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Effect of low-level laser therapy on the inflammatory response in an experimental model of ventilator-induced lung injury

Thaís Fernanda Fazza,[a,b] Bruno Valle Pinheiro,[a,b] Lídia Maria Carneiro da Fonseca,[a,b] Luiz Philippe da Silva Sergio,[c] Mateus Pinto Botelho,[a] Gabrielle de Moura Lopes,[a] Flavia de Paoli,[d] Adenilson de Souza da Fonseca,[c] Leda Marília Fonseca Lucinda[a,b,d] and Maycon Moura Reboredo *[a,b]

The effect of low-level laser therapy (LLLT) on an experimental model of ventilator-induced lung injury (VILI) was evaluated in this study. 24 adult Wistar rats were randomized into four groups: protective mechanical ventilation (PMV), PMV + laser, VILI and VILI + laser. The animals of the PMV and VILI groups were ventilated with tidal volumes of 6 and 35 ml kg^{-1}, respectively, for 90 minutes. After the first 60 minutes of ventilation, the animals in the laser groups were irradiated (808 nm, 100 mW power density, 20 J cm^{-2} energy density, continuous emission mode, and exposure time of 5 s) and after 30 minutes of irradiation, the animals were euthanized. Lung samples were removed for morphological analysis, bronchoalveolar lavage (BAL) and real time quantitative polynucleotide chain reaction (RT-qPCR). The VILI group showed a greater acute lung injury (ALI) score with an increase in neutrophil infiltration, higher neutrophil count in the BAL fluid and greater cytokine mRNA expression compared to the PMV groups ($p < 0.05$). The VILI + laser group when compared to the VILI group showed a lower ALI score (0.35 ± 0.08 *vs.* 0.54 ± 0.13, $p < 0.05$), alveolar neutrophil infiltration (7.00 ± 5.73 *vs.* 21.50 ± 9.52, $p < 0.05$), total cell count (1.90 ± 0.71 *vs.* $4.09 \pm 0.96 \times 10^5$, $p < 0.05$) and neutrophil count in the BAL fluid (0.60 ± 0.37 *vs.* $2.28 \pm 0.48 \times 10^5$, $p < 0.05$). Moreover, LLLT induced a decrease in pro-inflammatory and an increase of anti-inflammatory mRNA levels compared to the VILI group ($p < 0.05$). In conclusion, LLLT was found to reduce the inflammatory response in an experimental model of VILI.

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Introduction

Mechanical ventilation (MV) is commonly used as a life-sustaining therapy in critically ill patients with acute respiratory failure. However, it is related to several complications, such as ventilator-associated pneumonia, diaphragm dysfunction, and ventilator-induced lung injury (VILI).[1]

VILI results from excessive stress (transpulmonary pressure) and strain (deformation caused by volume variation relative to the volume of the lung at rest) applied to the lungs during MV.[1–3] When the forces exceed the capacity of the distention of the lung skeleton, composed mainly of elastin and collagen fibers, damage may occur.[1–3] Due to the rupture of the interstitium, the cells anchored to it (*e.g.*, epithelial, endothelial cells or resident inflammatory cells), suffer deformation, causing mechanotransduction, cytokine production, recruitment and activation of polymorphonuclear cells, and, finally, inflammatory response.[1–3]

From a clinical point-of-view, the use of protective MV has been associated with a reduction in mortality in patients with acute respiratory distress syndrome (ARDS), probably by reducing the occurrence of VILI.[4–6] As VILI is an inflammatory process, the use of anti-inflammatory strategies to mitigate it seems reasonable. In fact, some experimental studies have demonstrated the efficacy of anti-inflammatory therapies, such as the inhalation of interleukin (IL)-10 and intravenous anti-tumor necrosis factor (TNF) antibodies in reducing VILI.[7–10] In this context, the anti-inflammatory effect of low-level laser therapy (LLLT) may represent an option for VILI treatment.

