

Laser therapy, used in a specific dose, modulates pulmonary inflammatory processes in an experimental model of sepsis in rats

O uso da laserterapia em dose específica modula o processo inflamatório pulmonar em um modelo experimental de sepse em ratos

El uso de la láserterapia en dosis específica modula el proceso inflamatorio pulmonar en un modelo experimental de sepsis en ratones

Caroline Bublitz¹, Livia Assis², Ana Cláudia M. Rennó³, Vivian Cury⁴, Gabriela Harada⁵, Aya H. Hirai⁶, Liria Yuri Yamauchi⁷

ABSTRACT | This study aimed to determine the effectiveness of LLLT in decreasing the lung inflammatory process in septic rats. A total of 32 male Wistar rats were divided into four groups (n=8): control group (CG), sepsis 24h (S24), sepsis and LLLT with 30 J/cm² (S24L30); sepsis and LLLT with 65 J/cm² (S24L65). The irradiation was performed immediately after surgery in the anterior region of the trachea and ventral regions of the chest, bilaterally, just below the ribs. Histological analysis of lung tissue was performed and the number of inflammatory cells was quantified. The S24 group showed an increase of inflammatory cells compared to the CG (p <0.01); S24L30 increased the number of inflammatory cells, while S24L65 decreased this number compared to S24 (both p<0.05); S24L65 had a lower number of inflammatory cells compared to S24L30 (p<0.01). In conclusion, LLLT at a specific energy dose (30J / cm²) was capable of decreasing the number of

inflammatory cells in acute lung tissue inflammation due to sepsis.

Keywords | Laser Therapy, Low-Level; Sepsis; Lung.

RESUMO | O objetivo do estudo foi determinar a eficácia da LLLT na diminuição do processo inflamatório pulmonar em ratos sépticos. Foram utilizados 32 ratos machos Wistar divididos em quatro grupos (n=8): controle (CG); sepse 24h (S24); sepse e tratamento com LLLT 30 J/cm² (S24L30); sepse e tratamento com LLLT 65 J/cm² (S24L65). A irradiação foi realizada imediatamente após a cirurgia na região anterior de traqueia e nas regiões ventrais do tórax, bilateralmente, logo abaixo das costelas. Foi realizada análise histológica do tecido pulmonar e o número de células inflamatórias foi quantificado. O grupo S24 apresentou um aumento de células inflamatórias comparado ao CG (p <0,05); S24L30 aumentou o número de células inflamatórias, enquanto S24L65 diminuiu este

Study developed in Celular and Molecular Biology Laboratory, Biological Sciences Department, Universidade Federal de São Paulo, Campus Baixada Santista - Santos (SP), Brazil

¹PhD student. Participated in the conception of the project, design and execution of the research, data analysis and writing of the manuscript - Universidade Federal de São Paulo, Campus Baixada Santista - Santos (SP), Brazil.

²Post-doctoral student. Participated in the conception of the project, design and execution of the research, and writing of the manuscript - Universidade Federal de São Paulo, Campus Baixada Santista - Santos (SP), Brazil.

³Associate professor. Participated in the conception of the project, critical review and approval of the final version of the manuscript - Universidade Federal de São Paulo, Campus Baixada Santista - Santos (SP), Brazil.

⁴Post-doctor. Participated in the design of the research and data analysis - Faculdade de Medicina da Universidade of São Paulo - São Paulo (SP), Brazil

⁵Bachelor in Physiotherapy. Participated in the conception of the project, execution of the research and data analysis - Universidade Federal de São Paulo, Campus Baixada Santista - Santos (SP), Brazil.

⁶Bachelor in Physiotherapy. Participated in the conception of the project, execution of the research and data analysis - Universidade Federal de São Paulo, Campus Baixada Santista - Santos (SP), Brazil.

⁷Adjunct professor. Participated in the intellectual conception of the project, critical review of the manuscript and approval of the final version of the manuscript - Universidade Federal de São Paulo, Campus Baixada Santista - Santos (SP), Brazil.

numero em relação ao S24 (ambos $p < 0,05$); S24L65 diminui o numero de células inflamatórias comparado ao S24L30 ($p < 0,01$). Concluiu-se que LLLT em dose específica de energia ($30\text{J}/\text{cm}^2$) foi capaz de diminuir o número de células inflamatórias no tecido pulmonar em fase aguda da sepse.

Descritores | Terapia a Laser de Baixa Intensidade; Sepse; Pulmão.

