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Low intensity laser therapy (LILT) *in vivo* acts on the neutrophils recruitment and chemokines/cytokines levels in a model of acute pulmonary inflammation induced by aerosol of lipopolysaccharide from *Escherichia coli* in rat

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ABSTRACT

It has been suggested that low intensity laser therapy (LILT) acts on pulmonary inflammation. Thus, we investigate in this work if LILT (650 nm, 2.5 mW, 31.2 mW/cm^2, 1.3 J/cm^2, laser spot size of 0.08 cm^2 and irradiation time of 42 s) can attenuate edema, neutrophil recruitment and inflammatory mediators in acute lung inflammation. Thirty-five male Wistar rats (n = 7 per group) were distributed in the following experimental groups: control, laser, LPS, LPS + laser and dexamethasone + LPS. Airway inflammation was measured 4 h post-LPS challenge. Pulmonary microvascular leakage was used for measuring pulmonary edema. Bronchoalveolar lavage fluid (BALF) cellularity and myeloperoxidase (MPO) were used for measuring neutrophil recruitment and activation. RT-PCR was performed in lung tissue to assess mRNA expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin (IL-10), cytokine-induced neutrophil chemoattractant-1 (CINC-1), macrophage inflammatory protein-2 (MIP-2) and intercellular adhesion molecule-1 (ICAM-1). Protein levels in both BALF and lung were determined by ELISA. LILT inhibited pulmonary edema and endothelial cytoskeleton damage, as well as neutrophil influx and activation. Similarly, the LILT reduced the TNF-α and IL-1β, in lung and BALF. LILT prevented lung ICAM-1 up-regulation. The rise of CINC-1 and MIP-2 protein levels in both lung and BALF, and the lung mRNA expressions for IL-10, were unaffected. Data suggest that the LILT effect is due to the inhibition of ICAM-1 via the inhibition of TNF-α and IL-1β.

1. Introduction

Acute Respiratory Distress Syndrome (ARDS) is a critical illness characterized by acute lung injury (ALI), leading to pulmonary permeability, edema and respiratory failure [1]. There is nonspecific therapy, and mortality caused by this disease still remains high [2]. Circulating neutrophils consideration is important to define both clinical settings and experimental animal models [3].

The mechanism of polymorphonuclear leukocytes (PMNs) migration in LPS inhaled rats has been partially characterized, it involves airway cells production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), as well as neutrophil-specific chemoattractants known as CXC chemokines, which include the family of cytokine-induced neutrophil chemoattractants (CINCs) and macrophage-inflammatory protein-2 (MIP-2), also known as CINC-3 [4]. CD18 integrins have also been shown to play a role on PMN migration. Expression of CD18 adhesion molecules on the PMNs surface can be induced by exposure to LPS or TNF-α and to chemokines such as CINC-1 and MIP-2 [5]. In rats, treatment with Abs to CD11a/CD18 or CD11b/CD18, as well as the intercellular adhesion molecule-1 (ICAM-1) endothelial cell inhibitor, attenuated the migration of PMNs to intrapulmonary LPS [6].

Traditional treatments of ALI and asthma include a variety of pharmacological corticosteroids, methotrexate, and other disease-modifying agents such as cyclosporine and intravenous immunoglobulin, as well as novel treatments, such as TNF-α and IL-1β [7]. The low intensity laser therapy (LILT) has been used in inflammatory pathologies as a new anti-inflammatory therapy, without provoking side effects [8].

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Indeed, LILT has been employed in the treatment of patients with asthma [9]. It have been reported that LILT can relief the late and early symptoms of airway and lung inflammations by reducing the mRNA expression of inflammatory mediators, such as TNF-α and IL-1β [10,11]. Although it is well established that PMNs and inflammatory mediators are important in the development of ALI, the *in vivo* LILT effect on lung inflammation after stimulation by LPS inhalation is not yet completely explored.

Among the pro- and anti-inflammatory mediators that participate of the development and perpetuation of ALI, there is no enough information about on which mediators of ALI the LILT exerts its anti-inflammatory effect.

Keeping this in mind, the present study was developed specifically to investigate which are the inflammatory mediators that are driven for LILT effect on ALI induced by LPS inhalation.

2. Material and methods

2.1. Animals

All animal care was in accordance with the guidelines from the Camilo Castelo Branco University (UNICASTELO) 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 Institute of the Camilo Castelo Branco University (UNICASTELO). All rats were placed in a common box and divided randomly into groups of seven animals each.