[a]*Pulmonary Research Laboratory, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil. E-mail: mayconreboredo@yahoo.com.br; Fax: +55 (32) 2102 -3841; Tel: +55 (32) 2102 -3841*
[b]*Center of Reproductive Biology, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil*
[c]*Department of Biophysics and Biometry, Institute of Biology Roberto Alcantara Gomes, Rio de Janeiro State University, Rio de Janeiro, Rio de Janeiro, Brazil*
[d]*Department of Morphology, Institute of Biological Sciences, Federal University of Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil*

Previous studies that evaluated the effects of LLLT in experimental models of acute lung injury (ALI) have shown a reduction in the expression of inflammatory cytokines, oxidative stress, neutrophil activation, myeloperoxidase activity, and the generation of pro-inflammatory mediators.[11–20] In these studies, ALI was induced by intraperitoneal and intratracheal lipopolysaccharide (LPS),[11,12,15,16,19] intestinal ischemia and reperfusion,[13,14,17,20] and formaldehyde exposure.[18] To the best of our knowledge, there are no studies that have evaluated the effect of LLLT on an experimental model of VILI with MV at high tidal volumes. The ALI model induced by VILI represents an appropriate model to evaluate the mechanisms of lung injury and the inflammatory response.[21] Moreover, the concepts derived from the experimental protocols of VILI has resulted in a reassessment of the use of MV in clinical practice, especially in patients with ARDS.[21,22]

Thus, the aim of this study was to assess the effect of LLLT on an experimental model of VILI in Wistar rats. It was hypothesized that the LLLT would decrease the inflammation response of VILI measured by the ALI score, cell counts in bronchoalveolar lavage (BAL) fluid and cytokine mRNA expressions.

Methods

Animal preparation

This study was approved by the Ethics Committee in Animal Experiments of the Federal University of Juiz de Fora, Minas Gerais, Brazil. Twenty four adult male Wistar rats (weighing 309.4 ± 16.5 g) were obtained from the Reproduction Biology Center, Federal University of Juiz de Fora vivarium (Brazil).

Experimental protocol

The animals were randomized into four groups: protective MV (PMV, $N = 6$), PMV with laser (PMV + laser, $N = 6$), VILI (VILI, $N = 6$) and VILI with laser (VILI + laser, $N = 6$).

The animals were anesthetized using an intraperitoneal bolus of Anasedan® from CEVA, xylazine (8 mg kg^{-1}) and Ketamin® from Crisalia, and dextroketamine hydrochloride (80 mg kg^{-1}). After confirmation of the depth of the anesthesia, the trachea was cannulated with a 14-gauge tube, and then the animals were ventilated based on their group allocations for 90 minutes (Inspira ASV; Harvard Apparatus, Holliston, MA, USA). The animals of the PMV groups were ventilated using a tidal volume of 6 ml kg^{-1}, positive end-expiratory pressure (PEEP) of 5 cmH_2O, a respiratory rate (RR) of 80 breaths per min, and a fraction of inspired oxygen (FiO_2) of 1. The VILI groups were ventilated using a tidal volume of 35 ml kg^{-1}, a PEEP of 0 cmH_2O, a RR of 18 breaths per min, and a FiO_2 of 1. After the first 60 minutes of ventilation, the animals of the laser groups were irradiated, according to the LLLT protocol.

After the total period of ventilation (90 minutes), the animals were euthanized by exsanguination of the abdominal aorta. A laparotomy was performed, the trachea was clamped at end-inspiration, and the lungs were removed for further analysis.

LLLT protocol

After the first 60 minutes of ventilation, a therapeutic low-level infrared laser (Photon Lase III, aluminum gallium arsenide – AlGaAs) purchased from D.M.C. Equipamentos Ltda (São Paulo, Brazil) was used. Animals from the PMV + laser and VILI + laser groups were irradiated using the physical parameters presented in Table 1.[15,16]

On the dorsal decubitus position, the thoracic region was shaved, and low-level laser irradiation was performed only one time, quickly and perpendicularly to the skin in three points (upper, medium and lower) of each side of the anterior thoracic region. After 30 minutes of LLLT, the animals were euthanized.

