Dual Effect of Low-Level Laser Therapy (LLLT) on the Acute Lung Inflammation Induced by Intestinal Ischemia and Reperfusion: Action on Anti- and Pro-Inflammatory Cytokines

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Background and Objective: It is unknown if pro- and anti-inflammatory mediators in acute lung inflammation induced by intestinal ischemia and reperfusion (i-I/R) can be modulated by low-level laser therapy (LLLT).

Study Design/Material and Methods: A controlled ex vivo study was developed in which rats were irradiated (660 nm, 30 mW, 0.08 cm^2 of spot size) on the skin over the right upper bronchus 1 hour post-mesenteric artery occlusion and euthanized 4 hours later. For pretreatment with anti-tumor necrosis factor (TNF) or IL-10 antibodies, the rats received either one of the agents 15 minutes before the beginning of reperfusion.

Methods: Lung edema was measured by the Evans blue extravasation and pulmonary neutrophils influx was determined by myeloperoxidase (MPO) activity. Both TNF and IL-10 expression and protein in lung were evaluated by RT-PCR and ELISA, respectively.

Results: LLLT reduced the edema (80.1 ± 41.8 $\mu g\ g^{-1}$ dry weight), neutrophils influx ($0.83 \pm 0.02 \times 10^6$ cells ml^{-1}), MPO activity (2.91 ± 0.60), and TNF (153.0 ± 21.0 pg mg^{-1} tissue) in lung when compared with respective control groups. Surprisingly, the LLLT increased the IL-10 (0.65 ± 0.13) in lung from animals subjected to i-I/R. Moreover, LLLT (0.32 ± 0.07 pg ml^{-1}) reduced the TNF-α level in RPAECs when compared with i-I/R group. The presence of anti-TNF or IL-10 antibodies did not alter the LLLT effect on IL-10 (465.1 ± 21.0 pg mg^{-1} tissue) or TNF (223.5 ± 21.0 pg mg^{-1} tissue) in lung from animals submitted to i-I/R.

Conclusion: The results indicate that the LLLT attenuates the i-I/R-induced acute lung inflammation which favor the IL-10 production and reduce TNF generation. Lasers Surg. Med. 43:410–420, 2011.

Key words: phototherapy; cytokines; mesenteric ischemia; reperfusion injury; IL-10; TNF; inflammation

INTRODUCTION

Abdominal trauma causing intestinal ischemia and reperfusion (i-I/R) may induce remote organ injury, being the lung the first one to be affected [1]. The i-I/R is also associated to induction of systemic inflammatory response, a fact that may indicate a casual link between mediators released during systemic inflammation and the pulmonary dysfunction in adult respiratory distress syndrome (ARDS) [2]. ARDS is a critical illness characterized by acute lung injury, leading to pulmonary permeability, edema, and respiratory failure [1,2]. There is no specific therapy for ARDS, and mortality caused by this disease still remains high [3]. Circulating neutrophils play a major role in the development of both clinical settings and experimental animal models of acute lung inflammation [4–6]. Data obtained from experimental models showed that the interaction neutrophil–endothelial cell adhesion may be a rate-limiting step in the pathogenesis of acute lung inflammation induced by i-I/R [3]. The mechanisms that regulate neutrophil accumulation in the lungs and increase of microvascular permeability, as well as those that exert protective effects against the severity of such lung dysfunction, has been reported [4,5]. Many inflammatory mediators are released during i-I/R, such as tumor necrosis factor (TNF) and IL-10 [7,8].

Indeed, treatment of rats with anti-TNF antibodies prevented neutrophil influx, tissue injury, and lethality after i-I/R [7]. In transgenic mice, there is a greater production of TNF and greater lethality as compared to their

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wild-type counterparts [8]. The enhanced lethality correlated with greater TNF in serum and lung was blocked by treatment of animals with a soluble chimeric form of TNF receptor, confirming the essential role of TNF for enhanced neutrophil influx, tissue injury, and lethality [8].

Several studies have demonstrated that IL-10 modulates pro-inflammatory cytokines production and tissue injury following i-I/R injury [9,10]. There are evidences showing that the balance between the TNF and IL-10 production determines tissue injury and lethality during i-I/R [11]. In fact, some authors have reported that the acute ling inflammation derived from i-I/R is the result of the unbalance between this cytokines [12]. On this context, treatment with anti-IL-10 was associated with increased TNF concentration, tissue injury, and lethality, demonstrating a role of the endogenous production of IL-10 in modulating exacerbated tissue pathology and lethality [12]. Additionally, IL-10 appears to be involved with the preconditioning.

Based on the studies above mentioned it is clear that the strategies which favor IL-10 production and/or prevent TNF generation or function are effective to prevent i-I/R-induced lung injury.

Therapeutic advantages of low-level laser therapy (LLLT) for inflammatory pathologies have been suggested by several authors [13–15]. Some reports have referred that laser therapy can interfere positively in order to relief the clinical signals and the late and early symptoms of lung inflammation [16–18]. All treatments with LLLT on lung inflammation described above were done in a non-invasive way; thus it is reasonable to suggest that LLLT would bring beneficial results on non-allergic lung inflammation such as that induced by i-I/R.

There are some studies performed to understand which cellular signaling is responsible by LLLT anti-inflammatory action in lung and airways disorders. In acute lung inflammation elicited by immune-complex reaction in rat, irradiation with laser light reduced the MPO activity and the TNF level, beside it attenuated the pulmonary hemorrhagic lesion [19,20]. In respiratory distress induced by exposition of airways smooth muscle to TNF, Aimbire et al. [21] demonstrated that LLLT restored the relaxation capacity of rat bronchi segments by increasing cAMP. Still about TNF-induced acute lung inflammation, LLLT reduced the bronchial hyperreactivity by a mechanism that involves reductions of the calcium sensitivity as well as the inositol triphosphate receptor expression [22] and the RhoA mRNA expression in bronchi segments [23]. Moreover, Mafra de Lima et al. [24] reported the efficiency of LLLT in reducing both the cholinergic hyperreactivity and TNF mRNA expression by a mechanism dependent of NF-κB in rat bronchi in a model of LPS-induced acute lung inflammation. In the same experimental model, Mafra de Lima et al. [25] demonstrated that LLLT reduces in vivo the lung edema and neutrophil influx and in vitro attenuated the TNF-induced disruption of pulmonary endothelial barrier restoring the endothelial integrity.

