndrome (ARDS) and asthma [5]. In lungs, cytokines not only orchestrate and perpetuate the inflammatory response, but also cause functional alterations, such as bronchoconstriction and airway hyper-responsiveness that are observed in acute lung injury (ALI) [6]. Among the pro-inflammatory mediators that participate in lung and airway inflammation during endotoxemia induced by LPS, interleukin-1β (IL-1β) plays a pivotal role in the development of ALI. IL-1β participates in both early and later inflammatory response, being able to initiate and perpetuate the inflammation [7, 8].

The disruption of lung endothelial barrier integrity with paracellular gap formation is an ultrastructural hallmark of ALI and its more severe form, the ARDS [9, 10]. The conformational changes in the lung microvasculature are a consequence of lung endothelial activation by diverse bioactive and biophysical stimuli, resulting in high permeability pulmonary, edema and alveolar flooding [11].

IL-1 contributes to alveolar barrier dysfunction in experimental ventilator-induced lung injury by promoting lung neutrophil recruitment, and by increasing epithelial injury and permeability. Because preserved alveolar barrier function is associated with better outcomes in patients with ALI, these data support further testing of IL-1β receptor antagonist for the treatment of ALI. In anesthetized rats, LPS caused systemic hypotension and increased biochemical factors, such as nitrate/nitrite and methyl guanidine, tumor necrosis factor (TNF-α), and IL-1β [12]. In isolated lungs, IL-1β increased lung weight to body weight ratio, lung weight gain, protein and dye tracer leakage, and capillary permeability [13].

Traditional treatments of ARDS and asthma include a variety of pharmacological corticosteroids, methotrexate, and other disease-modifying agents such as cyclosporine and intravenous immunoglobulin, as well as newer treatments such as TNF-α and IL-1β [14, 15]. The low level laser therapy (LLLT) has been used in inflammatory pathologies as a new anti-inflammatory therapy absent of side effects [16].

LLLT has been used in the treatment of patients with pleurisy [17] and asthma [18]. In addition, some authors have demonstrated that this therapy is also beneficial in experimental models of airway and lung inflammation. The anti-inflammatory effect of LLLT on the TNF-α level in bronchoalveolar lavage fluid after inflammation induced by immune-complex reaction was observed in rats [19]. Moreover, LLLT restores the relaxation response of trachea smooth muscle segments after incubation with TNF-α [20]. However, the action mechanism of LLLT on pulmonary diseases, such as asthma or endotoxemia, is still poorly elucidated.

Considering the IL-1β effects on lung and airway described above, in the present work, we studied the effect of LLLT on lung permeability and bronchoalveolar lavage and IL-1β mRNA expression in trachea from rats with systemic inflammation induced by LPS.

MATERIAL AND METHODS

Animals. All experiments were carried out in accordance with the guidelines of Vale do Paraíba University for animal care. The experiments were carried out on Male Wistar rats weighting between 230 and 250 g each, maintained under standard conditions of temperature (22–25°C), relative humidity (40–60%) and light/dark cycle with access to food and water ad libitum. The animals were provided by Central Animal House of the Research and Development Institute of Vale do Paraíba University (UNIVAP). All rats were placed in a common box and divided randomly into groups of seven animals each.

Anaesthesia. In all experimental groups the rats were pre-anaesthetized with butorfanol (acepromazine, 0.1 mg/kg) and anesthetized with zoletil (Chloridrate of zolazepam, 0.1 mg/kg+Tiletamine Chloridrate 0.1 mg/kg).

Experimental Groups

All experimental groups content seven animals. The design of all experimental groups studied herein is equal to that described in subsection "Acute lung injury induced by LPS".

Pharmacological Intervention

In order to investigate the participation of IL-1β on lung capillary leakage and myeloperoxidase (MPO) activity was used an IL-1 β receptor antagonist (AF12198; 16 mg/kg, i.v) 30 min before LPS intravenous injection like described by [21].