2.2. Anesthesia

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

2.3. Induction of lung inflammation and dexamethasone treatment

In order to induce the lung inflammation male Wistar rats received inhalation for 10 min of lipopolysaccharide from negative-gram bacteria *Escherichia coli* dissolved in 2 mL saline solution at the concentration of 0.3 mg mL^{-1}. Briefly, rats ($n = 7$ per group) were allocated in a 16 cm side cubic box hermetically closed. An aerosolizator pumps the LPS solution in a controlled manner into box through a hole in the box. For glucocorticoid treatment, the rats were administered with dexamethasone (1 mg kg^{-1} p.o.) or vehicle (0.5% methylcellulose/0.2% Tween 80 p.o.) 1 h before LPS challenge. Four hours after vehicle or LPS challenge, the inflammatory parameters were analyzed.

2.4. LILT

A continuous wave low intensity GaAsAl diode laser (model Thera lase, Brazil) operating in the wavelength of 650 nm was employed. Laser parameters were 2.5 mW output power and laser spot size of 0.08 cm^2, giving an optical power density of 31.2 mW/cm^2. Animals were irradiated during 42 s with a laser dose of 1.3 J/cm^2, 1 h after LPS challenge. The optical power was calibrated by a Newport Multifunction Optical Meter model 1835C. The laser power stability during irradiation was determined collecting light with a partial reflecting mirror (4%). All animals were irradiated on the skin over the upper bronchus at the site of the tracheotomy.

2.5. Experimental groups

The experimental groups consisted of 35 male Wistar rats randomly allocated into five groups, named as:

Control – animals challenged with vehicle or saline.
Laser – animals challenged with vehicle or saline and irradiated with laser.
LPS – animals challenged with LPS.
LPS + laser – animals challenged with LPS and treated with laser.
Dexa + LPS – animals pretreated with dexamethasone and challenged with LPS.

2.6. Reagents

Acepromazine, zolazepam chloride and tiletamine chloride were purchased from Cristalia (São Paulo, Brazil). LPS from *E. coli* serotype 0111:B4 and dexamethasone were purchased from Sigma (St. Louis, MO, USA). The culture medium (RPMI 1640) was obtained from Invitrogen (São Paulo, Brazil) and the reagents for PCR of TNF-α, IL-1β, IL-10, ICAM-1 and chemokines were obtained from R&D Systems (Minneapolis, MN, USA).

2.7. Assessment of lung capillary leakage

The extravasated Evans blue dye albumin (EBA) concentration in lung homogenate was calculated against a standard curve using the Evans blue dye. The lung permeability data were expressed as μg g^{-1} dry weight.

2.8. Isolation of rat pulmonary arterial endothelial cells (RPAECs) and cell culture

Endothelial cells were collected from rat truncus pulmonalis 4 h after inhalation with saline or LPS by digestion with 0.05% collagenase (Biochrom, Berlin, Germany) and cultured in M199 medium (Gibco/Life Technologies, USA), supplemented with 10% heat-inactivated fetal calf serum, 100 mg mL^{-1} streptomycin, and 100 U/mL penicillin (Sigma, USA) 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 will have been washed off.

2.9. RPAECs damage

Rhodamine-phalloidin staining of a RPAECs monolayer was performed to enable visualization of actin filaments. The PAECs were obtained from rats exposed or not to LPS (0.3 mg mL^{-1}). After 4 h of inhalation with LPS, the RPAECs were morphologically analyzed, photographed and counted using a microscope (model Leica DMLB) equipped with an HBO 100 W mercury lamp (model HBO 100 W) and the corresponding filter set for fluorescence microscopy: blue-FITC excitation filter 450–490 nm; barrier 510 nm; emission 520–560 nm. The quantification of RPAECs was done by counting the number of cells in twenty fields randomized chosen and then the mean was calculated. The TNF-α level in supernatant of RPAECs culture was determined by ELISA using commercially available kits according to the manufacturer's instructions.

2.10. Quantification of bronchoalveolar lavage fluid (BALF) cellularity

Four hours after LPS challenge, animals anesthetized with sodium pentobarbital (200 mg kg^{-1} i.p.) were euthanized and the trachea was cannulated. BALF cells were collected from the airway