Measurements

Lung histology

The lungs were removed in blocks and the lower right lobes were separated, fixed in 10% buffered formaldehyde, and then processed for paraffin embedding. Four-micrometer thick slices were cut and stained with hematoxylin–eosin. Morphological examinations were performed by a pathologist blinded to the experimental groups, using a conventional light microscope (Zeiss, Hallbergmoos, Germany).

Lung injury was quantified using a weighted scoring system, as described elsewhere.[21] Briefly, 20 random fields at a magnification of 400× were independently scored. Values of zero, one or two were used to represent the severity based on the following findings: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces, and alveolar septal thickening. The neutrophils were differentiated from the other inflammatory cells based on their nucleus morphology in the form of a ring. The neutrophils in the interstitial space include cells that have entered the interstitium and those that are circulating or adhere to the alveolar capillaries. Hyaline membranes could be identified as a single well-formed eosinophilic band of fibrin within the airspace. Regarding the evaluation of

Table 1 Physical parameters of the laser

Wavelength (nm)	808
Diode laser	AsGaAl
Power (mW)	100
Spot area (cm^2)	0.028
Power density (W cm^{-2})	3.571
Energy per point (mJ)	5
Energy density (J cm^{-2})	20
Time per point (s)	5
Wave emission mode	continuous
Number of points per lung	3
Application technique	Punctual by skin contact
Number of treatment sessions	one

septal thickening, only in cases that were equal to or greater than twice the normal size were considered.

To generate a lung injury score, the sum of five variables was weighted according to the relevance attributed to each one. The resulting score was a continuous value between zero (normal) and one (the most severe injury). Additionally, the extent of each lung injury score component was calculated based on the sum of the values (zero, one, or two) of each of the 20 analyzed fields.

Bronchoalveolar lavage

The left lung was washed three times *via* the tracheal tube with 4 ml of phosphate-buffered saline (PBS) solution containing ethylenediaminetetraacetic acid (10 nM). The fluid was centrifuged at 1500*g* for 10 minutes to separate the cellular elements from the non-cellular elements. Total leukocyte numbers and differential cell counts were measured, as described elsewhere.[10]

IL1-β, IL-6, TNF-α, chemokine ligand 2 (CXCL2) and IL-10 mRNA expressions

Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure IL-1β, IL-6, TNF-α, CXCL2 and IL-10 mRNA gene expressions. The right middle lobe was cut and transferred to microcentrifuge flex tubes with Trizol® reagent for total RNA extraction using a standard procedure.

cDNA synthesis. RNA concentration and purity were determined using a spectrophotometer. cDNA synthesis was carried out using a two-step cDNA synthesis kit (Promega, USA). 1 μg of RNA was reverse transcribed into cDNA using GoScriptTM reverse transcriptase (Promega, USA) according to the manufacturer's protocol using a total reaction amount of 20 μl. Real time quantitative polynucleotide chain reaction (RT-qPCR) was performed using 5 μl of concentrated SybrGreen for a final volume of 10 μl containing 50 ng of cDNA. For determination of the initial relative quantity of cDNA, samples were amplified with IL1-β, IL-6, TNF-α, CXCL2, IL-10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Reactions were run on an AppliedBiosystems 7500 RT-qPCR machine (Applied Biosystems, USA). An internal standard, GAPDH, was set up to normalize the relative gene expression level and this standard was ran with each different experiment. Melt curve analyses were performed for all genes and the specificity, as well as the integrity, of the PCR products were confirmed by the presence of a single peak. Relative expression was calculated from differences in the cycle time of the internal standard GAPDH compared to the target mRNA. Duplicate cycle threshold (CT) values were analyzed in Microsoft Excel (Microsoft) using the comparative CT (2-ΔΔCT) method (Applied Biosystems, USA).