Acute lung injury induced by LPS. In order to investigate the effect of LLLT on acute lung injury (ALI) induced by LPS intravenous injection (5 mg/kg) [22] is important to consider the time evolution of the inflammation after LPS administration. Rats were divided into 12 groups (*n=7 for each group*): groups 1, 2 and 3 received an i.v. injection of PBS (50 μl) and rats were euthanized, 4, 12 and 24 h after the injection, respectively; groups 4, 5 and 6 received LPS and, 4, 12 and 24 h after that the rats were euthanized, respectively. Groups 7, 8 and 9 were irradiated, without receiving any drug, and they were euthanized 4, 12 and 24 h after irradiation, respectively. Group 10 was challenged with LPS and 1 h later treated with LLLT and euthanized 4 h after LPS; group 11 received LPS and it was irradiated twice, at 1 and 6 h after LPS injection, and then euthanized 12 h after LPS. Group 12 received LPS and it was irradiated twice, in the first and 24th hour after LPS, and then euthanized 24 h after LPS.

Assessment of lung capillary leakage. The assessment of lung permeability was analyzed, 6, 12 and 24 h after LPS injection by a method yet described by [23]. The extravasated EBA concentration in lung homogenate was calculated against a standard curve using the Evans blue dye.

Bronchoalveolar lavage (BAL). Shortly after each temporal profile (4, 12 or 24 h) after LPS injection and using the same anesthesia protocol previously described, the rat bronchoaveolar lavage was measured like described by [24]. The data were expressed as BAL (cells/ml).

Activity of myeloperoxidase (MPO). The neutrophil accumulation inside the lungs was evaluated by measuring the myeloperoxidase (MPO) activity like described by [25]. The MPO activity in the supernatants was assayed by measuring the change in A_{460} resulting from the decomposition of H_2O_2 in the presence of *o*-Dianisidine [29].

Determination of IL1-β in Broncoalveolar Lavage (BAL)

Under anaesthetize the rats were tracheostomised with a gauge catheter. The lungs were washed with 3× 5 ml sterile Hank's balanced salt solution (HBSS) without Ca^{+2} or Mg^{+2} (GIBCO). Levels of IL-1β in BAL were determined by ELISA technique 4, 12 and 24 h after LPS intravenous injection.

Determination of IL-1β mRNA Expression in Trachea Tissue

Total RNA was isolated from trachea tissues by TRIzol reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer's instructions. The RNA was subjected to DNase digestion, Applied Biosystems, Foster City, CA) using the SYBRGreen core reaction kit (Applied Biosystems). The primer used for real time PCR was: rat IL-1β-793–871 (GenBank™ accession number M98820), forward primer 5′-CACCTCTCAAG CAGAGCACAG-3′ and reverse primer 5′-GGGTTC CATGGTGAAGTCAAC-3′; rat Exon (GenBank™ accession number NW_047658). The PCR primer efficiencies were calculated using standard curves, and the relative expression levels of IL-1β in real time were analyzed, 4, 12 and 24 h after LPS injection, using the 2^{CT} method, presented as the ratio to the expression of the housekeeping gene-GAPDH. Each sample was replicated twice from three independent sets of RNA preparations.

Laser irradiation. A diode laser with an output power of 30 mW and a wavelength of 660 nm (model: laser unit, Kondortech) was used. The laser beam covered an area of 0.785 cm^2, resulting in an energy dosage of 7.5 J/cm^2. The rats were irradiated on the skin over the upper bronchus at the site of tracheotomy in according to time course described in subsection of "Material and methods" named "Acute lung injury induced by LPS."

Statistical Analysis

Statistical differences were evaluated by analysis of variance (ANOVA) and Tukey–Kramer Multiple Comparisons Test to determine differences between groups. The results were considered significant when $P<0.05$. In order to determine the analysis of variance between the experimental groups studied was used the two-way ANOVA statistical analysis to determine the *F* score.