Considering the LLLT effects on TNF cytokine and the balance that exists between the actions of TNF-α and IL-10 in reperfusion-induced acute lung inflammation, the present work investigated if LLLT could modulate the acute lung inflammation by up-regulating the IL-10 anti-inflammatory cytokine and down-regulating the TNF pro-inflammatory protein in a model of i-I/R in rat.

MATERIALS AND METHODS

Animals

All animal care was in accordance with the guidelines from the Camilo Castelo Branco University for animal care. The experiments were carried out on Male Wistar rats weighting 150–180 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 the Central Animal House of the Biomedical Engineer Center of the Camilo Castelo Branco University. All rats were placed in a common box and divided randomly into groups of seven animals each.

Anesthesia

Rats were pre-anaesthetized with acepromazine (0.1 mg kg^{-1}) and anesthetized with chloridrate of zolazepam (0.1 mg kg^{-1}) + tiletamine chloridrate (0.1 mg kg^{-1}).

Intestinal Ischemia/Reperfusion (i-I/R) Rat Model

Laparotomy was done under anesthesia and then the rats were submitted to occlusion of superior mesenteric artery with a microsurgical clip (Vascu-statt no 1001-531; Scalan International, St. Paul, MN) during 45 minutes, as described by Cavriani et al. [5]. After the occlusion period, the clip was removed and intestinal perfusion was re-established. Animals were sacrificed under deep anesthesia 4 hours after reperfusion by exsanguination via the abdominal aorta.

Laser Irradiation

A 660 nm laser diode (MM Optics, CW diode laser, São Carlos, SP) with an output power of 30 mW and 0.08 cm^2 of spot size was employed. The optical power was calibrated utilizing a Newport Multifunction Optical Meter model 1835C. The stability of laser during the laser irradiation was measured collecting light with a partially reflecting surface (4%). Laser irradiation dose was fixed at 5.4 J, applied punctually lasted along 3 minutes and at half an hour after the beginning of reperfusion. All animals were irradiated on the skin over the upper bronchus at the site of tracheotomy.

Anti-TNF or Anti-IL-10 Antibody

In order to investigate if the effect of laser irradiation on i-I/R-induced lung inflammation is a consequence of its action on each cytokine independently, the animals received an intravenous injection of rat anti-TNF antibody (0.25 mg kg^{-1}) 15 minutes before the beginning of reperfusion. The treatment with anti-TNF antibody was

based on studies by Flory et al. [26] and Isobe et al. [27]. In a parallel series of experiments for studying IL-10 the animals were pretreated with anti-IL-10 antibody (500 $\mu g\ kg^{-1}$) 15 minutes before the beginning of reperfusion. The treatment with anti-IL-10 antibody was based on studies by Kono et al. [28].

Experimental Groups

Sixty-three male Wistar rats were randomly allocated into nine groups ($n = 7$) named as shown in Table 1.

Lung Myeloperoxidase (MPO) Activity

MPO was measured as an index of the presence of neutrophils. Lung tissue samples were obtained from rats killed after intestinal reperfusion. The lungs were perfused via the pulmonary artery with pH 7.0 phosphate-buffered saline (PBS) containing 5 IU ml^{-1} heparin. Briefly, to normalize the pulmonary MPO activity among the group, whole lung was homogenized with 3 ml g^{-1} PBS containing 0.5% of hexadecyltrimethylammonium bromide and 5 mM EDTA, pH 6.0. The homogenized samples were sonicated (Vibra Cell, Sonics Materials, Newtown, CT) for 1 minute and were then centrifuged at 37,000*g* for 15 minutes. Samples of lung homogenates (20 μl) were incubated for 15 minutes with H_2O_2 and ortodianisidine; the reaction was stopped by the addition of 1% $NaNO_3$. Absorbance was determined at 460 nm using a microplate reader (Bio-Tek Instruments, Synergy™ H4, Winooski, VT). Lung MPO activity was also measured in basal and sham-operated rats.

Pulmonary Microvascular Leakage

Pulmonary vascular permeability was assessed by Evans blue dye extravasation. In brief, Evans blue dye (25 mg kg^{-1}) was given intravenously to rats 5 minutes before the animals were killed. Four hours after intestinal reperfusion, the rats were killed and the lungs perfused as described above and two samples of lung parenchyma removed. Both were weighted and then one was placed in formamide (4 mg ml^{-1} wet weight) at 20°C for 24 hours and the other was put to dry in oven (60°C) till constant weight. The concentration of Evans blue dye extracted in formamide was determined by spectrophotometry at a wavelength of 620 nm (Bio-Tek Instruments, Synergy 2) using standard dilution of Evans blue in formamide (0.3–100 $\mu g\ ml^{-1}$). The dry/wet ratio of each lung sample was determined (index of edema) and used in the final calculation of Evans blue extravasation which was expressed as μg Evans blue/g of dry weight. The expression of the results as a function of dry weight of tissue avoided under-evaluation of changes due to edema.

Isolation of Rat Pulmonary Arterial Endothelial Cells (RPAECs) and Cell Culture

Endothelial cells were collected from rat truncus pulmonalis 4 hours after intestinal reperfusion by digestion with 0.05% collagenase (Biochrom, Berlin, Germany) and cultured in M199 medium (Gibco/Life Technologies, Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum, 100 mg ml^{-1} streptomycin, and 100 U/ml penicillin (Sigma, St. Louis, MO) at 37°C and 5% CO_2. Cell monolayers were identified as endothelial cells by a pavement-like appearance in phase contrast microscopy. Cells used for our experiments were taken from the first or second passage; therefore, possible contaminations have been washed off.

