Background and Objective: The results of low-level infrared laser (LLL) systemic action on inflammatory modulation process, specifically diminishing pro-inflammatory and producing anti-inflammatory cytokines are extremely controversial in the literature. More studies are necessary to clarify the biomodulation process. The main objective was to investigate the effect of a single session of an AsGaAl laser on spleen cells interleukin-6 (IL-6) and tumor necrosis factor - alpha (TNF-α) release, in vivo, in mice.

Study Design/Materials and Methods: In a pilot study, 18 isogenic mice were distributed in three groups: control (no surgical procedure, n = 6), sham (surgical procedure with three standard cutaneous incisions, followed by abdominal muscle incision followed by suture, n = 6) and LLL (same procedure followed by a single LLL exposure 12 hours after the procedure, n = 6). The animals in the LLL group received a single infrared continuous laser session (780 nm wavelength, power of 20 mW, energy density of 0.4 J). All animals of the sham and LLL groups were sacrificed 36 hours after surgical procedure; the spleen mononuclear cells were isolated and cultivated for 48 hours. The IL-6 and TNF-α were measured by the ELISA method.

Results: IL-6 and TNF-α concentrations released by the mononuclear cells showed significant differences between the control and sham group (P < 0.07). However, there were no differences between the control and LLL group and between the sham and LLL groups (P > 0.07).

Conclusion: The single session of infrared LLL showed a tendency of decreasing the IL-6 and TNF-α release by mononuclear spleen cells in mice after application, although there was not a significant difference between the sham and LLL group. Conclusions regarding effectiveness of a single session procedure cannot be made due to the low statistical power of this pilot study. Lasers Surg. Med. 42:584–588, 2010. © 2010 Wiley-Liss, Inc.
Cytokine is the generic term used to assign a group of molecules (proteins) involved in the signals emission between the cells during the development of immune and repair responses. The cytokines can be allocated in several categories: interleukins (IL), interferon (IFN), transforming growth factor (TGF), tumor necrosis factor (TNF) and others [13].

The TNF-α and some IL’s are known as proinflammatory cytokines. These have an important role as mediators in inflammatory and immunological processes, proteolysis, cell recruitment, and healing. The TNF-α has an essential position in the cytokines release cascade, therefore it also promotes stimulation of other cytokines release such as IL-6. The IL-6 has been associated with several diseases that unchain inflammatory processes such as rheumatoid arthritis, acute pancreatitis, viral infections, bacterial meningitis, and Alzheimer disease [14]. Moreover, these inflammatory mediators sensitize the primary afferent nociceptors, increasing pain sensitivity [15].

Several studies in the literature show the LLL irradiation effects in cells associated to inflammatory reply as in lymphocytes, fibroblasts, macrophages, endothelial cells. Furthermore, there are blood plasma components such as platelets, red blood cells, hemoglobin, immunoglobulin and plasmatic proteins, cell growth factors, and cytokines [5,12,16–18].

However, there is still a lack of research to validate the LLL systemic effects due to a single laser application on cytokines release. Similarly, there is not a consensus regarding to the dosage to be used in the clinical practice.

There are studies showing that, after using the red LLL with energy density below 18.9 J/cm², the cytokine levels decreased, and others with the same result with approximately 38.0 J/cm² [16,19–21]; infrared laser irradiation (850 nm, energy of 0.3 a 1.5 J) should also control the induction of proinflammatory cytokines [22]. However, other studies did not find inflammatory modulation when using 904 nm LLL and energy density of 9.0 J/cm²[17].

Therefore the purpose of this article was to analyze the role of one single session of a low level infrared laser on the IL-6 and TNF-α proinflammatory cytokines release from spleen mononuclear cells in mice, in vivo.

METHODS

This study was approved by the Research Ethics Committee of the Federal University of São Paulo according to protocol 2038/07.

The experiments were performed on 18 Balb-C isogenic male mice weighing 25–30 g each. The animals were maintained in appropriate cages with a 12-hour light/dark cycle, with a temperature around 20°C, with a relative humidity of (65%), and with access to food and water ad libitum.

Surgical Procedure

Anesthesia protocol consisted of ketamine (0.06 ml) and xylazine 2% (0.015 ml) application by intraperitoneal injection.

The animals were positioned on a plane surface with their limbs in extension position and shaved. A skin square flap measuring 2 cm each side was created, the cranial base of the flap was kept intact. The abdominal muscle under the flap was exposed and incised in a 1 cm extension; both muscle and skin flap were sutured with a monofilament thread (mononylon 6–0) (Fig. 1A).