RESULTS

Effect of LLLT or AF12198 on Pulmonary Microvascular Leakage

Figure 1 represents the acute lung injury in response to LPS injection. In this assay, we determined

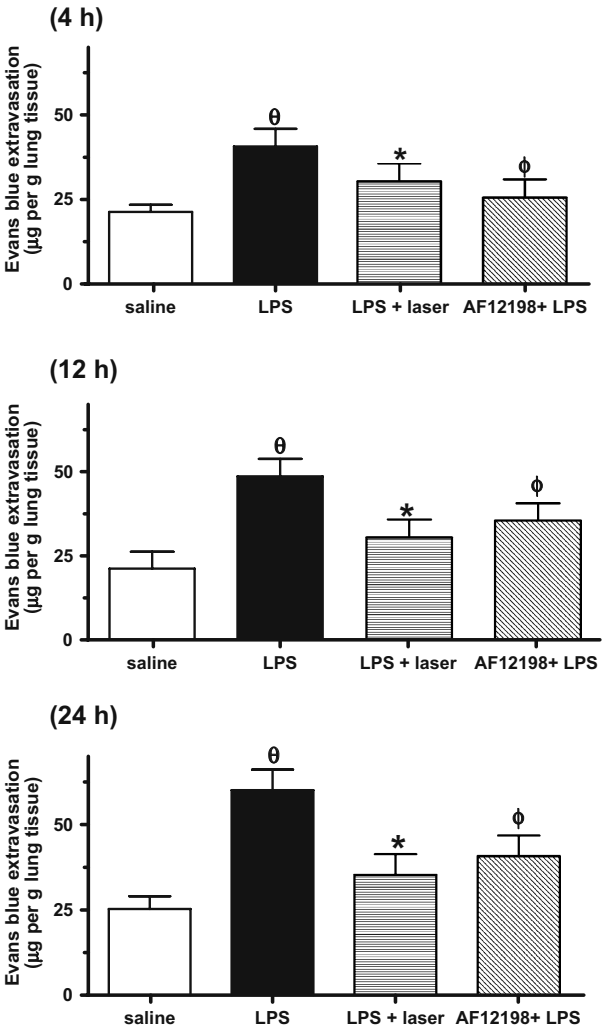

Fig. 1. We determined vascular permeability in the lung using the Evans blue extravasation technique. LLLT and AF12198 reduced pulmonary microvascular leakage at 4, 12 and 24 h. Statistically significant differences are indicated by *theta* ($P<0.001$) comparing data to saline or by *asterisk* ($P<0.001$) or *phi* ($P<0.001$) comparing data to LPS.

vascular permeability in the lung using the Evans blue extravasation technique 4, 12 or 24 h after LPS. After LPS, Evans blue extravasation into the lung was significantly higher (LPS 4 h, 40.7±5.2 μg Evans blue per gram lung, $P<0.001$, $F=18640$; LPS 12 h, 48.6±5.2 μg Evans blue per gram lung, $P<0.001$; LPS 24 h, 60.1±6.0 μg Evans blue per gram lung, $P<0.001$) in comparison with saline groups (saline 4 h, 21.3±2.1 μg Evans blue per gram lung; saline 12 h, 21.2±5.0 μg Evans blue per gram lung; saline 24 h, 25.3±3.7 μg Evans blue per gram lung). In order to show the involvement of IL-1β in acute lung inflammation, the rats were pre-treated with AF12198 and it reduced the lung microvascular leakage from rats inflamed with LPS at the 4 h (AF12198, 25.4±5.3 μg Evans blue per gram lung, $P<0.001$), 12 h (AF12198, 35.5±5.1 μg Evans blue per gram lung, $P<0.01$) and 24 h (AF12198, 40.7±5.3 μg Evans blue per gram lung, $P<0.001$) after LPS. LLLT reduced pulmonary microvascular leakage at the time 4 h (LLLT, 28.1±5.3 μg Evans blue per gram lung, $P<0.001$; $F=18640$), 12 h (LLLT, 30.5±5.3 μg Evans blue per gram lung, $P<0.001$; $F=45350$) or 24 h (LLLT, 35.2±6.1 μg Evans blue per gram lung, $P<0.001$; $F=165800$).