RPAECs Damage

The PAECs were obtained from rats submitted to intestinal reperfusion. Rhodamine–phalloidin staining of a RPAECs monolayer was performed to enable visualization of actin filaments (original magnification 320×). After 4 hours of reperfusion, the RPAECs were morphologically analyzed, photographed, and counted using a microscope (Leica Microsystems, DMLB, Wetzlar, Germany) equipped with an HBO 100 W mercury lamp (Leica Microsystems, HBO 100 W) and the corresponding filter set for fluorescence microscopy. The quantification of RPAECs was done by counting the number of cells in 20 fields randomized chosen and then the mean was calculated.

Lung Histology

The organs were fixed in 4% buffered formaldehyde overnight and embedded in paraffin. Three-micrometer lung sections were stained with H&E and examined with a Leica microscope (20× magnification).

TABLE 1. Description of the Experimental Groups

Group	Description
Basal	Non-manipulated animals
Sham operated	Manipulated animals which were not submitted to occlusion or reperfusion
i-I/R	Animals submitted to occlusion of superior mesenteric artery and reperfusion
Sham + laser	Manipulated animals which were irradiated with laser light
i-I/R + laser	Animals submitted to i-I/R and irradiated with laser light
Anti-TNF + i-I/R	Animals which were pretreated with anti-TNF antibody and submitted to i-I/R
Anti-IL-10 + i-I/R	Animals which were pretreated with anti-IL-10 antibody and submitted to i-I/R
Anti-TNF + i-I/R + laser	Animals which were pretreated with anti-TNF antibody, submitted to i-I/R and laser-irradiated
Anti-IL-10 + i-I/R + laser	Animals which were pretreated with anti-IL-10 antibody, submitted to i-I/R and laser-irradiated

Lung Tissue Sampling and Processing Mediators

After BALF was performed, the thoracic cavity was exposed and, the heart and lung were removed in bloc. The two major lung lobes were dissected out, and the pulmonary vasculature of the lobes was perfused with ice-cold sterile PBS, using a peristaltic pump (Thermo Fisher Scientific, Suwannee, GA), for removing the blood poll of cells. Then, lobes were cut into 5-mm pieces using a tissue chopper, flash frozen in liquid nitrogen, and stored at -80°C for real-time PCR (RT-PCR) analysis of gene expression.

Lung Cytokines Protein

Mediators level in lung homogenates were determined by enzyme linked immunosorbent assay (ELISA) using commercially available kits according to the manufacturer's instructions. TNF-α was determined by a rat-specific sandwich immunoassay kit obtained from R&D Systems (Minneapolis, MN). The IL-10 concentration was determined using a solid phase sandwich ELISA kit, which used peroxidase and tetramethylbenzidine as a detection method (BioRad, Protein Assay, Mississauga, ON, Canada). The detection limit of those assays was found to be in the range of 1–5 pg ml^{-1}. For lung tissue, cytokine levels were further corrected for protein content using the assay of Lowry. The protein data in lung tissue were expressed as pg mg^{-1}.

Lung Cytokine mRNA Expression

Total cellular RNA from rat lung recovered 4 hours post-intestinal reperfusion was isolated by guanidium thiocyanate–phenol–chloroform extraction. The polymerase chain reaction was performed on a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) employing a reaction kit (Applied Biosystems, SYBR-Green core). Real-time PCR (RT-PCR) was performed with specific primers for rat *TNF* 195–305 (GenBank™ accession number X66539), forward primer 5′-AAATGGGCTCCCTCTATCAGTTC-3′ and reverse primer 5′-TCTGCTTGGTGGTTTGCTACGAC-3′ rat Exon (GenBank™ accession number D00475); *IL-10* primer 402–485 (GenBank™ number M58810), forward primer 5′-TGACAATAACTGCACCCACTT-3′ and reverse primer (GenBank™ number NW036214) 5′-TCATTCATGGCCTTGTAGACA; and rat *β-actin* −3474–3570 (GenBank™ accession number J00691), forward primer 5′-AAGTCCCTCACCCTCCCAAAAG-3′ and reverse primer 5′-AAGCAATGCTCACCTTCCC-3′ as basal. One microliter of RT reaction was utilized for RT-PCR. The PCR primer efficiencies were calculated using standard curves, and the relative expression levels of TNF and IL-10 in real-time were evaluated applying the 2^{C_T} method, presented as the ratio associated to the expression of the housekeeping gene—actin.

Reagents

Acepromazine, zolazepam chloride, and tiletamine chloride were purchased from Cristalia (São Paulo, Brazil). The culture medium (RPMI 1640) and rhodamine–phalloidin were obtained from Invitrogen (São Paulo, Brazil). The antibodies for TNF and IL-10 were purchased from Sigma and the reagents for ELISA were obtained from R&D Systems and the reagents for PCR came from Applied Biosystems.

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 experimental groups was used the two-way ANOVA statistical analysis to determine the *F*-score.

RESULTS

Effect of LLLT on Pulmonary Leakage Microvascular and Endothelial Cytoskeleton

Figure 1A represents the pulmonary edema in response to intestinal i-I/R. In this assay, we determined vascular permeability in the lung using the Evans blue extravasation technique 4 hours after reperfusion. The Evans blue extravasation into the lung was significantly higher (355.6 ± 41.67 μg g^{-1} dry weight) in comparison with basal group (40.1 ± 2.1 μg g^{-1} dry weight). It is observed that LLLT significantly reduced pulmonary microvascular leakage 4 hours after i-I/R (80.1 ± 41.8 μg g^{-1} dry weight). The anti-TNF antibody (122.5 ± 21.2 μg g^{-1} dry weight) also reduced the pulmonary edema after i-I/R but, it is less effective than LLLT. Otherwise, laser irradiation of the sham group had no effect on pulmonary microvasculature when compared with basal group. Figure 1B displays images showing the morphological changes in RPAECs 4 hours after intestinal reperfusion. Control cells show characteristic peripheral bands and close cell–cell contact but, the i-I/R caused a significant change in this pattern. The i-I/R results in the development of randomly oriented stress fibers, disappearance of peripheral bands, and loss of cell-to-cell contact. The LLLT reduced the morphological changes caused by intestinal reperfusion as it can be seen in Figure 1B. Table 2 shows the total counts and percentage of RPAECs before and 4 hours after intestinal reperfusion when treated or not with laser. Figure 1C represents the participation of TNF cytokine in i-I/R-induced RPAECs cytoskeleton damage in comparison with basal group (0.03 ± 0.01 pg ml^{-1}). LLLT (0.32 ± 0.07 pg ml^{-1}) reduced the TNF-α level when compared to i-I/R group (0.62 ± 0.05 pg ml^{-1}). The pretreatment with anti-TNF antibody abolished the participation of TNF in cytoskeleton damage of RPAECs. It is observed that laser radiation on animals non-submitted to reperfusion has no effect on pulmonary microvasculature, endothelial cytoskeleton, and TNF concentration.

