He-Ne Laser Effects on Blood Microcirculation During Wound Healing: A Method of In Vivo Study Through Laser Doppler Flowmetry

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Background and Objectives: Low-intensity laser therapy (LILT) is widely used for wound healing promotion and its mechanism of action may be due to an enhancement of blood supply. The aim of this study was to evaluate blood flow alterations in a wound healing model, using laser Doppler flowmetry (LDF) associated with a normalized perfusion parameter.
Study Design/Materials and Methods: An injury was provoked in 15 rats and blood flow was measured periodically over a period of 21 days. Control groups were established to evaluate LDF and He-Ne laser effects on microcirculation. A 1 J/cm^2 dose was utilized, with 6 mW/cm^2 irradiance.
Results: The results demonstrated flow alterations provoked by lesion, and inflammatory response ($P < 0.05$). There were no statistical differences between groups.
Conclusions: The results did not show a significant sustained effect on microcirculation with this He-Ne dose. Lasers Surg. Med. 35:363–368, 2004.

Key words: biomodulation; blood flow; laser Doppler; low-intensity laser therapy; skin repair

INTRODUCTION

There are several reports documenting significant positive effects of low-intensity laser therapy (LILT) on biological systems [1–7]. However, this therapy has yet to gain wide acceptance in the medical community. Sufficient differences of opinion seem evident between studies showing beneficial effects and those reporting no effects whatsoever. A good understanding of accurate mechanisms of action involved in this therapy could simplify the choice of treatment parameters, and clarify the appropriate clinical indication of this therapy.

A proposed mechanism of LILT is a vascular response, since blood flow is an important factor in wound healing and pain relief.

According to Bradley et al. [1] the ability of LILT to promote healing with conventional doses (less than 100 J/cm^2) is not due to immediate vascular effects, but could be due to a delayed angiogenic stimulus. On the other hand, Maegawa et al. [2] reported an immediately induced arteriolar vasodilatation in rat's mesentery, by the use of LILT, and this finding was not associated with a rise in temperature provoked by the irradiation.

The wavelength could be an important parameter. As reported by Baxter [3], the Helium-Neon laser (He-Ne, $\lambda = 632.8$ nm) has been the most popular laser with many studies reporting the use of this device, and the relative lack of penetration of this wavelength may have played an important role in the failure of laser therapy to produce analgesia in several cases.

Several reports demonstrated that LILT and blood flow effects in vivo are related to wavelengths longer than 780 nm [1,2,4–8]. In vitro studies with wavelengths between 632.8 and 660 nm showed effects on blood cells, such as changes in erythrocyte aggregation and increased proliferation of lymphocytes [9–11]. Moreover, several authors reported biological responses with red lasers and the observed effects could be associated with blood microcirculation alterations [12–16].

Given the large range of treatment parameters involved in this therapy, i.e., wavelength, temporal mode of operation (pulsed or continuous), radiant exposure, irradiance, exposure time, frequency and total duration of treatment, optical properties of tissues, etc., it is not difficult to understand that results differ from one study to the next.

Laser Doppler flowmetry (LDF) provides a noninvasive method of assessing cutaneous perfusion. Skin perfusion measurements using the laser Doppler technique depend on how the light interacts with moving blood cells and static tissue [17]. Laser Doppler measurements make use of wavelength-shifted photons (Doppler effect), scattered by moving particles during their propagation in a turbid medium [18]. The technique makes use of a coherent,

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monochromatic, low intensity radiation (1–2 mW), from a gas laser, such as He-Ne, or a diode laser, usually emitting in the range of 632.8 to 830 nm, and commonly delivered by an optical fiber.