Effect of LLLT on Neutrophils in Bronchoalveolar Lavage (BAL)

Figure 2 illustrates the BAL cellular profile of rats treated with laser irradiation and which did and did not receive LPS. The number of BAL cells per milliliter of BAL fluid increased for all times studied herein. At 4, 12 and 24 h after LPS (LPS 4 h, 25.1±2.2 cells/ml, $P<0.001$; LPS 12 h, 43.5±3.1 cells/ml $P<0.001$; LPS 24 h, 60.5±10.1 cells/ml, $P<0.001$) we observed an increase of BAL cells in comparison with saline group (saline 4 h, 2.3±0.3 cells/ml; saline 12 h, 2.1±0.3 cells/ml; saline 24 h, 2.8±0.4 cells/ml). In evaluation of polymorphonuclear leukocyte, the percentage of neutrophils (PMN/μl) accompanied the increase after LPS injection. In 4 and 12 h after LPS we observed approximately 96% of neutrophils and 98% of neutrophils 24 h after LPS. LLLT reduces the BAL cells number in all time investigated herein, 4 h (LLLT, 11.87±2.2 cells/ml, $P<0.001$, $F=15070$); 12 h (LLLT, 21.50±3.0 cells/ml, $P<0.001$) or 24 h (LLLT, 41.6±4.2 cells/ml, $P<0.001$; $F=870200$). These results showed the importance of neutrophils in ALI and the significant effect of laser therapy on this cell type.

Effect of LLLT or AF12198 on Myeloperoxidase (MPO) Activity

The Fig. 3 represents the lung neutrophil infiltration that was evaluated by measuring the levels of MPO in homogenates of lung tissue. Figure 3 shows the effect of LLLT or AF12198 on lung neutrophils influx 4, 12 and 24 h after LPS injection respectively. In order to show the involvement of IL-1β in acute lung inflammation, the rats were pre-treated with AF12198 and it reduced the lung microvascular leakage from rats inflamed with LPS at the 4 h (32.6%), 12 h (50.1%) and 24 h (65.3%) after LPS. LLLT reduced the MPO activity in homogenates of lung tissue 4, 12 and 24 h after LPS injection (35.2%, 55.4% and 71.3.5% of inhibition, respectively).