Effect of LLLT on Pulmonary Neutrophils

Forty-five minutes of mesenteric artery ischemia followed by 4 hours of intestinal reperfusion induces a

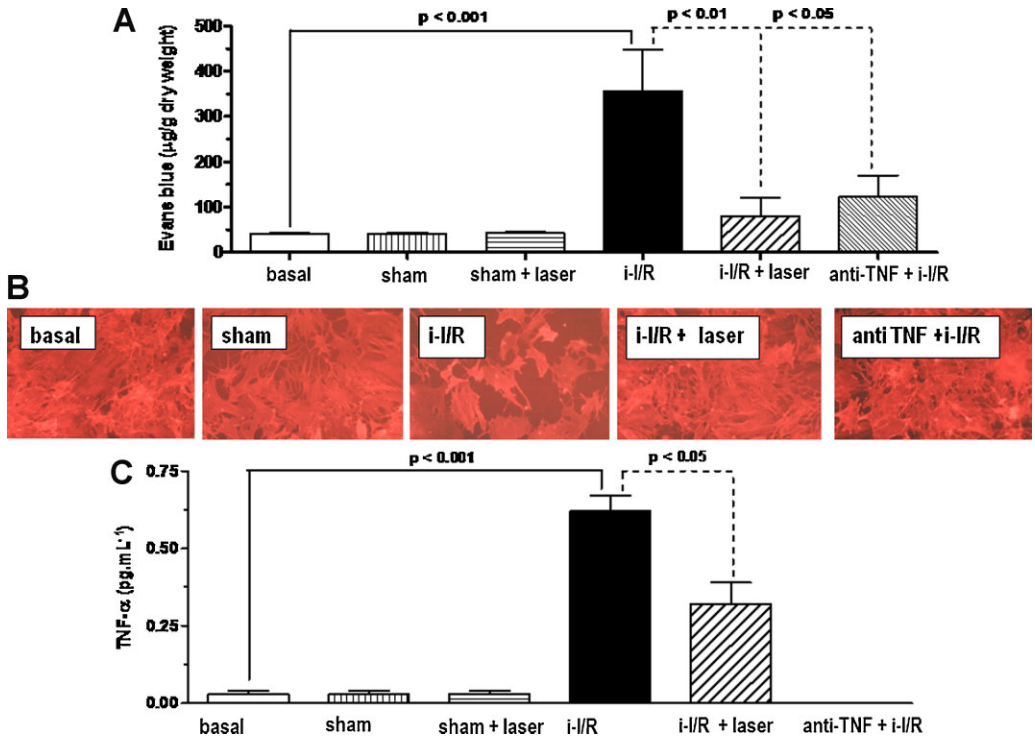

Fig. 1. Effect of LLLT on pulmonary microvascular leakage and pulmonary endothelial cytoskeleton from animals subjected to i-I/R. Rats were treated with laser 30 minutes after the beginning of reperfusion. The pulmonary microvascular leakage (**A**), the pulmonary endothelial cytoskeleton damage (**B**), and the levels of TNF in a culture medium (**C**) were measured 4 hours post-i-I/R. Data were expressed as mean ± SEM of seven animals. The results were considered significant when $P < 0.05$.

significant increase in the number of neutrophils recovered in lung ($1.55 \pm 0.10 \times 10^6$ cells ml^{-1}) in comparison with basal group ($0.04 \pm 0.03 \times 10^6$ cells ml^{-1}). The treatment with laser 30 minutes after beginning of reperfusion significantly attenuated the number of neutrophils in lung tissue ($0.83 \pm 0.02 \times 10^6$ cells ml^{-1}) as compared with that from i-I/R group (Fig. 2A). The anti-TNF antibody decreased more efficiently the neutrophils influx than LLLT ($0.37 \pm 0.04 \times 10^6$ cells ml^{-1}). Figure 2B illustrates the neutrophil infiltration into lung after i-I/R and the treatment with LLLT, clearly showing the LLLT effect in reducing inflammatory cells into lung. Laser irradiation of the sham group did not affect neutrophil influx when compared with basal group.

Effect of LLLT on MPO Activity

Figure 3 shows that i-I/R induces a large increase in MPO content in lung homogenates ($3.60 \pm\pm 0.60$) in

TABLE 2. Number and the Percentage of Pulmonary Endothelium Actin Filaments, Normal and Disrupted

	Normal filament	Disruption filament	Disruption/ (normal + disruption) (%)
Basal	115 ± 10.8	5.0 ± 2.0	4.2
Sham	115 ± 10.8	5.0 ± 2.0	4.2
Sham + laser	115 ± 10.7	5.0 ± 2.1	4.2
i-I/R	10.0 ± 1.1[a]	105 ± 1.7	91.3
i-I/R + laser	79.5 ± 2.0[b]	5.3 ± 3.2	6.2
Anti-TNF + i-I/R	82.7 ± 2.1[c]	8.0. ± 3.1	8.8

[a]i-I/R versus basal ($P < 0.05$).
[b]i-I/R + laser versus i-I/R ($P < 0.05$).
[c]Anti-TNF + i-I/R versus i-I/R ($P < 0.05$).

