Low level laser therapy reduces acute lung inflammation without impairing lung function

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1. Introduction

The use of low-level laser therapy (LLLT) as a therapeutic tool was first described almost 50 years ago [1]. Since then LLLT has been proven to be a treatment that is effective and with few collateral effects compared to other therapies prescribed for inflammatory and traumatic disorders [2]. However, even after decades of continuous research, the exact mechanisms of low energy laser radiation action on the human body is still unknown.

Several molecular targets have been described. LLLT seems to enhance the electron flow through the respiratory chain in the mitochondria, as a result from photon acceptance on cytochrome c oxidase, leading to increased production of reactive oxygen species (ROS) [3]; as a consequence of this redox signaling imbalance, the transcription factors HIF-1α, NRF2 and NF-kB may be activated, leading to a massive genic response [4] directed to inflammation, repair and proliferation [5–7]. Also in the mitochondria, LLLT may enhance the membrane potential...
The inhibition of IL-1β production and diminished neutrophils influx in alveolae and infiltration and cytokines (IL-1β, IL-6, TNF-α) release [24].

Nonetheless, some questions still remain regarding the effect of LLLT on inflammed lungs. Therefore, in this work, we addressed some of these unresolved issues. First, the time and moment of the LLLT application is different from other groups. We applied light therapy just once, six hours after the lung injury, when the inflammatory is already in progress. Second, we assessed the mechanisms responsible for the neutrophil accumulation in the lungs, a prominent feature in this experimental model. We focused our attention not only on cytokines, but also in chemokines. And finally, we evaluated the inflammatory reaction and the pulmonary mechanics one week after the injury and LLLT. This is important because, in other experimental models, LLLT promoted increased collagen deposition, a finding that could affect adversely whether it occurs in lungs.

2. Methods

2.1 Experimental procedures

All experiments were conducted according to international standards of ethics in animal experimentation and approved by the Ethics Committee of the School of Medicine, University of São Paulo (USP), protocol number 061/11.

C57BL/6 male mice, approximately 8-week old, were kept in cages suitable for the species covered with shavings (5 per cage), under controlled environmental conditions with light/dark cycles of 12 hours and temperature 24 ± 2 °C. They had free access to water and standard chow.

Forty animals were equally divided into 4 groups: a) animals that received PBS and no LLLT treatment (PBS Group); b) animals that received PBS and LLLT treatment (PBS+LLLT Group); c) animals that received LPS and no LLLT treatment (LPS Group); d) animals that received LPS followed by LLLT treatment (LPS + LLLT Group).

In order to induce ALI, all animals were anesthetized with a mixture of ketamine (150 mg/kg) and xylazine (5 mg/kg) and lipopolysaccharide (LPS, 5 mg/kg) was administered intratracheally. They were kept in individual cages for recovery.

2.2 Protocol of LLLT treatment

Laser irradiation was performed just once, six hours after the LPS or PBS instillation [25]. Animals from the PBS+LLLT and LPS+LLLT groups were irradiated using a gallium-aluminum-arsenide (GaAlAs) diode laser (PHOTON LASER II, DMC® Equipment Ltd, SP, São Carlos, Brazil), with the following parameters: 660 nm ± 10 wavelength, 30 mW power output, spot area of 0.028 cm² in continuous radiation mode, a technical point of contact, energy density 10 J/cm², 9 sec irradiation time and power density of 1.07 W/cm². Irradiation was performed on the skin (after shaving) in the region of the mid axillary line bilaterally. Laser was applied by contact technique, with the optical fiber kept perpendicular to the skin.
2.3 Pulmonary mechanics evaluation

Seven days after the intratracheal instillation of LPS or saline, mice from the four groups studied were anesthetized (50 mg/Kg i.p. thiopental), tracheostomized and connected to a rodent ventilator (FlexiVent; SCIREQ, Montreal, Canada) with the tidal volume and frequency set at 10 mL/kg and 2 Hz, respectively. Oscillatory lung mechanics was performed by producing flow oscillations at different prime frequencies (from 0.25 Hz to 19.625 Hz) for 16 s. Pressure and flow data were obtained and airway impedance was calculated at each frequency. Values of respiratory system resistance (Rrs) and elastance (Ers) were collected. Tissue damping (Gtis) and tissue elastance (Htis) parameters were obtained by applying the constant phase model [26].