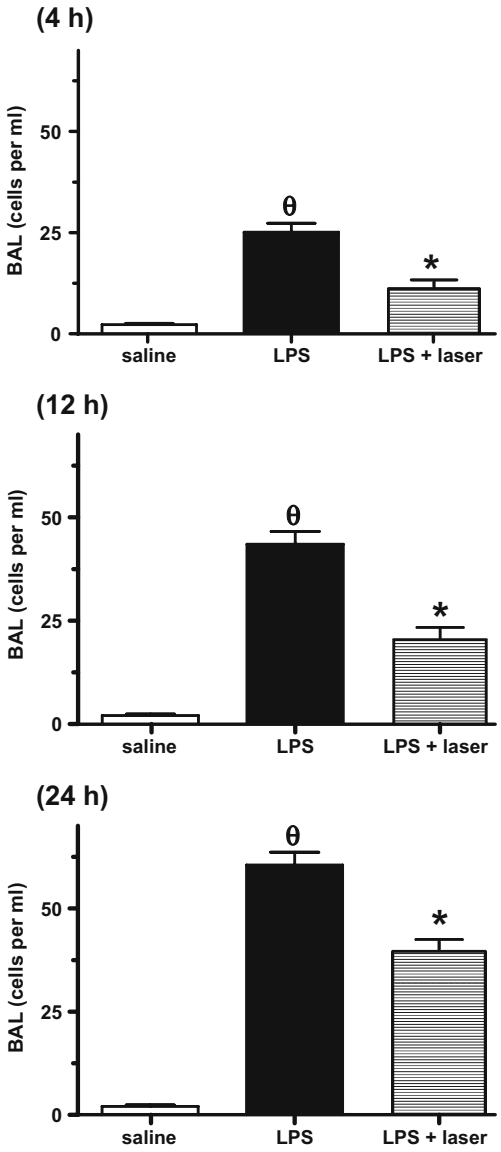

Fig. 2. BAL cellular profile of rats treated with laser irradiation and which did and did not receive LPS. LLLT reduces the BAL cells number at 4, 12 and 24 h after LPS. Statistically significant differences are indicated by *theta* ($P<0.001$) comparing data to saline or by *asterisk* ($P<0.001$) comparing data to LPS.

Effect of LLLT on Concentration of IL-1β in Bronchoalveolar Lavage (BAL)

The Fig. 4 shows a pronounced time-dependent increase of IL-1β concentration in BAL after LPS in all times studied. At 4, 12 and 24 h after LPS (LPS 4 h, 0.95±0.2 ng/ml, $P<0.001$; LPS 12 h, 1.32±0.15 ng/ml, $P<0.001$; LPS 24 h, 1.65±0.15 ng/ml, $P<0.001$) we observed an increase of IL-1β concentration in BAL in comparison with saline group (saline 4 h, 0.55±0.2 ng/ml; saline 12 h, 0.55±0.21 ng/ml; saline 24 h, 0.55±0.10 ng/ml). LLLT reduced significantly the IL-1β concentration in BAL fluid in all times after LPS (LLLT 4 h, 0.43±0.1 pg/ml, $P<0.001$, $F=2774$; LLLT 12 h, 0.91±0.11 pg/ml, $P<0.001$, $F=2926$; LLLT 24 h, 1.13±0.15 pg/ml, $P<0.001$, $F=6949$) when compared with saline group. These results indicate the anti-inflammatory effect of LLLT can be mediated by IL-1β activity.

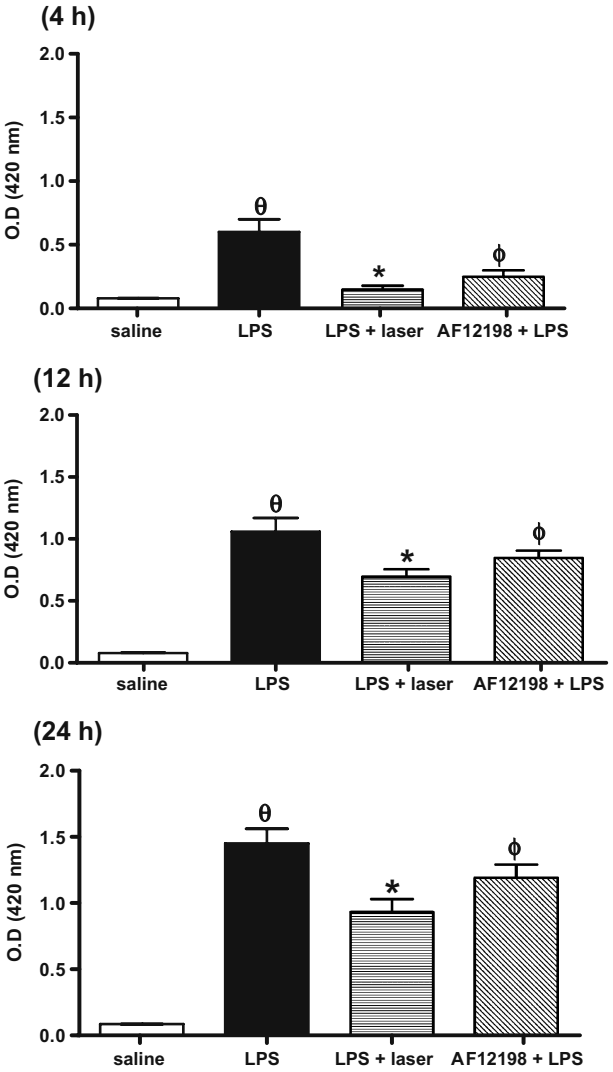

Fig. 3. Lung MPO activity at 4, 12 and 24 h after LPS injection. MPO activity in the rats at 4, 12 and 24 h after LPS was significantly elevated. The laser therapy and AF12198 significantly attenuated the increase in lung MPO activity induced by LPS. Statistically significant differences are indicated by *theta* ($P<0.001$) comparing data to saline or by *asterisk* ($P<0.001$) or *phi* ($P<0.001$) comparing data to LPS.