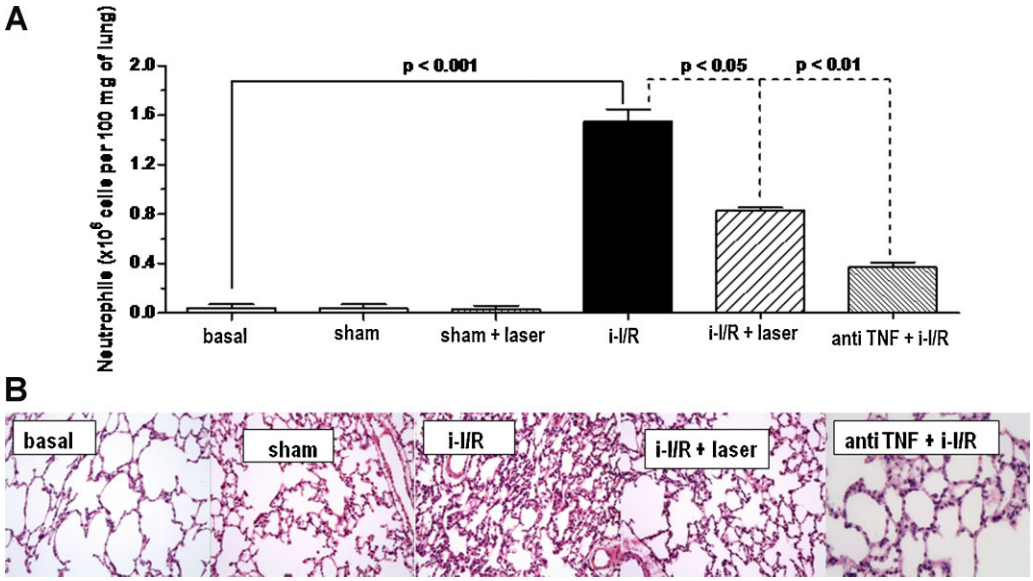

Fig. 2. Effect of LLLT on neutrophil influx from animals subjected to i-I/R. Rats were treated with laser 30 minutes after the beginning of reperfusion. The pulmonary neutrophil infiltration (**A**) and histological analysis (**B**) were measured 4 hours after i-I/R. Data were expressed as mean ± SEM of seven animals. The results were considered significant when $P < 0.05$.

comparison with animals from basal group (0.5 ± 0.04). This increase was significantly attenuated by the laser (2.91 ± 0.60) as well as by the anti-TNF antibody (1.33 ± 0.20) when compared with animals subjected to intestinal reperfusion but not irradiated. It is observed that laser irradiation on sham group did not affect neutrophil influx when compared with basal group.

Effect of LLLT on Pulmonary Cytokines Expression

We evaluated the TNF in vivo by assessing whole lung TNF-α mRNA expression and accumulation of TNF protein in lung. As demonstrated in Figure 4A, there was a significant increase in lung TNF mRNA in response to i-I/R (1.02 ± 0.10) in comparison with basal group (0.25 ± 0.09). In this condition, laser irradiation (0.39 ± 0.15) reduced the induction of TNF mRNA expression measured 4 hours after reperfusion. Figure 4B shows the up-regulation of TNF mRNA expression (655.0 ± 50.3 pg mg^{-1} tissue) in comparison with respective basal group (50.0 ± 20.1). The treatment with LLLT also inhibited the protein levels of TNF (153.0 ± 21.0 pg mg^{-1} tissue).

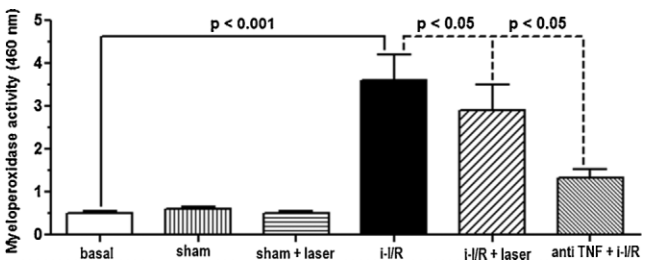

Fig. 3. Effect of LLLT on MPO content in lung tissue after i-I/R. Rats were treated with laser 30 minutes after the beginning of i-I/R. MPO activity was measured in lung homogenates to identify the neutrophils recruitment. Data were expressed as mean ± SEM of seven animals. The results were considered significant when $P < 0.05$.

The effect of phototherapy on anti-inflammatory cytokine IL-10 after i-I/R is disclosed in Figure 5. Figure 5A illustrates that the mRNA expression of IL-10 post i-I/R (0.51 ± 0.06) was significantly higher in comparison with that from animals of the basal group (0.21 ± 0.05). After treatment with laser light, there was an increase of IL-10 expression (0.65 ± 0.13) in comparison with animals submitted to i-I/R but not irradiated. Figure 5B is consistent with the up-regulated lung of IL-10 after intestinal reperfusion (510.0 ± 98.2 pg mg^{-1} tis-1 tissue) in comparison with respective basal group (200.0 ± 121.6). The levels for IL-10 were more pronounced when inflamed animals received LLLT (812.0 ± 98.2 pg mg^{-1} tissue). Laser irradiation of the sham group has not effect on cytokines when compared with basal group.