2.4 Bronchoalveolar lavage (BAL) and lung tissue collection

Twenty four hours after LPS instillation, under anesthesia, animals were exsanguinated via the abdominal aorta. BAL was collected by introducing 1.5 ml of sterile saline into the lungs via a tracheal cannula and withdrawing the fluid into a test tube on ice. Total cells were counted using a Neubauer hemocytometer chamber and differential cellcounts were performed according to standard morphologic criteria.

For lung removal, with the animal connected to the ventilator, a positive end-expiratory pressure of 5 cm H₂O was applied to the system and the airways were occluded at the end of expiration. Lungs were fixed in formaldehyde, cut and stained with H&E. Polymorphonuclear (PMN), and mononuclear (MN) cells were counted in terminal bronchioles using the point-counting technique [27]. All analyses were performed in 16 randomly selected transversely sectioned of terminal bronchioles. The slides were coded and the researcher who performed the measurements was unaware of the study groups.

2.5 Real time PCR

Messenger RNA was measured using real time PCR (RT-PCR). Total RNA was extracted from frozen mouse lung using TRIzol protocol (Invitrogen, Carlsbad, Calif). Real-time PCR was performed in a 15 μl reaction mixture containing 7.5 μl 2 × SYBR Green Reaction Mix (Invitrogen), 0.3 μl each primer, 0.3 μl Super Script III RT/Platinum Taq Mix (10 pmol/μl), 0.15 μl ROX Reference Dye, and 5 μl sample in water. The sequences of the specific primers (Invitrogen) and reaction conditions used for RT-PCR are presented in Table 1.

2.6 Statistical Analysis

Data were expressed as mean ± SEM. Data were tested for Levene and Shapiro-Wilk test to assess normality and homogeneity of the samples, respectively. Comparisons between experimental groups were performed by analysis of variance (one-way ANOVA). Bonferroni test was used as posttest to compare individual groups. P-value < 0.05 was considered significant.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Number of cycles</th>
<th>Annealing temperature</th>
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| TNF-α | F: 5′-TGAGCACAGAAAGCATGATCC-3′
R: 5′-AAGAGGCTGAGACATAGGCAC-3′ | 40 | 54.2 °C |
| IL-1β | F: 5′-GGGAGCTACCTGTTGTTTCCC-3′
R: 5′-ATATGGGTCGGACAGCCAGG-3′ | 40 | 60.0 °C |
| IL-6 | F: 5′-CGGAGGAGAGAGATACAGGAG-3′
R: 5′-GGTAGCATCCATCATTTTTG-3′ | 40 | 51.8 °C |
| MCP-1 | F: 5′-ACCTGGGATCAGGAACAAATGAG-3′
R: 5′-GAAATGTTCTTGAAGTTTTG-3′ | 40 | 57.1 °C |
| F4/80 | F: 5′-CCTGAACATGCAACTGCGAC-3′
R: 5′-GGGATGAGACACCTGCTGTA-3′ | 40 | 59.9 °C |
| TGF-β | F: 5′-GAGAGCAGGAATACAGGCTTTC-3′
R: 5′-CGGATTCTATGCTATGATGTTG-3′ | 40 | 56.0 °C |
| β2M | F: 5′-CATGCTCGTCTCGTGCTACC-3′
R: 5′-AATGTGGACGGCGGGTGAACTG-3′ | 40 | 60.0 °C |
3. Results

3.1 LLLT effect on inflammatory cells infiltration induced by intratracheal LPS inoculation

Intratracheal LPS inoculation induced, 24 h after the procedure, a strong inflammatory reaction on distal pulmonary tissue, particularly in alveolae and perivascular tissue. This reaction is characterized by intense immune cells infiltration, both in interstitium and inside the alveolae, perivascular edema and interalveolar septae enlargement, as observed in Figure 1 Panel C.