Statistical analysis

Normality of data was analyzed using the Kolmogorov–Smirnov test. Data were expressed as mean ± standard deviation or median (interquartile range) for normally and non-normally distributed data, respectively. One-way ANOVA followed by the Tukey test was used to compare the normally distributed data. For the non-normally distributed data, the Kruskal–Wallis test followed by the Mann–Whitney *U* test was used. Adjustments for repeated measures were performed according to the Bonferroni correction. Statistical analyses were performed using SPSS version 17.0. A p-value of <0.05 was considered to be statistically significant.

Results

Animals

Two animals died during the protocol and were replaced (one from the PMV + laser group and one from the VILI + laser group). There were no significant differences among the groups regarding body weight.

Lung histology

The animals of the VILI group showed a higher ALI score when compared to the two PMV groups. The analysis of each component of the score demonstrated that compared to the two PMV groups the VILI group had greater alveolar and interstitial neutrophil infiltration. However, when the VILI + laser group was compared to the VILI group it showed a lower ALI score and alveolar neutrophil infiltration (Table 2, Fig. 1).

Bronchoalveolar lavage

Inflammatory lung response was observed in the VILI group, as shown by the increased number of neutrophils in the BAL

Table 2 Acute lung injury score and its components

	Groups			
Score	PMV	PMV + laser	VILI	VILI + laser
Overall score	0.27 ± 0.09*	0.26 ± 0.09*	0.54 ± 0.13	0.35 ± 0.08*
Alveolar neutrophils	4.50 ± 4.50*	4.67 ± 4.03*	21.50 ± 9.52	7.00 ± 5.73*
Interstitial neutrophils	26.67 ± 5.09*	27.00 ± 7.51*	37.67 ± 2.25	34.67 ± 5.72
Proteinaceous debris	9.50 ± 5.99	7.00 ± 4.73	16.00 ± 5.40	9.17 ± 7.31
Hyaline membrane	0 (0)	0 (0)	0 (0.25)	0 (0.25)
Septal thickening	2.50 (6.25)	3.00 (2.50)	3.50 (16.00)	0 (1.00)

Values are expressed as the mean ± standard deviation or median (interquartile range) for normally and non-normally distributed data, respectively. PMV: Protective mechanical ventilation; VILI, ventilator-induced lung injury. * $p < 0.05$ compared to the VILI group.

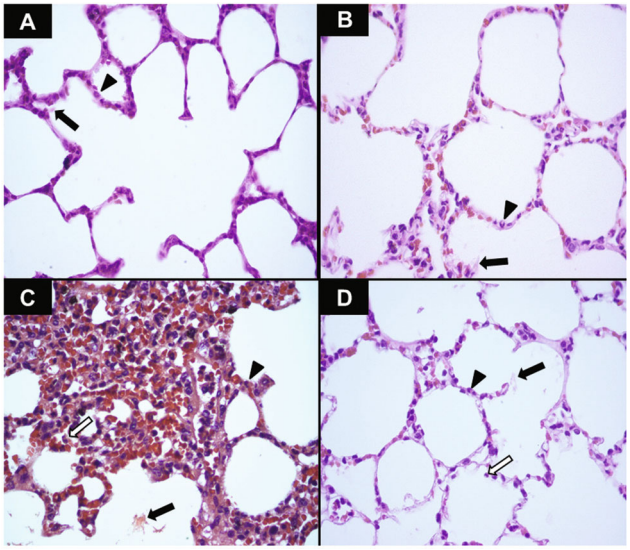

Fig. 1 Representative images of the histological analysis of the lower right lobe longitudinal section stained using hematoxylin and eosin (H&E. 400×). (A) Protective mechanical ventilation group; (B) protective mechanical ventilation with laser group; (C) ventilator induced lung injury group; (D) ventilator induced lung injury with laser group. Alveolar neutrophils (open arrows); interstitial neutrophils (arrowheads); proteinaceous debris (black arrows). Note that VILI group showed higher accumulation of neutrophils in the interstitium and alveoli when compared to the other groups.