Effect of LLLT on Cytokines in the Presence of Anti-TNF or Anti-IL-10 Antibodies

In order to verify if the low-level laser radiation acts by distinct mechanism on TNF and IL-10 proteins in lung homogenates, the effect of laser on animals treated with anti-TNF antibody 30 minutes before initial of reperfusion was initially evaluated (Fig. 6). The lung IL-10 concentration in animals submitted to i-I/R and treated with

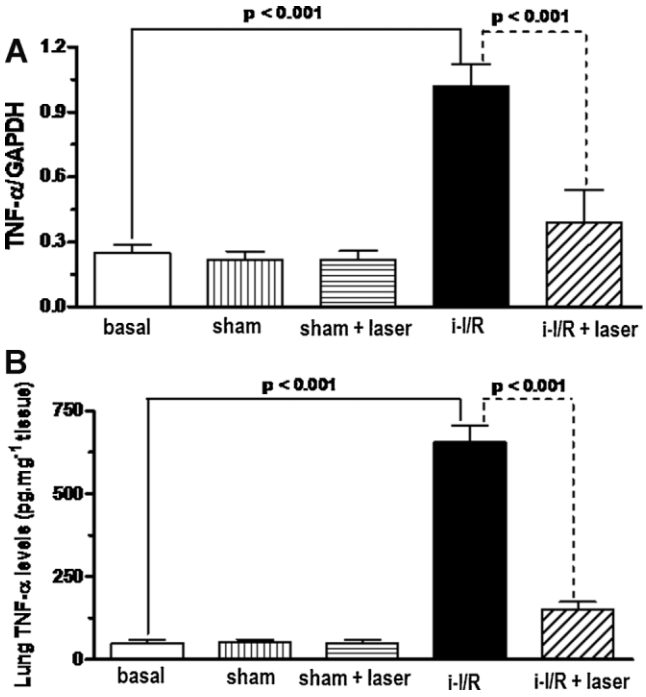

Fig. 4. Effect of LLLT on pro-inflammatory TNF mRNA expression and protein in rat lung. Rats were treated with laser 30 minutes after the beginning of reperfusion. A representative RT-PCR analysis showing TNF together with housekeeping expression GAPDH were used as internal control. The content of TNF protein in lung was measured by ELISA technique. TNF mRNA expression (**A**) and protein (**B**) were measured 4 hours post-i-I/R. Data were expressed as mean ± SEM of seven animals. The results were considered significant when $P < 0.05$.

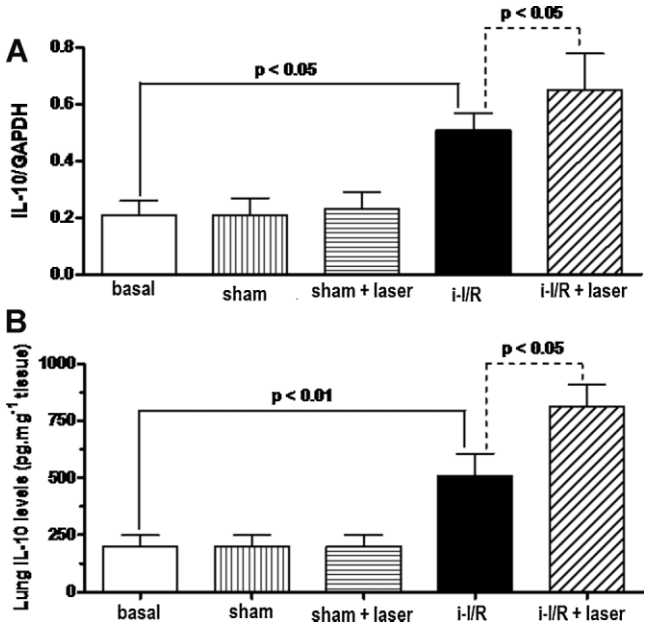

Fig. 5. Effect of LLLT on anti-inflammatory IL-10 mRNA expression and protein in rat lung. Rats were treated with laser 30 minutes after the beginning of reperfusion. A representative RT-PCR analysis showing IL-10 together with housekeeping expression GAPDH were used as internal control. The content of IL-10 protein in lung was measured by ELISA technique. IL-10 mRNA expression (**A**) and protein (**B**) were measured 4 hours post-i-I/R. Data were expressed as mean ± SEM of seven animals. The results were considered significant when $P < 0.05$.

anti-TNF antibody was higher (303.7 ± 50.3 pg mg^{-1} tissue) than that from the basal group (50.0 ± 20.2 pg mg^{-1} tissue), but less expressive when compared with IL-10 concentration from animals submitted to i-I/R but not treated with anti-TNF antibody (see Fig. 5B). In the presence of anti-TNF antibody the laser increased markedly the IL-10 protein concentration (465.1 ± 21.0 pg mg^{-1} tissue). The anti-TNF antibody treatment not influenced the effect of LLLT in increasing the concentration of IL-10. Likewise, the laser irradiation on the sham group promoted no effect on cytokines when compared with basal group.

Figure 7 illustrates the effect of the laser irradiation on TNF level in lung homogenates from animals treated with anti-IL-10 antibody 30 minutes before reperfusion. As expected, the TNF concentration in animals submitted to i-I/R and treated with anti-IL-10 antibody was markedly higher (775.1 ± 50.1 pg mg^{-1} tissue) compared with the basal group (52.0 ± 20 pg mg^{-1} tissue). In the presence of anti-IL-10 antibody, the TNF protein level in lung was increased in comparison with animals submitted to i-I/R but not treated with antibody (see Fig. 4B). The laser reduced significantly the TNF protein concentration when the anti-IL-10 antibody was administrated (223.5 ± 21.0 pg mg^{-1} tissue). The anti-IL-10 antibody treatment did not influence the action of LLLT in reducing the concentration of TNF. The laser irradiation on the sham group promoted no effect on cytokines when compared with basal group.

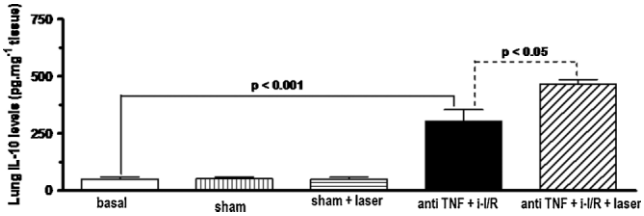

Fig. 6. Effect of LLLT on IL-10 anti-inflammatory protein in lung from rats treated with anti-TNF-α antibody. Rats were pretreated with anti-TNF antibody 15 minutes before the beginning of reperfusion. The content of IL-10 protein in lung was measured by ELISA technique 4 hours post-i-I/R. Data were expressed as mean ± SEM of seven animals. The results were considered significant when $P < 0.05$.