LDF is a suitable method to study skin microcirculation allowing continuous, noninvasive, real-time assessment of skin perfusion in a tissue volume of approximately 1–1.5 mm^3 under the measuring probe [19]. The recorded signal is related to the flow of the blood cells (mostly red blood cells), which is defined as the product of the number of blood cells and their velocities within the measured skin volume. The flow signal is generated by the movement of blood cells in both the sub-capillary thermoregulatory vascular bed and the nutritional capillaries. Blood flow in the microcirculation is difficult to monitor because of the small size of its component vessels that can be disturbed by the monitoring process. Therefore, the measurement techniques are usually restricted to a noninvasive optical technique such as LDF, which is able to detect microcirculatory changes with noncontact probes. The unknown orientation of the microcirculatory vessels and their branches beneath the measuring probe lead to a substantial variability in the flux measured in different subjects and even in the same subject if the probe is repositioned. LDF signals present also marked temporal fluctuations that are generated by cardiac pulsations, vasomotion, and the influence of the autonomic system on vascular tone [17].

In previous studies, an enhancement of wound healing was demonstrated, as well as an acceleration of granulation tissue formation and faster reepithelization in burned rat skin by 3 minute exposures of a polarized continuous mode He-Ne laser ($\lambda = 632.8$ nm), irradiance of 6 mW/cm^2, and a radiant exposure of 1 J/cm^2 [20,21]. Therefore, in this study, aforementioned parameters were used to investigate alterations in blood microcirculation that could be associated with laser radiation, enlightening one possible mechanism of action involved in this therapy.

In view of the complexity of interactions that determine blood flow, in the present study, a normalized perfusion parameter F(%) was used in order to obtain a lower sensitivity to systemic and environmental flow fluctuations, as well as, to the dispersion of values among subjects.

The purpose of this study was to evaluate blood flow alterations in response to the He-Ne laser irradiation during skin repair via LDF associated with a normalized perfusion parameter F(%). The ability of the proposed method to detect perfusion variations was assessed, and the effect of radiation emitted by LDF equipment was evaluated during the experimental period.

MATERIALS AND METHODS

The experiments were performed according to COBEA (Brazilian College of Animal Experience), an institute associated with ICLAS (International Council of Laboratory Animal Science).

All experiments were carried out on 15 adult healthy male Wistar rats. Initial body weight was approximately 300 g; the animals were housed in separate cages with free access to food and water and maintained in room temperature with a 12-h light/dark cycle. The animals were anesthetized with ketamine-xylazine mixture i.p., and the dorsum of each subject was shaved. The shaved surfaces were cleaned with a 2% clorhexidine pad for skin disinfection and did not present any clinical signals of damage caused by the razor. After these procedures, the animals were maintained in a controlled environment animal room for 24 hours, to allow sufficient time for equilibrium of the cutaneous microcirculation, due to a loss of heat promoted by the lack of hair in the dorsal area.

The animals were randomly distributed into groups of five animals. During the experiment, the animals of each studied group were analyzed in the same period of the day to avoid metabolic variations that could promote alterations on skin blood microcirculation. Upon arrival at the laboratory, a 10-minute stand by period was provided for acclimatization at room temperature. During all test times, the animals were anesthetized as previously described, and a normal respiratory rate (eupnea) was obtained in order to proceed with the measurements.

For the flux measurements, a Flolab® flowmeter (Moor Instruments Ltd., UK) was used equipped with a 1 mW laser, emitting at 780 nm. The probe was a MP13 (Moor Instruments, 1.5 mm external diameter, two 0.25 mm optical fibers, 0.5 mm spaced apart). MP13 is a noncontact probe that avoids flow alterations due to mechanical contact with the skin. The Doppler filter of the instrument was fixed at 15 kHz. The LDF laser output power was measured using a calibrated detector (LaserCheck®, Coherent, USA).

The flowmeter probe was fixed by a metallic arm, preventing involuntary movements due to manipulation, which could affect the recorded signals.