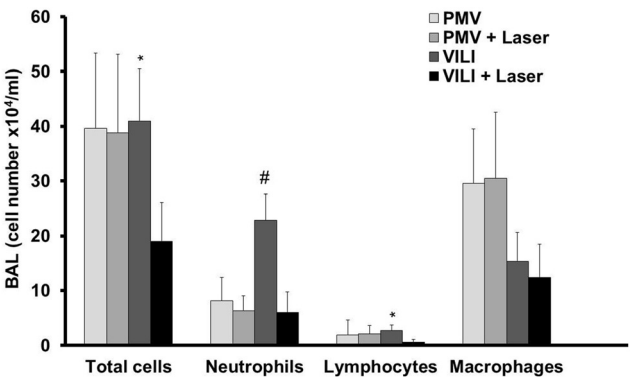

Fig. 2 Cell count in the bronchoalveolar lavage (BAL) fluid. PMV: Protective mechanical ventilation; VILI, ventilator-induced lung injury. * $p < 0.05$ compared to the VILI + laser group. # $p < 0.05$ compared to the PMV, PMV + laser and VILI + laser groups.

fluid compared to the two PMV groups. The VILI + laser group showed a lower number of total cells, neutrophils and lymphocytes compared to the VILI group (Fig. 2).

IL1-β, IL-6, TNF-α, CXCL2 and IL-10 mRNA expressions

The mRNA expression of acute pro-inflammatory cytokines IL1-β, IL-6, TNF-α, CXCL2 chemokine, and the anti-inflammatory cytokine IL-10 were strongly expressed in the lungs of the rats exposed to VILI, when compared to the two PMV groups. The LLLT induced a significant decrease in the mRNA

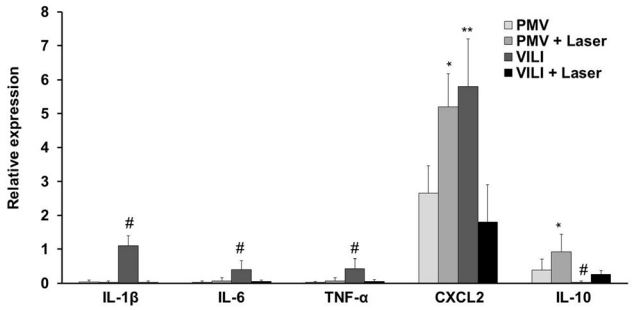

Fig. 3 mRNA expressions of IL-1β, IL-6, IL-10, CXCL2, and TNF-α. PMV: Protective mechanical ventilation; VILI, ventilator induced lung injury. * $p < 0.05$ compared to the VILI + laser group. # $p < 0.05$ compared to the PMV, PMV + laser and VILI + laser groups. ** $p < 0.05$ compared to the PMV and VILI + laser group.

expression of these pro-inflammatory cytokines and an increase in anti-inflammatory cytokines when compared to the VILI group (Fig. 3).

Discussion

Considering that previous studies showed that one hour of MV with a high tidal volume was able to induce lung injury and an inflammatory response, we evaluated the effects of LLLT on inflammatory response after one hour of VILI.[10,23] There was a reduction in the lung injury score, alveolar neutrophil infiltration, the number of neutrophils in the BAL fluid, and the inflammatory cytokine mRNA expression in the VILI + laser group, suggesting that the use of LLLT was able to decrease the lung inflammation in this animal model.