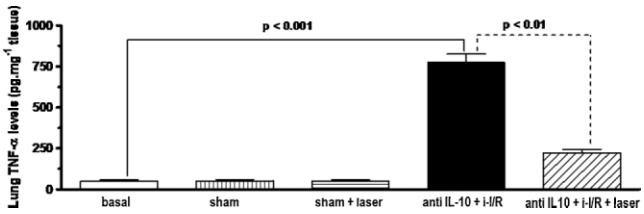

Fig. 7. Effect of LLLT on TNF inflammatory protein in lung from rats treated with anti-IL-10 antibody. Rats were pretreated with anti-IL-10 antibody 15 minutes before the beginning of reperfusion. The content of TNF protein in lung was measured by ELISA technique 4 hours post-i-I/R. Data were expressed as mean ± SEM of seven animals. The results were considered significant when $P < 0.05$.

DISCUSSION

The present manuscript reports by the first time the effect of LLLT on pulmonary inflammation in a model of intestinal i-I/R in rat. The contribution of this investigation was to demonstrate the capability of the laser therapy in a non-invasive manner and applied in a few minutes in modulating the pro- and anti-inflammatory cytokines expression in lung that facilitates the restoration of the balance between the actions of those cytokines, which is fundamental for maintaining the homeostasis.

An interesting point in the present manuscript is that laser effect was tested in a model of i-I/R, which differently of those models of acute lung inflammation elicited by LPS, is characterized by the existence of an inflammatory focus, since the ischemia and posterior reperfusion state provoked an important lesion on the intestinal mesenteric arteries. Furthermore, the acute lung inflammation induced by LPS has low indices of mortality [29]; whereas a high lethality is a common outcome in i-I/R models [8].

Other question to be addressed is the effect of phototherapy on pulmonary microvascular leakage. The pulmonary leakage depends of the integrity of the endothelial barrier and somehow represents the result of i-I/R process in which the leukocyte–endothelium interaction also contributes to the development of acute lung inflammation, particularly due to the massive production of cytokines such as TNF and IL-1β [30]. Our results demonstrated that the laser therapy was able to diminish in a significant manner the pulmonary edema in animals from the group subjected to i-I/R when compared with those animals inflamed but not treated with laser. Considering the LLLT anti-inflammatory effect on pulmonary microvascular leakage is reasonable suggesting that the action mechanism of the laser therapy in i-I/R-induced acute lung inflammation model involves the participation of endothelial cytoskeleton that is the molecular motor that drives non-muscle actin cytoskeleton rearrangement. In fact, it was reported that the endothelial cell damage is decreased after LLLT in a model of acute lung injury induced by *Escherichia coli* lipopolysaccharide in rat [25]. Although the experimental model of acute lung inflammation above mentioned had been induced by LPS injection, that is, a different model of that presented here, it is important to stand out that the LPS reproduces almost all the effects induced by i-I/R, including the pulmonary edema. Herein, the attenuation of the pulmonary endothelial cytoskeleton damage suggests a modulator action of the LLLT on i-I/R-induced acute lung inflammation through a signaling pathway that involves TNF, since the laser irradiation reduced the TNF levels collected from the supernatant of culture medium of RPAECs. This result is very interesting because showed that laser irradiation is able to reach lung structures involved with the vascular permeability. Thus, it is probable that LLLT effect in restoring the pulmonary microvascular leakage can be directly associated to its effect on the endothelial cytoskeleton. Admitting this, it is reasonable to suppose that the LLLT effect on neutrophils influx could be privileged by reducing the after reperfusion damage in RPAECs.

Our results showed that after i-I/R there was a significant augment of neutrophils influx into lung tissue, since the MPO activity was increased. These results evidenced the participation of neutrophils and their role in the development of i-I/R, which is in agreement with Souza et al. [7] findings. However, when the animals were treated with laser radiation, the MPO activity was significantly reduced in comparison with those animals submitted only to i-I/R. These preliminary results allow to hypothesize that LLLT may interfere with one of the processes linked to cellular migration; although the LLLT effect on MPO catalytic activity was not measured in the present work. Thus, from these results is reasonable to suggest that the reduction of pulmonary neutrophil number elicited by laser can be directly related to the effect of this therapy on the interaction of leukocytes with endothelial cells. However, we cannot affirm it due to fact that adhesion molecules were not assessed in the present study. Further studies are necessary to clarify if there is such interaction.

Regarding to the LLLT effect on TNF production induced by intestinal reperfusion our results showed that LLLT reduced the TNF mRNA expression in lung tissue from animals inflamed after i-I/R. The mRNA expression measured by RT-PCR technique does not detect the presence of the protein in lung due to the fact that although mRNA was expressed there is a natural delay in the protein synthesis process; because of that, in the present manuscript was also evaluated the concentration of TNF protein in lung homogenates assessed by ELISA, which indicates that LLLT is truly efficient in reducing this cytokine.

An interesting study described by Frangogiannis et al. [31] showed that mast cells are an important early source of TNF during i-I/R. In this regard, an initial tissue release of TNF, possibly mast cell-derived, may be essential and sufficient for an early wave of neutrophil influx to occur and a feedback process is then installed in which neutrophil influx facilitates TNF production and at the

same time TNF production facilitates neutrophil influx [7,8]. Knowledge of pharmacological strategies in modulating this feedback may be of interest for the treatment of i-I/R. We cannot discard the hypothesis that LLLT effect on neutrophil influx would be a secondary response to inhibition of TNF expression and protein in lung. The results described herein showed that the laser was more efficient on TNF expression and protein than on MPO activity. This result suggests that the reduction of neutrophil influx by LLLT is a mechanism dependent of LLLT action on TNF. Otherwise, it is possible that the beneficial effect of LLLT in reducing the neutrophil influx observed lately (4 hours after reperfusion) could be initiated by reduction of TNF-α which is produced by the mast cells; to probe that, the laser must be applied in an early phase of i-I/R.

IL-10 is an anti-inflammatory cytokine [32,33] that is presents with elevated levels in plasma for animal models of acute lung inflammation and inhibits the release of pro-inflammatory cytokines (IL-6, IL-1β, and TNF) from monocytes/macrophages, thus preventing subsequent tissue damage [34,35]. These findings highlighted the potential importance of the pro-inflammatory versus the anti-inflammatory imbalance in acute lung inflammation, which is corroborated by the ratio of IL-10 and TNF in the lung.