In animals that were submitted to LLLT 6 h after inoculation, the pulmonary inflammatory response was much less evident, particularly regarding cell infiltration. As presented in Figure 1, Panel D, there was a marked decrease in the number of inflammatory cells inside the alveolae and in the lung interstitium. LLLT did not affect the pulmonary tissue (Figure 1, Panel B) when applied to lungs that were not exposed to LPS (Figure 1, Panel A).

Further, we observed that the LPS-induced inflammatory infiltrate in lung tissue was composed by both polymorphonuclear (Mean = 53.41; SEM 1.604) and mononuclear cells (Mean = 16.21; SEM 1.721) (Figure 2, Panel B and C). LLLT was able to reduce infiltration of both cell types after LPS inoculation. Moreover, we confirmed the presence of macrophages in lungs using expression of F4-80 as a marker. F4-80 is a transmembrane protein found exclusively on macrophage, being therefore, a specific marker of infiltration by these cells. As observed in Figure 2, Panel D, there was a marked increase in F4-80 mRNA levels in animals exposed to LPS (Mean = 3.52, SEM 0.54) compared to Control mice. This phenomenon was completely abolished in animals submitted to LLLT (Mean = 1.10; SEM 0.044; p < 0.003).

We also checked whether LLLT could decrease cell infiltration inside the alveolae. As observed in Figure 2, Panels E and F, LPS inoculation induced migration of macrophages (Mean = 2.82; SEM 0.5) (Figure 2, Panel B and C). LLLT was able to reduce infiltration of both cell types after LPS inoculation.
and neutrophils (Mean = 2.26; SEM 0.84) to alveolar space. In animals that received LLLT, the amount of macrophages cell types was reduced (Mean = 1.43; SEM 0.4; \( p < 0.01 \)).

### 3.2 LLLT effect on inflammatory markers

Acute proinflammatory cytokines, like TNF-\( \alpha \), IL-1\( \beta \) and IL-6 were strongly expressed in lungs of mice exposed to LPS (TNF-\( \alpha \) Mean = 14.62; SEM 0.39; IL-1\( \beta \) Mean = 7.25; SEM 0.74; IL-6 Mean = 16.39; SEM 0.27). Treatment of these animals with LLLT induced a significant decrease on these cytokines levels, as observed in Figure 3, Panels A (Mean = 6.44; SEM 0.38; \( p < 0.0001 \)), B (Mean = 2.43; SEM 0.79; \( p < 0.001 \)) and C (Mean = 7.20; SEM 0.67; \( p < 0.0001 \)).

We also tested the effect of LLLT in MCP-1 expression, a chemokine capable of inducing macrophage chemotaxis. As observed in Figure 3, Panel D, MCP-1 levels were markedly decreased in animals submitted to laser therapy (LPS Mean = 22.15; SEM 0.33 vs. LPS+LLLT Mean = 12.10; SEM 0.4; \( p < 0.001 \)), a result that can, at least partially, explain the diminished infiltration of inflammatory cells presented earlier. Interestingly, no LLLT effect was detected on ICAM-1 expression (Figure 3, Panel E).

Together, these results suggest that animals exposed to LPS and submitted to laser therapy present a milder lung inflammatory response, characterized by decreased cell infiltration, decreased cytokines and chemokines levels.

### 3.3 Lung function one week after ALI induction

Animals were exposed to LPS and treated, or not, with LLLT, a single application 6 h after the inoculation. One week later we assessed the resistance and elastance of respiratory system. As observed in Figure 4, Panels A and B, LLLT did not affect either of these parameters. Further, we evaluated also the tissue elastance (Htis) and tissue damping (Gtis) in the same animals. Again, as observed in Figure 4, Panels C and D, laser treatment did not cause any impairment on lung mechanics.

Additionally, we did not detect any difference in collagen content, in a later stage, in animals that received laser treatment compared to animals that were exposed only to LPS (Figure 4, Panel E). In order to explain this phenomenon, we assessed the expression of TGF-\( \beta \), a growth factor known to stimulate collagen secretion. Early after LPS inoculation (24 h), we observed high levels of TGF-\( \beta \) expression (Mean = 2.75; SEM 0.13) that was inhibited by LLLT (Mean = 1.09; SEM 0.22; \( p < 0.001 \) – Panel F).