The model of VILI has been widely used in the literature, and many experimental studies have demonstrated that inflammation plays an important role in the pathogeneses of VILI.[7,10,21–25] Similarly, this study showed that a model of VILI increased the number of neutrophils detected in the BAL fluid, and mRNA expression of acute pro-inflammatory cytokines, but decreased the expression of anti-inflammatory cytokine mRNA in rats receiving high tidal volume ventilation. Moreover, these animals exhibited lung injury, as demonstrated by the higher ALI scores in the VILI groups. The inflammatory process of VILI is associated with an increase in numerous pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-33) and the chemoattractant CXCL2 (macrophage inflammatory protein – MIP-2).[24,25] Nonetheless, this study consistently showed that the great mechanical stress of VILI increases the expression of MIP-2 mRNA by tissue macrophages, stimulating neutrophil recruitment, which plays an initial important role in the control of lung injury. It was also observed that the transcription of TNF-α, IL-1β and IL-6 was increased. In general, tissue damage is recognized by immune cells such as macrophages and monocytes, which could lead to toll-like receptor (TLR)-9 activation and stimulation of the factor nuclear kappa B (NF-

κB) signaling pathway, enhancing the transcription of the inflammatory cytokines.[26]

Regarding the pharmacological treatment of VILI, the use of corticosteroids is the most studied method.[9] In this context, it was previously demonstrated that dexamethasone was able to attenuate the VILI when administered before the induction of injury.[10] Although corticosteroid benefits have been seen in other experimental studies, their clinical use in conditions such as ARDS is still under debate, in part due to the several side effects that are associated with this treatment.[1,3] Therefore, new strategies should be developed to prevent and reduce VILI.

Considering that LLLT is used for the treatment of a variety of inflammatory diseases and side-effects have not been described, this therapy could be implemented for VILI treatment.[11,12,27,28] A low power infrared laser (808 nm) has a penetration capacity of 3.4 cm into tissue, which can possibly reach rat lung tissue.[29] The anti-inflammatory effects of LLLT can be explained by cellular and molecular mechanisms that were first proposed by Karu.[30] In summary, this author reported that laser characteristics (monochromatic and collimated beams) induce alterations in the potential and permeability of the mitochondrial membrane. As a consequence, ATP synthesis increased, calcium ion homeostasis changed, and an increase in reactive oxygen species (ROS) production was observed immediately (*i.e.*, occurred some minutes after laser irradiation), leading to cellular signaling cascade and molecular responses, and finally, culminated in changes to gene expressions.[28,30–33] In this context, the animals were euthanized after 30 minutes of LLLT in our study.

In this study, the potential anti-inflammatory effect of LLLT in an experimental model of VILI was evaluated. The LLLT decreased the neutrophil migration to the lungs, as demonstrated by the lower alveolar neutrophil infiltration in the lung histology and by the smaller number of neutrophils in the BAL fluid. These effects on neutrophil recruitment were confirmed by a decrease in the CXCL2 chemokine mRNA expression.[34] Unexpectedly, the PMV + laser group showed higher CXCL2 chemokine mRNA levels when compared to the VILI + laser group.

The LLLT also decreased the expressions of IL1-β, IL-6 and TNF-α mRNA. This result could explain the reduction of neutrophils since IL1-β, IL-6 and TNF-α releases are important to neutrophil proliferation, recruitment and activation.[35] Moreover, LLLT induced an increase in IL-10 mRNA expression, which is an interesting result considering a clinical study showed that the GG genotype of the IL-10 promoter polymorphism in position -1082 (*-1082GG*) was associated with greater production of IL-10, decreasing the severity of illness, organ dysfunction, and mortality in ARDS patients.[36] IL-10 also plays an important role as an anti-inflammatory cytokine that suppresses macrophage activation and modulates pro-inflammatory cytokines, such as IL1-β, IL-6, TNF-α.[20,37] Nevertheless, de Lima *et al.* demonstrated that LLLT appeared to have an additive effect on upregulated IL-10 mRNA expression after two and four hours in a model of ALI induced by intestinal ischemia and reperfusion.[20]