The LDF output signal is named Flux (F) or Perfusion, a quantity proportional to the blood flow into the sample volume. Since the actual blood volume is unknown, arbitrary units (a.u.) are suggested for Flux or Perfusion measurements by using a calibration standard [22]. The used equipment was calibrated prior to measurements using a calibration kit supplied by the manufacturer of the flowmeter.

The flow registers were performed at two selected sites on the skin, over the vertebral column in the anterior–posterior direction. The first site, here denoted as lesion site (LS), was located at the middle of the back at 3 cm from the base of the tail and received an injury (see below). The second site, here denoted as control site (CS), was located at 1 cm from the base of the tail. In this site the control measurements of healthy skin blood flow were performed. Lesion and CSs were standardized due to the great spatial variation of blood flow even in close anatomic areas [17,19]. Thus, randomized sites could make the analyses of the results impractical, because of the dispersion of flow values. The probe distance from the skin was adjusted using a 1 mm spacer.

For both sites (LS and CS) a 6 mm diameter area was selected, in which three 30 second-distinct measurements were carried out, to compute a mean blood flow of each site.

In the LS, 6 mm diameter lesions were produced using a cylindrical brass rod cooled to 77 K. The contact was made in two sequences of 15 seconds each with an interval of 5 minutes.

The laser of the flowmeter produces a small spot over the measured area, resulting in an irradiance of 2.0 W/cm^2 (due to the small diameter of the optical fiber). Although this irradiation occurred at only 0.2% of total lesion area during each measurement (30 seconds), the averaged irradiance over the entire lesion area is 3.5 mW/cm^2. Thus, it was decided to investigate possible effects caused by this irradiation over these areas. Therefore, five animals here denoted as Flowmeter Control Group (FCG) had their blood flow monitored on the 1st day before lesion (BL), immediately after lesion (Initial Register—IR), and then on the 14th and 21st days (Single Register—SR).

Five animals denoted as Laser Group (LG) received laser treatment at days 1, 2, and 3. The light source was a polarized, continuous mode He-Ne laser (Coherent, USA), output power of 10 mW. The irradiation was performed with an irradiance of 6 mW/cm^2, radiant exposure of 1 J/cm^2, and a constant spot of 6 mm diameter. The exposure time was 3 minutes. The output power of the laser was measured prior to irradiations, using a power meter (LaserCheck®, USA).

The skin blood flow registers on LG were performed before the lesions (BL), immediately after lesions prior to the irradiation (IR), 7 minutes after laser irradiation (7 minute), and 20 minutes after laser irradiation (Final Register—FR) on the 1st day. On the 2nd and 3rd days the measurements performed were IR, 7 minutes and FR; and then SR were performed on the 7th, 14th, and 21st days. The measurements performed at 7 and 20 minutes after laser exposure were performed to investigate immediate effects of laser [2].

The five animals of the Laser Control Group (LCG) received the same procedure as the LG, with the exception of the He-Ne laser treatment. After registering the flow in the lesion and control areas (IR), measurements were performed 27 minutes delayed (FR) providing means of comparison with the LG final recordings, since the necessary time to position and irradiate the animals was 7 minutes. These procedures were executed on days 1, 2, and 3, and SR were obtained on days 7, 14, and 21. The animals in LCG differed from those in FCG only in the time of exposures, during experimental period, to the flowmeter laser. Table 1 displays the experimental timeline of measurements.

The perfusion registers were stored in a personal computer and were analyzed using software supplied by the manufacturer of the flowmeter (MoorSoft Windows®/moorLAB, v1.2).

Laser Doppler values are presented as percentage changes from initial blood flow obtained on day 1, before carrying out any procedures, which means that the data obtained on day 1, from healthy skin site (LS), and the healthy skin CS, were computed as follow: $F(\%) = 100 \cdot (F_{LS}/F_{CS})/F_0$, where F_{LS} and F_{CS} are the mean blood flow from LS and CS, respectively. F_0 is F_{LS}/F_{CS}, both measured prior to injury, i.e., the baseline. F(%) means the percentage of perfusion variation of the LS, referred to the CS, and referred to the measurements on day 1. Thus, computed values of F(%) from the day 1 prior to lesion placement are always 100%. Detailed mathematical modeling of the normalized perfusion parameter F(%) reveals its lower sensitivity to systemic and environmental flow fluctuations, to instrument calibration errors, as well as to the dispersion of values among specimens, when compared to the commonly used hemodynamic parameter F (measured flow value in a.u.) [23].