RESUMEN | El objetivo del estudio es determinar la eficacia de la LLLT en la disminución del proceso inflamatorio pulmonar en ratones sépticos. Se utilizaron 32 ratones machos Wistar divididos en cuatro grupos ($n=8$): control (CG); sepsis 24h (S24); sepsis y tratamiento con LLLT $30\text{ J}/\text{cm}^2$ (S24L30); sepsis y tratamiento con LLLT $65\text{ J}/\text{cm}^2$ (S24L65). Se realizó la

irradiación inmediatamente después de la cirugía en la región anterior de la tráquea y en las regiones ventrales del tórax, bilateralmente, debajo de las costillas. Se realizó un análisis histológico del tejido pulmonar y se cuantificó el número de células inflamatorias. El grupo S24 presentó un aumento de células inflamatorias en comparación al CG ($p < 0,05$); S24L30 aumentó el número de células inflamatorias, mientras S24L65 disminuyó este número en relación al S24 (ambos $p < 0,05$); S24L65 disminuyó el número de células inflamatorias en comparación al S24L30 ($p < 0,01$). Se concluye que la LLLT en dosis específica de energía ($30\text{J}/\text{cm}^2$) ha sido capaz de disminuir el número de células inflamatorias en el tejido pulmonar en fase aguda de la sepsis.

Palabras clave | Terapia Láser de Baja Intensidad; Sepsis; Pulmón.

INTRODUCTION

Sepsis is a potentially life-threatening complication of an infection and it is a frequent cause of death in critically ill patients. It is also related to the interaction of different events involving inflammatory and anti-inflammatory processes, humoral and cellular reactions and circulatory abnormalities¹. As a consequence, sepsis leads to different system function impairments, putting a series of organs at risk, including liver, kidneys and lungs². One of the most common types of lung injury related to sepsis is the adult or acute respiratory distress syndrome (ARDS) and it is responsible for a high mortality rate³. ARDS is characterized by acute inflammation, with neutrophil migration, interstitial edema, diffuse alveolar wall thickening with increased collagen and fibrin deposition as well as alveolar hemorrhage⁴. Upregulation of proinflammatory cytokines occurs in the presence of ARDS, with a marked upregulation of interleukin (IL-6, IL-8) and tumor necrosis factor (TNF- α) expression⁵. Moreover, neutrophils also release damaging proinflammatory and pro-apoptotic mediators that act on adjacent cells to create ulcerating lesions⁶. In this context, it is extremely important to develop treatments capable of modulating the inflammatory process related to sepsis, to try to avoid the development of ARDS.

The stimulatory effects of low level laser therapy (LLLT) on tissues and its ability to modulate the

inflammatory process have been highlighted by many authors⁷⁻⁹. It seems that LLLT regulates the expression factors related to inflammation such as COX-2, cytokines, interleukins, prostaglandins and the reduced number of inflammatory cells such as neutrophils in many experimental models such as acute injuries¹⁰, osteoarthritis⁷ and lung inflammation¹¹⁻¹⁴. Mafra et al.^{13,14}, demonstrated that LLLT reduced the cholinergic hyper reactivity and tumor necrosis factor (TNF)mRNA expression in rats exposed to lipopolysaccharides via an NF-kappaB-dependent mechanism. Furthermore, Silva et al. (2014)¹⁵ demonstrated that LLLT (660 nm, 30 mW and 5.4 J) reduced bronchial hyper responsiveness and decreased allergic lung inflammation in an experimental model of allergic asthma. Also, de Lima et al. (2010)¹⁶ observed a reduction in edema, neutrophils influx and decreased TNF in the inflammatory process in rat lungs.

Although the positive effects of this therapeutic intervention on the acceleration of inflammatory modulation, there is a lack of information about the interaction of LLLT in the inflammatory process within the lungs. In this context, the hypothesis that LLLT would modulate the lung inflammation process in an experimental model of sepsis, improving lung function was introduced. Thus, the aim of this study was to determine the effectiveness of LLLT on the lung inflammatory process in septic rats.

METHODOLOGY

Animals and Experimental design

In this study, 32 male Wistar rats (weighing 300 ± 20 g) were used. They were maintained under a controlled temperature ($22 \pm 2^\circ\text{C}$), light-dark periods of 12 hours, and with free access to water and a commercial diet. All animal handling and surgical procedures were strictly conducted according the Guiding Principles for the Use of Laboratory Animals. This study was approved by the Institutional Experimental Animal Use Committee.

The animals were randomly distributed into the following experimental groups ($n=8$): control group (CG); sepsis group: rats submitted to the experimental model of sepsis without treatment (S24G); and sepsis laser treated groups: rats submitted to the experimental model of sepsis treated with laser therapy at 30 J/cm^2 (S24L30G) and rats treated with laser therapy at 65 J/cm^2 (S24L65J). All the animals were euthanized 24 hours post-surgery.