Nevertheless, we could still observe a persistent presence of inflammatory cells inside the alveolae of animals one week after they were exposed to LPS (BAL Mean = 3.35; SEM 0.25; Macrophage Mean = 3.03; SEM 0.30). Early treatment of these animals (6 h after LPS inoculation, the same protocol used during this whole study) was able to reduce this late alveolar inflammation, even one week after the procedure (Figure 5, Panels A – Mean = 1.10; SEM 0.15 \( p < 0.001 \) and C – Mean = 0.95; SEM 0.15; \( p < 0.001 \)).
4. Discussion

Low-level laser therapy has been a valuable treatment for inflammatory disorders, notably in joints, muscle and skin. Here we report that LLLT could also be useful for the treatment of pulmonary disorders. In an experimental model of acute lung injury, a single early LLLT application not only improved inflammatory infiltrate (for at least one week) and cytokines secretion, but also did not affect lung mechanics in later follow-up.

Our experimental model is widely used to study acute inflammatory injury and its pros and cons have been discussed elsewhere [28]. It is characterized by an initial phase with increased PMN cells in interstitial and bronchoalveolar space, albumin leakage from capillaries and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, MCP-1, MIP-1) secretion. In a later phase (24–48 h after insult) this model is characterized by normalization of cytokines concentrations in BAL, while maintaining increased numbers of PMN, monocytes, macrophages and lymphocytes [29] and changes in respiratory function associated with collagen deposition [30].

We observed here that LLLT had beneficial effects over all these phases of the lung inflammation. Some of these findings have been observed in other studies before, however some differences are noteworthy. First, while most studies regarding LLLT and lung inflammation utilized the application of irradiation directly on the bronchi [31–33], we chose to apply the laser radiation in two points, one on each side of the midline of the body, in order to the pulmonary radiation to reach the greatest extent of lung parenchyma. Second, we decided to irradiate the animals 6 hours after the initiation of inflammation, a time when most of the pro-inflammatory signals have already been triggered by LPS inoculation [25]. Third, the energy density selected in the present study was based on some scientific studies with positive outcomes [34–36]. All published studies which present good results in lung inflammation used visible laser, however the energy density applied was much higher or lower than 10 J/cm². Therefore, we decided to use this parameter combination in the current study.

The timeline of the laser application provides one of the most interesting findings of this study. LLLT

Figure 4 LLLT did not change the respiratory mechanic of lungs exposed to LPS. Lungs from mice exposed to LPS were evaluated ex-vivo for resistance and elastance (Panels A and B). No difference was observed between this group and the one obtained from animals that received LPS plus LLLT. Also the tissue elastance (Htis) and the tissue damping (Gtis) were not affected by LLLT treatment after LPS inoculation (Panels C and D). These findings are corroborated by the fact that LLLT treatment did not change collagen deposition in lungs from animals that were exposed to LPS or exposed to LPS and irradiated with LLLT (Panel E). Part of the explanation for this finding may come from the fact that LLLT inhibited the expression of TGF-β earlier (24 h after LPS inoculation) (Panel F). * p < 0.05 vs. PBS; ** p < 0.05 vs. LPS.

Figure 5 LLLT anti-inflammatory effect lasts at least one week in mice lungs exposed to LPS. One week after mice lungs were exposed to LPS, they were washed and the amount of cells present inside the alveolae (bronchoalveolar lavage – BAL) were counted. We could still observe a large number of cells in BAL, notably macrophages (Panels A to C). A single LLLT early irradiation (6 h after LPS exposure) was able to greatly diminish by 70% the number of cells, mainly macrophages (Panels A and C). N = 8 in each group. ** p < 0.05 vs. LPS.
exerts not only an inhibitory effect on the chemotaxis of inflammatory cells, as described by other authors [37], but also decreases the number of leukocytes already present at the injury site. Moreover, immune cells were described separately, and LLLT seems to block the infiltration of all cell types, both in interstitial and in alveolar space. These results were confirmed by detection of F4-80 expression, a reliable method to detect the presence of macrophages. This finding is interesting because neutrophils and macrophages, for example, respond to distinct signals when they migrate inside an injury site [38].