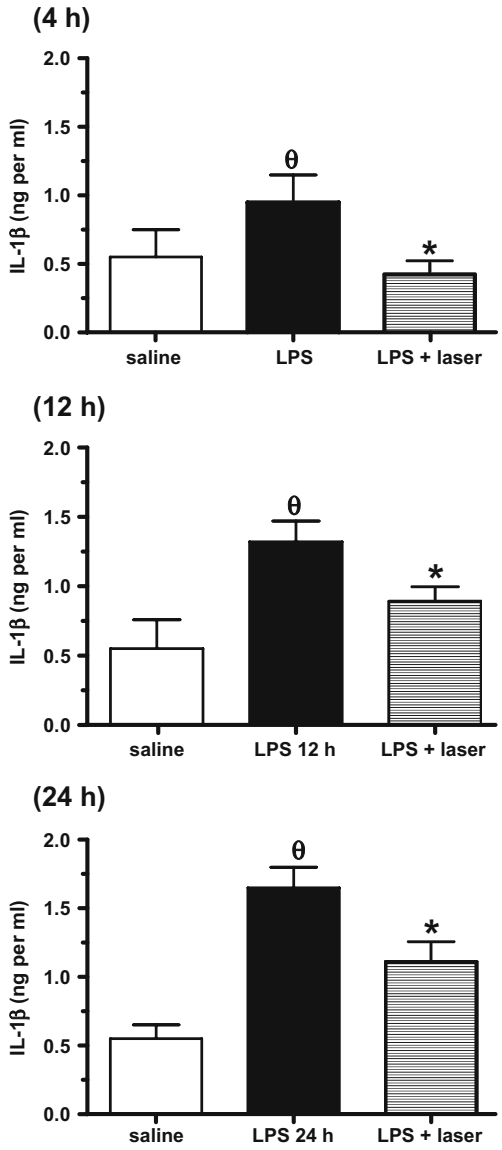

Fig. 4. IL-1β level in BAL at 4, 12 and 24 h after LPS injection. After LPS the IL-1β level in BAL was significantly increased. LLLT presented a significant effect on IL-1β level in BAL in all periods studied. Statistically significant differences are indicated by *theta* ($P<0.001$) comparing data to saline or by *asterisk* ($P<0.001$) comparing data to LPS.

Effect of LLLT on IL-1β mRNA Expression in Tracheal Tissue

Figure 5 represents the effects of LPS or LLLT on IL-1β mRNA expression in rat tracheal tissue. LPS caused a significant increase of rat trachea IL-1β mRNA expression related to time of exposure to LPS (LPS 4 h, 5.48±0.40, $P<0.01$), (LPS 12 h, 12.30±0.45, $P<0.001$), (LPS 24 h, 14.22±0.45, $P<0.001$) when compared to saline group (saline 4 h, 0.98±0.18), (saline 12 h, 0.98±0.20), (saline 24 h, 0.98±0.20). The treatment with laser diminished the rat trachea IL-1β mRNA expression 4, 12 and 24 h after LPS (LLLT 4 h, 2.13±0.17, $P<0.001$, $F=47360$), (LLLT 12 h, 7.13±0.45, $P<0.001$, $F=129200$),