Experimental model of sepsis

Sepsis was produced by cecal ligation and puncture (CLP) as previously described¹⁷. Rats were anesthetized intraperitoneally with a mixture of ketamine-xylazine (Ketamine 50 mg/Kg and Xylazine 10 mg/Kg), and a midline laparotomy was performed. The cecum was ligated at its base and punctured twice with a 22-gauge needle. The cecum was then returned to the peritoneal cavity, the muscle and skin layers were then closed. Rats were resuscitated with 10 ml of 0.9% sterile saline administered subcutaneously. All animals were injected subcutaneously with the analgesic buprenorphine (0.2 mg/kg) immediately after surgery.

Laser therapy

Laser irradiation was made immediately after the surgery. A low-energy GaAlAs laser (Photolase DMC Ltda[®]) was used with an 808 nm, continuous wavelength, 30 mW, 3.8 W/cm^2 , beam diameter 0,028 mm, fluence of 30 J/cm^2 with irradiation time of 28seconds (energy per point 0.8J – totaling 2.4J per session) or 65 J/cm^2 with an irradiation time of 1.01 seconds (energy per point 1.8J – totaling 5.4J per session)¹⁸. The irradiation was performed once, using the punctual contact technique, at three points: anterior region of the trachea

and bilaterally on the ventral regions of the chest (just below the ribs).

Euthanasia

At the end of the experimental period (24 hours), rats were deeply anesthetized by urethane (1.25 mg/kg) and euthanized individually by transcardial perfusion with 100 ml isotonic saline at room temperature (containing heparin 0.2%) followed by pulmonary perfusion with 500 ml of fixative fluid (4°C) over a period of 20 min¹⁹. The lungs were removed for analysis.

Histological procedures

After surgical resection of the specimens of each experimental group, the right lung was immediately washed with saline and fixed in 10% buffered formalin (Merck, Darmstadt, Germany) for 12 hours, followed by dehydration in a graded series of ethanol and then embedded in paraffin. Thin sections ($5 \mu\text{m}$) in the longitudinal axis of the lung were obtained using a microtome (Leica Microsystems SP 1600, Nussloch, Germany). Sections of each specimen were stained with hematoxylin and eosin (HE) (Merck).

Morphometric evaluation

Histological characterization of the lung tissue was performed using $5 \mu\text{m}$ thick sections stained with H&E and the total cells (polymorphonuclear and mononuclear) in terminal bronchioles were determined by the point-counting technique. Using a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany), the cells were determined by counting the number of points of the integrating eyepiece falling on areas of tissue inflammation in four areas of each airway wall and the number of cells in this same area. All analyses were performed in 8 randomly selected transversely sectioned, terminal bronchioles. The slides were coded and the researcher who performed the measurements was unaware of the study groups.

Statistical analysis

All variables were organized into mean and standard deviation. The distribution of all variables was tested for normality by using Shapiro–Wilk's W test. For the variable manifesting a normal distribution, comparisons among the groups were made via one-way analysis of variance

(ANOVA), complemented by Tukey HSD post-hoc analysis. STATISTICA version 7.0 (data analysis software system – StatSoft Inc.) was used to carry out statistical analysis. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Morphometric evaluation (inflammatory cells)

Figure 1 shows the morphometric evaluation of the number of total cells (polymorphonuclear and mononuclear) in terminal bronchioles.

The CG was statistically different from the non-treated group (S24) and from the group treated with laser therapy at 30 J/cm² (S24L30), both showed increased inflammatory cells ($p = 0.0025$ and $p = 0.0001$, respectively; Figure 1). Moreover, S24L30 increased the number of inflammatory cells compared to S24 ($p = 0.042$; Figure 1), and S24L65 decreased this number compared to S24 ($p = 0.0089$; Figure 1). When the treated groups were compared, S24L65 had a lower number of inflammatory cells compared to S24L30 ($p = 0.0001$; Figure 1). No other significant difference was observed between groups.