We investigated some of the common mechanisms involved in this process in both cell types. We assessed the chemokine monocyte chemotactic protein –1 (MCP-1 or CCL2), which is a potent factor involved in monocyte recruitment into inflamed lung [39]. There was a marked increase in the expression of MCP-1 in the animals exposed to LPS, which was significantly reduced by laser irradiation. We suggest that this was one of the mechanisms by which LLLT reduced the number of inflammatory cells in the lung, however, not the only one.

We further investigated the effect of LLLT on the complex cascade of pro-inflammatory cytokines and other compounds that initiate and amplify the inflammatory response causing acute lung injury and acute respiratory distress syndrome [40]. Similarly to other authors, we detected a significant increase in the expression of several pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), that was inhibited by laser treatment [31, 41, 42]. Similar findings can be observed in studies using other models of inflammation, performed by us and other groups [43–46].

Altogether these initial results suggest a potent antiinflammatory effect of LLLT, acting both on cellular and humoral immunity. We look further into the consequences of this treatment, mainly whether lung fibrosis was resulting.

Laser treatment has been extensively used for enhancing healing [47–50]. In some of these experimental models, LLLT induces fibroblasts migration and collagen deposition [51], a phenomenon that would be detrimental, since it could lead to pulmonary fibrosis and loss of function. Therefore, we assessed pulmonary mechanics one week after animals were exposed to LPS, with or without LLLT. Elastance is a measure of the lung resistance to deformation (inflation), while resistance relates pressure to flow. Both parameters are impaired in fibrotic lungs [52]. We observed that LLLT treatment did not affect either of these variables one week after LPS exposure. The same results were obtained when we evaluated the tissue elastance (Htis) and tissue damping (Gtis), measurements that characterize the viscoelastic properties of lung tissue. Together with the finding that LLLT did not enhance collagen deposition in lungs, we may affirm that laser therapy neither cause fibrosis nor disturb pulmonary mechanical function when applied to inflamed lungs. Part of the explanation for this phenomenon may come from the finding that LLLT blocked the up-regulation of TGF-β expression induced by LPS.

Interestingly, one week after LPS exposure we could still detect an increased number of inflammatory cells, mainly macrophages, inside the alveolar space in animals exposed to LPS. A single early application of LLLT after LPS inoculation was able to reduce this later inflammatory reaction. This finding leads us to believe that LLLT alters beneficially the beginning of the inflammatory response and that this effect is long lasting.

From these results, we can apprehend that LLLT exerts a potent and long-lasting antiinflammatory effect. It is still a subject of controversy the molecular intracellular targets of laser. There are evidences suggesting that LLLT may act on the mitochondria [53], increasing adenosine tri-phosphate (ATP) production [54]. LLLT was also implicated on the activation of transcription factors [5], and in modulating the intracellular redox balance [55]. Actually, it seems probable that laser intracellular targets may vary from one tissue to other, depending on the cellular types and the characteristics of the light.

Although very encouraging, we need to be very cautious before advocating an immediate translation of these findings to a clinical research. First, we do not know whether the method of application we used in our mice can be utilized in humans with the same effects. Second, patients usually come to medical attention with pulmonary diseases long after the beginning of the inflammatory process and we do not know yet whether LLLT would be effective in these later periods. Finally, the dynamics of the human inflammatory response is different from the one found in mice. Although the medical use of low energy laser irradiation has been occurring for decades, primarily in the area of tissue healing and inflammatory conditions, light therapy use for pulmonary pathologies in humans has remained speculative [56], however, evidences of its benefits, like the ones presented in this study, may lead to further clinical trials soon.

Therefore, in this study we presented evidences that one single transthoracic application of low energy laser is able to reduce inflammation in an experimental model of acute lung injury. This phenomenon occurs even whether LLLT was applied after the inflammatory process is established and occurs through inhibition of cytokines and chemokines secretion, leading to a marked decrease in immune cells infiltration in inflamed lungs. LLLT effect lasts for at least one week and, although improving healing, did not provoke any deleterious effect in pulmonary function. This kind of experimental evidence is needed for the design of further clinical trials in-
volving the use of LLLT in pulmonary diseases in humans.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher’s website.

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References