From the 18 animals studied, 12 were submitted to this surgical procedure and afterwards they were randomly assessed to a sham or to a LLL group. The six remaining animals served as a control group.

- Control group: 06 animals without surgical procedure.
- LLL group: 06 animals with laser exposure 12 hours after surgical procedure.
- Sham group: 06 animals with the same surgical procedure, but without laser exposure.

LLL Irradiation

An infrared AsGaAl diode laser (model Twin Laser) with λ of 780 nm, spot size of 0.04 cm² and an output power of 20 mW was used.

Three punctual laser applications were performed on the exposed area 12 hours after the surgical procedure (Fig. 1B). A laser exposure simulation was performed with the equipment in “stand by” for the sham group. The contact technique was used in all exposures [23,24].

The single exposure to the LLL group was continuous with an energy density of 10 J/cm², an application time of 20 seconds, and a final energy of 0.4 J per point.

Separation of Mononuclear Cells

The animals were euthanized 36 hours after the surgical procedure in the sham and LLL groups, the spleen was dissected and macerated and later mononuclear cells were isolated using Ficoll-Hypaque at a density of 1.095. These cells were counted in hematologic automated equipment.
and the concentration was adjusted to $2 \times 10^6$ cells/ml. The cells were incubated in 48 culture plate wells and stimulated with concanavalin mitogen (ConA) for 48 hours. After this period, the supernatant was removed and frozen to $-80 \degree C$ until the dosage of cytokine was made. Results from the control group were considered as baseline standard.

**Enzyme Linked Immunosorbent Assay (ELISA) Method Analyze**

Analyzees were carried out using specific antigens of IL-6 and TNF-$\alpha$ cytokines dosage based on the ELISA test (eBioscience, Inc., San Diego, CA) in the Laboratory of Medical Investigation in Dermatology and Immunodeficiency—LIM-56, FMUSP, São Paulo, Brazil.

The plates were coated with capture antibody and incubated overnight at $4 \degree C$. The wells were blocked with assay diluent. All samples were incubated overnight at $4 \degree C$. The wells were washed out and the detection antibody was added and incubated at room temperature for 1 hour. The wells were washed out and avidin-HRP complex was added and incubated at room temperature for 30 minutes. After that, the substrate solution was added and incubated at room temperature for 15 minutes. The stop solution was added and the plate read at 450 nm.

**Analyzes of the Data**

The Graph Pad statistical program was used for data processing. In the beginning, a normality test (Kolmogorov–Smirnov) was performed followed by analysis of variance (ANOVA) with the Kruskal–Wallis multiple comparison test. The data demonstrate a mean ($\pm$ SEM) and the statistical significance was considerate at $P < 0.07$.

**RESULTS**

Regarding to the IL-6 concentration in pg/ml, the averages ($\pm$ SEM) of the studied groups were: control of 38.0 ($\pm$ 4.7), sham of 57.8 ($\pm$ 8.8), and LLL of 53.1 ($\pm$ 8.2). The TNF-$\alpha$ concentration in pg/ml were: control of 51.3 ($\pm$ 6.1), sham of 64.6 ($\pm$ 6.3), and LLL of 54.8 ($\pm$ 4.2).

The results observed in the IL-6 and TNF-$\alpha$ concentration among the three groups were similar. The sham group showed significant difference when compared to control group ($P < 0.07$), meaning that there was expressive proinflammatory cytokines release by mononuclear spleen cells in mice with the surgical proceeding. The difference between LLL group compared to control and sham groups was not significant ($P > 0.07$) (Figs. 2 and 3).

**DISCUSSION**

The LLL generates photochemical, photophysical and photobiological effects, affecting not only the application area, but also the surrounding region. Thus, the effect of a laser beam is not only limited to the diffusion optic area.

The effect of the metabolic mediators can reach the most distant areas of the body, generating systemic effects [23], however few studies prove this hypothesis. A possible explanation is the fact that the tissue submitted to laser produces signaling factors that, after irradiation, will circulate into the blood vessels and lymphatic system [6].

According to Rochkind et al. [23] the LLL systemic effects in the repair of the nervous system injuries, as well as the wound healing and repair of bilateral cutaneous burns induced in rat paws, demonstrated that the LLL irradiation accelerated the repair of the irradiated limb as well as the contralateral limb.