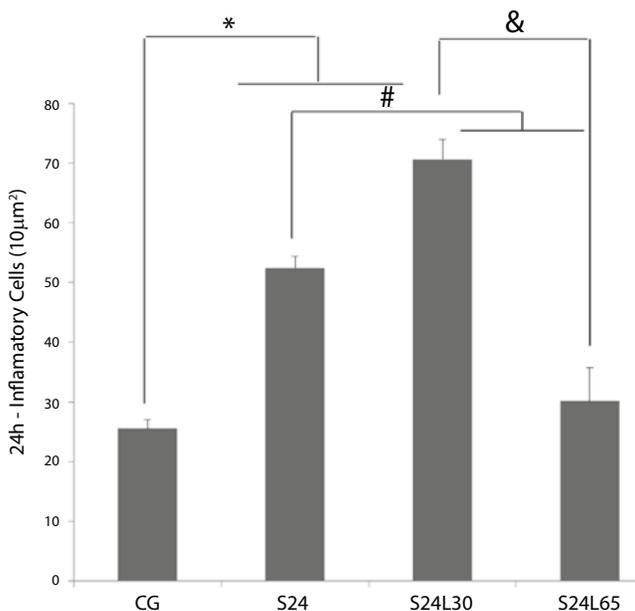


Figure 1. Number of inflammatory cells in the different groups euthanized 24h post-surgery; Control group (CG); Sepsis group: rats submitted to the experimental model of sepsis without treatment (S24G); Sepsis laser treated groups: rats submitted to the experimental model of sepsis treated with laser therapy at 30 J/cm² (S24L30G) and rats treated with laser therapy at 65 J/cm² (S24L65J). (* $p = 0.0025$ (S24) and $p = 0.0001$ (S24L30) vs CG; # $p = 0.042$ (S24L30) and $p = 0.0089$ (S24L65) vs S24; & $p = 0.0001$ (S24L65) vs S24L30

DISCUSSION

This study aimed to determine the effectiveness of LLLT on the lung inflammatory process in experimental, septic rats induced by the cecal ligation and puncture (CLP) technique. The main findings showed that in the 24h experimental time, the group treated with laser therapy at 65 J/cm² (S24L65G) had a lower number of inflammatory cells compared to the sepsis group (S24G) and for the group treated with laser therapy at 30 J/cm² (S24L30G), similarly for the control group (CG).

Sepsis is a potent instigator of multiple organ system failure, and most commonly affects the lungs²⁰. Acute lung injury can occur as a consequence of the inflammatory response accompanied by a depression in the immunological function, which is well known in sepsis physiopathology²¹.

The morphometric evaluation of lung tissue inflammation showed that the number of inflammatory cells was higher in the groups submitted to sepsis induction by CLP compared to the control group. These findings corroborate with the literature that indicates an immediate lung tissue inflammatory phase in sepsis induced by CLP²².

In addition, the sepsis group treated with laser therapy at 65 J/cm² (S24L65G) had a lower number of inflammatory cells when compared with the sepsis group (S24G) and with the group treated with laser therapy at 30 J/cm² (S24L30G). Therefore, our data supports the idea that laser therapy improved the inflammatory condition in lung tissue depending on the amount of energy that was taken in by the tissue.

Although the effects of LLLT have been widely demonstrated, it is important to highlight that the parameters have been highly variable in laser therapy studies related to anti-inflammatory results, with a wide range of energy doses being used by different authors using different tissues²³⁻²⁵. Some studies have compared different parameters of laser therapy and different results in the same tissues and conditions have been observed^{19, 26}. Aimbire et al.²⁷ compared a 685nm *Ga-Al-As* laser in different energy densities: 1.0, 2.5 and 5 J/cm² in a model of inflammation of the airways and lungs induced by the Gram-negative bacterial lipopolysaccharide (LPS) and observed that only the energy density of 2.5 J/cm² was effective in reducing lung neutrophils influx in association with the inhibition of COX-2-derived metabolites in the lung.

Specifically in lung tissue, De Lima et al.¹⁷ used a laser irradiation dose of 5.4J in a model of sepsis induced by Intestinal Ischemia/Reperfusion and demonstrated a reduced pulmonary neutrophils influx. In 2013, another study by De Lima et al.,²⁸ showed that a laser applied at an energy dose of 5.4J has an effect in attenuating acute lung inflammation by restoring the balance between the pro- and antioxidant mediators. Also, corroborating with those studies, the results of our study demonstrated a reduction in the number of inflammatory cells in the group treated with laser therapy at 65 J/cm² that received the total laser irradiation dose of 5.4J (the laser irradiation was performed in 3 points with 1.8 J of energy per point).

In addition, the author's hypothesis is that the differential effect of therapies in the acute phase of lung inflammation after sepsis induced by CLP may be due to the lack of energy offered to the tissue following laser irradiation in the group treated with laser therapy at 30 J/cm² (S24L30G). At this time, it is difficult to define an ideal laser treatment protocol and so further investigation is required to elucidate the mechanisms responsible for the stimulatory and inhibitory effects of laser irradiation on the acute inflammation of lung tissue.

Consequently, these data highlight the potential of LLLT to be used as a therapeutic approach to reduce acute lung inflammation, such as sepsis. Also, our study demonstrated the importance of studies that investigate the effects of different laser parameters to determine the efficiency and safety of laser irradiation and the treatment paradigms required for optimal stimulation.

CONCLUSION

In conclusion, LLLT in a specific dose (65 J/cm²) of energy was capable of decreasing the number of inflammatory cells in acute lung tissue inflammation due to sepsis.

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