Our results about IL-10 mRNA expression in lung from animals subjected to i-I/R showed that this cytokine is significantly increased in comparison with those animals inflamed but non-irradiated. The same response was observed in IL-10 protein in lung homogenates. These results are in accordance with those reported by Souza and Teixeira [11] that evidenced the enhancement of this anti-inflammatory cytokine in lung of animals submitted to severe i-I/R. Surprisingly, the treatment with LLLT induced a level of IL-10 higher than that found in animals with i-I/R-induced lung inflammation. The LLLT effect on IL-10 levels was similar for mRNA expression and for the homogenate protein in lung. These results are interesting due to the fact that, as previously reported herein, the presence of TNF in conditions of inflammation leads to an increase of IL-10. Moreover, most of the studies using animal models recognize that the rise of TNF is ordinarily accompanied by increasing of IL-10 [36]. Our results corroborates with these authors, since it was observed that TNF as well as IL-10 increased 4 hours after reperfusion. This effect is an intention of the IL-10 in counterbalancing the deleterious effect of TNF. Considering this observation, what was expected with LLLT treatment is that the IL-10 level would be also reduced, since with less TNF, the IL-10 anti-inflammatory in lung should be also reduced. However, the LLLT presents a opposite outcome. This result supports the beneficial effect of LLLT in i-I/R by other mechanism.

The fact that LLLT reduces the TNF level at the same time that increases the IL-10 into lung can suggest a non-specific effect of the laser therapy on these cytokines. Perhaps, the LLLT is acting by distinct mechanisms on each one. Therefore, in the present work the animals submitted to i-I/R were treated with anti-TNF antibody and then the IL-10 protein was measured in lung. As it was expected, our results showed that the IL-10 concentration into lung was markedly reduced by anti-TNF antibody; however, the IL-10 cytokine concentration was higher when compared to animals from basal group. This can be explained by the fact that TNF is not the only cytokine capable to induce the IL-10 formation. Indeed, Souza and Teixeira [11] demonstrated that the pro-inflammatory cytokine IL-1β is very important for generation of IL-10. These results showed that despite of treatment with anti-TNF antibody the effect of LLLT is maintained, increasing the IL-10 protein concentration into lung 4 hours after reperfusion. That reinforces our hypothesis that the laser acts on IL-10 by an independent mechanism of the TNF effect.

Considering that the laser therapy would reduce the concentration of TNF via the rise of IL-10, we blocked the participation of IL-10 in i-I/R-induced acute lung inflammation by using the anti-IL-10 antibody. Curiously, our results demonstrated that in absence of IL-10, the TNF concentration in lung was up-regulated post i-I/R in comparison with basal group. Moreover, the TNF level in lung from animals pretreated with anti-IL-10 antibody and subjected to i-I/R was markedly higher than the level in animals with i-I/R but not treated with anti-IL-10 antibody. The results suggest that LLLT acts on TNF and IL-10 in an independent manner, that is, the laser therapy reduced the TNF effect in acute lung inflammation by a cellular signaling that does not depend on its effect in increasing the IL-10 level. Based on these results, the LLLT seems to work as a facilitator of homeostasis, since it acts without specificity on anti- and pro-inflammatory cytokines.

Numerous investigators have employed specific antagonists to TNF in experimental models of acute lung inflammation. Examples of such approach include anti-TNF antibody or soluble type TNF receptor [37]. However, anti-TNF strategies have failed to prove as beneficial in the clinical treatment [38]. This may be due to the difficulty in designing clinical trials in patients. A practical problem is that patients often come to medical attention when the disease is relatively advanced and blocking these early cytokines may be too late. Although results presented herein were performed in an experimental model, it provides information that allows proposing the laser therapy as a co-adjuvant for a non-invasive treatment of acute lung inflammation. The evidence of the beneficial effects of laser therapy in treatment of patients with airway and lung compromised by acute inflammation cannot be discarded regardless the fact that there is little information about how the light can modulate the inflammatory process with only a few minutes of radiation.

Because low-level laser light is an alternative therapy, and although the results described in the present manuscript are based on an experimental model, it is important to consider the clinical implications of such treatment. Regarding to the light target, we know that the laser is

pointed to an area of the skin situated over the upper bronchus; however, we know neither what exactly the light target is nor what fraction of the delivered radiation attains the airways and lung. Certainly, those facts difficult even more the choice of the laser dosimetry that may be effective in clinical therapy. One possible way to overcome such problems would be to irradiate airways and lung by inserting an optical fiber via an endotracheal tube. However, for this intervention the patient needs to be anesthetized and it would limit the therapy in case of one more irradiation by day, beside others complications linked to this procedure. Therefore, considering that the current study evidenced the LLLT beneficial effect on lung inflammation in a non-invasive, it draws us to better study the dosimetry of the laser in order to characterize the non-invasive form laser therapy in the treatment of pulmonary disorders.

The studies assessing the LLLT clinical effect in respiratory disorders demonstrated that the laser therapy is effective with a non-invasive irradiation procedure, however, these same studies do not explain or propose how the laser light can promotes it [39–43].

Understanding of the action mechanism of LLLT on acute lung inflammation after i-I/R may be useful for the establishment of phototherapy as a novel co-adjuvant therapeutic strategy that limits the lung injury caused by the reperfusion process. The availability, good reproducibility, cost-effect efficacy, absence of side-effects, and safety make LLLT one of the most promissory non-pharmacological treatment for pulmonary diseases related to i-I/R, since it is associated to a mechanism involving the reduction of TNF and increase of IL-10 in lung.

CONCLUSION

We conclude that LLLT can attenuate the i-I/R-induced acute lung inflammation by modulating pro (TNF) and anti-inflammatory (IL-10) cytokines. Additional studies at other timepoints post i-I/R injury are warranted in this novel area of LLLT research.

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