Still among these probable systemic effects, there are theories of a significant decrease in the hemosedimentation speed (HSS) with the LLL action after 12 exposures, but changes in the hemoglobin rate had not been noticed [24]. However, some studies did not observe systemic effects, such as modifications of HSS, hemoglobin rate, leukocytes, and platelets counting [25,26]. This divergence in the obtained results can be associated with the standardization of LLL irradiation, beyond methodological problems of the studies that were performed with different samples, types

![Fig. 2. Average ($\pm$ SEM) of the IL-6 concentration in pg/ml released by the mononuclear spleen cells of the control, sham, and LLL groups.](image2)

![Fig. 3. Average ($\pm$ SEM) of the TNF-$\alpha$ concentration in pg/ml released by the mononuclear spleen cells of the control, sham, and LLL groups.](image3)
of laser, wave lengths, mean power, and non-specified energy doses [27,28].

The ELISA assay was employed for analysis of the IL-6 and TNF-α levels, according to Yamaura et al. [15] and Aimbire et al. [29]. These studies showed decrease of the proinflammatory cytokines with the laser application immediately after intervention. The ELISA is a standardized and reliable method to measure the cytokine concentration and for this reason, it was employed in the present study. Our preliminary results showed that the surgical proceeding generates increase of cytokines release because the sham group showed significant increase of the IL-6 and TNF-α concentration when compared to control. The LLL probably can modulate this process because changes in the cytokine levels release by spleen mononuclear cells in mice were not observed compared to the control group.

Thus, we looked for evidence of the laser effects after single or multiple treatment sessions. Studies have shown the effectiveness of a single laser exposure for pain relief [8], creatine kinase (CK) levels decrease and cell apoptosis [9], repair of ligament injuries [11] and prevention of muscle tissue damage [10], but others did not state the same conclusions for the healing of ligament injuries [30,31]. Then, the present study is justified by the lack of evidence of a single LLL session for the proinflammatory cytokines control.

According to the literature, the peak of inflammatory process maybe occurs around 4 hours after injury [29]. In the study conducted by Aimbire et al. [29] the laser was employed immediately after induced inflammation and, therefore, the animals did not suffer traumatic injury and probably vascular damage. The experimental model of the present study was applied trying to simulate surgical proceedings with strong vascular damages in humans. We hypothesized that the LLL application in the peak of inflammatory process could increase the hemorrhage.

The decrease of the TNF-α release can be promoted by several kinds of treatment. Among these, the LLL have characteristics favorable to its use, as a simple application and a non-invasive method of inflammation control [32].

The IL-6 is a pleiotropic cytokine with a wide field of biological activities. Therefore, this helps the erythropoietic function, controls the immune system response, and the production of acute phase reactions, such as the stimulation of C-reactive protein release. This cytokine also has an inductive metabolic role in the cortisol contra-regulator hormone [33]. For these important functions, both the IL-6 as well as the TNF-α were analyzed from a single LLL session during a 12-hour period after the surgical procedure. Because they are proinflammatory cytokines, there is a probability of a higher release in the initial stages of the healing process.

There are no standard values regarding to laser parameters in the modulation of inflammatory processes, particularly for the infrared laser, which is widely used in clinical practice due to its greater tissue penetration [34]. There are some studies demonstrating that the application of infrared LLL can inhibit the TNF-α with doses between 5 and 25 J/cm² [32]. Correa et al. [34] showed a migration control of the inflammatory cells with energy of 0.24 J. In the present article, the continuous infrared LLL (780 nm) with an output power of 20 mW and dose equal to 10 J/cm² was utilized. Based on these parameters, an application time of 20 seconds was added, which generated a final energy of 0.4 J per point. Therefore, the total exposure time of 60 seconds was equal to that used by Correa et al. [34]. We did not find strong modulation of one single session laser application when compared to sham group but the laser probably decreased the proinflammatory cytokines because there was not significant difference between LLL and control group in this experimental model.

Some factors probably influenced these results, such as an insufficient injury of the abdominal muscle of mice in order to generate a strong inflammatory process that could provide control of the cytokines release in circulating spleen cells. Perhaps a more aggressive injury with a stronger inflammatory stimulus could induce more evident systemic changes. Finally, it is important to highlight that we used a single laser session, and maybe 2 or 3 sessions could unchain a stronger healing response, once the IL-6 and TNF-α expression was already lower after a single laser pass.

CONCLUSION

According to the obtained results, we concluded that a single session of infrared LLL showed a tendency of decreasing the IL-6 and TNF-α release by the spleen mononuclear cells in mice despite the low statistical power of this pilot study. Thus, further studies are necessary with a larger sample to test our hypothesis. Another possibility is to study how the cumulative effect of the LLL might influence the cytokine release and the inflammatory process.

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