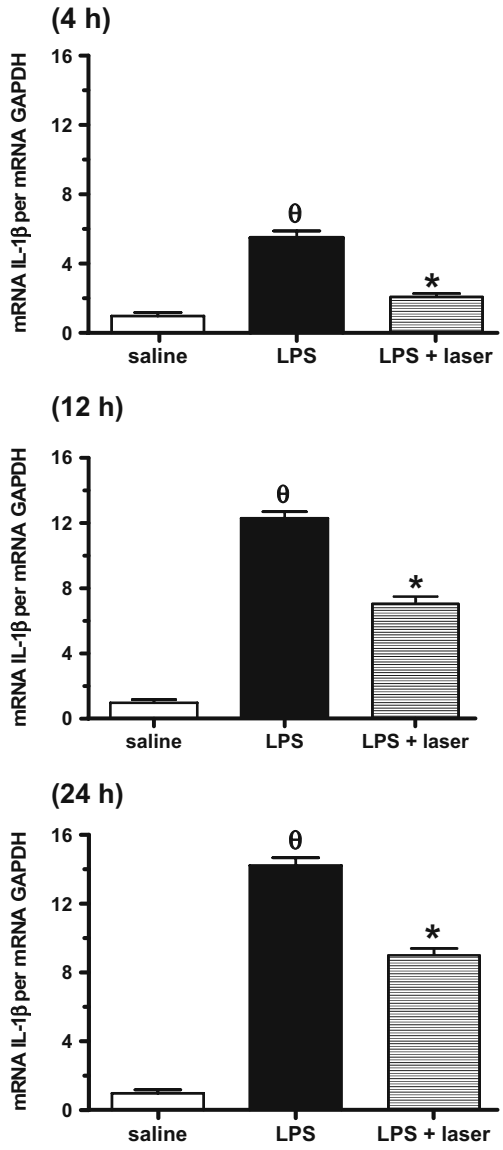

Fig. 5. IL-1β mRNA expression in trachea after LPS injection. The IL-1β mRNA expression in trachea tissue was significantly increased at 4, 12 and 24 h after LPS. LLLT diminished significantly the expression IL-1β mRNA expression in trachea after LPS injection. Statistically significant differences are indicated by *theta* ($P<0.001$) comparing data to saline or by *asterisk* ($P<0.001$) comparing data to LPS.

(LLLT 24 h, 9.15±0.40, $P<0.001$, $F=1188000$) in comparison with LPS group.

DISCUSSION

Interleukin (IL)-1β is one of the major cytokines involved in initiation and persistence of lung inflammation [26]. Increased expression of IL-1β by bronchial epithelium, increased numbers of IL-1β producing macrophages in the bronchial submucosa and elevated levels of IL-1β in bronchoalveolar lavage fluid as well as in tracheal biopsy samples have been reported in asymptomatic and symptomatic individuals with asthma [27].

In the present study we have shown that LLLT delivered at dose of 7.5 J/cm^2 and wavelength of 660 nm was able to relieve the LPS-induced airway inflammation through of a mechanism that involves the reduction of pro-inflammatory cytokine IL-1β level. It represents an important effect of LLLT since it is well known that IL-1β has a pivotal role in inflammatory processes [7].

It is now well recognized that increased vascular leakage and leukocyte diapedesis, essential features of the inflammatory response, contribute significantly to the multisystem organ dysfunction involving ALI [28]. Considering these provocative *in vitro* observations, this study sought to explore the potential anti-inflammatory property of LLLT *in vivo* using a LPS-induced rat model of lung inflammation verified by MPO activity and by BAL cellular parameters.

The endothelial cells are responsible by integrity of vascular wall and also participate of inflammatory response. The vasoactive effect of chemical mediators in the early phase of inflammation alters the vascular permeability permitting the infiltration of inflammatory cells into lung [26]. Thus, it is possible that the impaired infiltration of inflammatory cells into lung caused by LLLT can reduce the IL-1β level produced by these cells.