Despite the effects that LLLT has on cytokine and chemokine mRNA levels, other effects of laser therapy could explain the earlier modulation of the inflammatory response in the present study. The main effects of LLLT are caused by increased local microcirculation, the promotion of angiogenesis, vasodilation, inhibition of inflammatory mediators such as prostaglandin E2, activation of defense cells, and the antioxidant effects and promotion of healing.[13,35,38,39] Regarding the antioxidant effects of LLLT, a study of de Lima *et al.* evaluated the effects that laser therapy has on alveolar macrophages stimulated with LPS. They observed an increase in ROS intracellular generation in alveolar macrophages in response to LPS when compared to the control. In contrast, LLLT reduced ROS generation in alveolar macrophages stimulated with LPS.[40] Moreover, in the present study, the increase in IL-10 mRNA expression in the VILI + laser group attenuated ROS secretion.[41]

The positive clinical results of LLLT could also be a result of its effects being related to the modulation of biochemical reactions leading to cellular function modifications. The photochemical interactions act directly on cells, suggesting the involvement of the mitochondria, which plays an important role in energy generation through alteration of cytochrome C oxidase, thus increasing the ATP level.[30,42] Therefore, some of these effects of LLLT may be quicker acting than the transcription and translation of cytokines, and after, their functional effects in cell attraction and modulation.

The benefits of LLLT in the inflammatory response have been shown in other studies, with experimental models of ALI. Cury *et al.*[12] induced ALI by intratracheal instillation of LPS, where the animals were irradiated just once with a laser (AlGaAs, 660 nm, 30 mW, energy density of 10 J cm^{-2}, power density of 1.07 W cm^{-2}), six hours after the instillation of LPS. This irradiation was capable of inducing significant decrease in the inflammatory cell influx and in the secretion of cytokines (TNF-α, IL-1β, IL-6) and a chemokine (MCP-1).[12] Similarly, de Lima *et al.*[19] evaluated the effect of LLLT (AlGaAs, 650 nm, 2.5 mW, energy density of 1.3 J cm^{-2}, power density of 31.2 mW cm^{-2}), applied once and 1 h after ALI induction by aerosol of LPS, and found that laser therapy reduced TNF-α and IL-1β preventing lung ICAM-1 up-regulations as well as inhibiting endothelial cytoskeleton damage, and neutrophil influx and activation. Also, the LLLT (830 nm laser, 35 mW, 9 J cm^{-2}, 1 h after LPS administration) was able to decrease pulmonary and extrapulmonary inflammation in LPS-induced ALI, as confirmed by the lower levels of cytokines and reduced number of neutrophils present in the BAL fluid and in lung parenchyma.[11] Irradiation with LLLT with similar parameters in this study was performed in LPS-induced ALI and it was demonstrated that the mRNA levels of gene-related apoptosis were altered after LLLT and TUNEL assay, showing positive-labeled cells in lung parenchyma, including polymorphonuclear cells.[16]

Nevertheless, there are some limitations in this study that should be taken into consideration to qualify our results. Since the LLLT was applied only once, 30 minutes before euthanasia,

the long-term effects of laser therapy should be evaluated in an experimental model of VILI. Second, the evaluation of the protein levels of the pro-inflammatory cytokines, chemokines CXCL2 and IL-10 was not performed. The effect of LLLT on the protein levels of the cytokines would more reliably demonstrate the anti-inflammatory effect of the laser. Finally, the anti-inflammatory effects of laser therapy should be confirmed in clinical trials, including in ARDS patients, as the application of LLLT cannot yet be extrapolated to humans in clinical practice. However, in regard to the potential for the human application of LLLT in the lungs, it may not possible because of penetration capacity; but the use of a fiber laser intratracheally could be an alternative to this treatment.

In conclusion, LLLT reduced the inflammatory response in an experimental model of VILI by reduction of the lung injury score, the number of neutrophils in the BAL fluid, and the inflammatory cytokine mRNA expression.

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Ethical statement

All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Conselho Nacional de Controle de Experimentação Animal (CONCEA) – Brazil and approved by the Ethics Committee in Animal Experiments of Federal University of Juiz de Fora. All procedures were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflicts of interest

The authors declare that they have no conflict of interest.

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