Using this reliable model of ALI, we demonstrated a significant effect of LLLT on pulmonary inflammation and capillary leakage. A single irradiation of LLLT, 1 h after LPS injection, produced significant reduction in multiple indices of LPS-induced inflammatory lung injury, including vascular leak (reduction in EBA leakage), BAL neutrophils and phagocyte lung infiltration. Regarding the LLLT anti-inflammatory effect on a later time is possible to suggest that laser therapy has the potential to control important features that occurred not only at 4 but also 12 and 24 h after LPS exposure. Despite intense research and multiple diverse therapeutic trials [29, 30], specific effective therapies in preventing or reversing the severe pulmonary inflammation and increased capillary permeability remain elusive.

In the series of experiment using the IL-1β receptor antagonist (AF12198) it was confirmed, as previously described by other authors, that IL-1β is involved in the inflammatory process that leads to increases of pulmonary microvascular leakage and myeloperoxidase activity. These results showed that the LLLT has a more pronounced anti-inflammatory effect when compared to AF12198. It occurred because AF12198 is a specific antagonist to IL-1β receptor, differently of LLLT that is also able to interact with other inflammatory mediators, as TNF-α.

The mobilization, activation and trafficking of effectors cells to the airway are controlled by a complex pleiotropic cytokine milieu derived from resident airway cells including epithelial and airway smooth muscle cells [31]. In fact, smooth muscle cells are source of increasing amounts of pro-inflammatory cytokines, especially TNF-α and IL1-β after inflammatory stimuli [32].

In the preset work, the IL-1β level in trachea tissue increased at 4, 12 and 24 h after LPS injection. The excess of IL-1β can impair the function of pulmonary cytoskeleton contributing to the increase of pulmonary microvascular leakage, resulting in edema [22]. An increase of IL-1β level in trachea and BAL can stimulate these cells to express adhesion molecules, which certainly facilitate the migration of neutrophils and formation of edema. LLLT reduced the concentration of IL-1β in trachea and BAL in all time periods studied in the present manuscript. This result showed that low level laser can interact with inflammatory mediators produced in different sites into respiratory system, but it does not mean that this anti-inflammatory action of laser therapy is the better effect, because is possible that the anti-inflammatory effect would be more pronounced if laser was irradiated around a more vascular part of the lung. On the contrary, the local treatment with laser seems to be an advantage because it attains only to airway and lung; thus, a possible toxic effect of LLLT can be better controlled and even avoided [35–38]. Our results reinforce the proposal of a local treatment of lung inflammation induced by LPS. Anyway, the LLLT effect on ALI could be significant even when acting locally.

From these results it is reasonable to suggest that laser could be acting directly on IL-1β effects produced by inflamed lung with LPS. Considering the efficiency of LLLT in reducing IL-1β activity in BAL and IL-1β

level in trachea tissue seems that the anti-inflammatory action of laser therapy involve the participation of IL-1β either early (4 h) as later (24 h).

The migration of active neutrophils and macrophages directly from blood to lung is commonly evaluated by measurement of myeloperoxidase (MPO) activity in BAL [25]. The MPO activity was reduced after LLLT in BAL of rats inflamed with LPS. Although there is a significant participation of macrophages in inflammatory response induced by LPS, these cells suffer lesser anti-apoptotic effects in the presence of cytokines in comparison to neutrophils [33]. High levels of cytokines induce a cellular signaling pathway impairing the apoptosis of neutrophils, which can contribute to perpetuation of inflammatory response [34].

The enzymatic activation responsible by increasing of IL-1β mRNA expression has been described by some authors [39]. Some reports showed that LLLT interfere on mRNA of different mediators in diverse models of inflammation [40].

Unfortunately, we can still not affirm, which enzymatic pathway LLLT activates in trachea tissue and how much energy reaches the airways. However, the fact is that LLLT reduced IL-1β mRNA expression and it we make suggest that this therapy can interact with local production of IL-1β.

Taken together, these results suggest that LLLT may be a new alternative and co-adjuvant therapy in the treatment of airway diseases related to sepsis, associated to a mechanism involving both the reduction of the synthesis of IL-1β in BAL and IL-1β mRNA expression in trachea from rats systemically inflamed with LPS.

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