Statistical analyses were accomplished using the Student's *t*-test to compare percentage changes of blood flow in the skin microcirculation at the different times within groups as well as among the studied groups. $P < 0.05$ was considered significant.

RESULTS

The mean value of F(%) of each group, and its respective standard deviations for all measured moments are plotted in Figure 1.

TABLE 1. Experimental Timeline of Measurements

	Time					
Event	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21
Initial Register (IR)	LG LCG FCG	LG LCG	LG LCG			
7 minutes after He-Ne exposure (7 minute)	LG	LG	LG			
Final Register (FR)	LG LCG	LG LCG	LG LCG			
Single Register (SR)				LG LCG	LG LCG FCG	LG LCG FCG

Skin perfusion registers were carried out on all groups on the 1st day before the lesions (BL) to compute the baselines.
FCG, Flowmeter Control Group; LG, Laser Group; LCG, Laser Control Group.

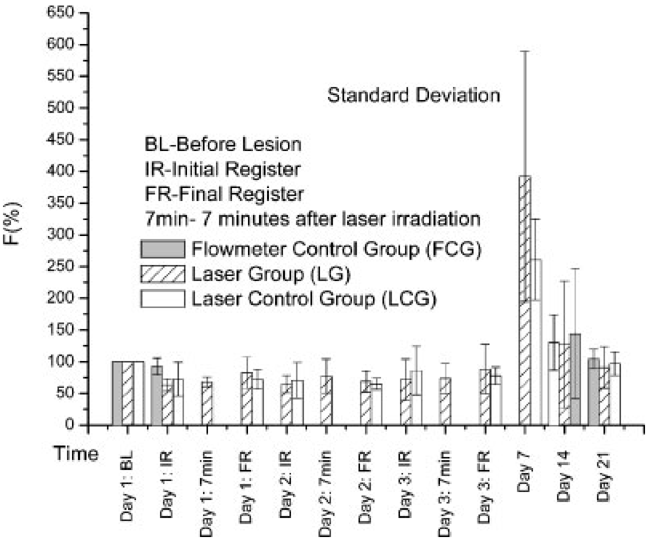

Fig. 1. Histogram of mean values of F(%) of the Flowmeter Control Group, Laser Group, and Laser Control Group, measured before lesion (BL) placement and at days 1, 2, 3, 7, 14, and 21 afterwards the lesions. Bars are standard deviation. Abscissa is in a nonlinear scale.

To verify the ability of the monitoring technique to detect flow alterations during the tissue repair process, for each group separately, a statistical test of significance of the computed values of F(%) of all measured moments was performed, referenced to its initial baselines. A statistically significant decrease of F(%) for all groups ($P < 0.01$) was verified immediately after the lesions, remaining below its initial values during the first 48 hours after injury.

On day 7, a statistically significant increase of F(%) of the LG and of the LCG was observed, when compared to the initial moments ($P < 0.05$): F(%) in LG rose to 392.2%, while LCG rose to 260.9%.

On day 14, the F(%) values of the LG and LCG remained higher than its initial values, and on day 21 the obtained F(%) values from all groups were close to its baselines obtained on day 1 before injury (Fig. 1). For both groups (LG and LCG), there was no evidence of statistical differences of the perfusion values between these moments at days 14 and 21.

To verify the influence of the dose from the laser of the flowmeter on skin microcirculation, statistical tests of significance were performed between the FCG and the LCG, and no evidence of differences of F(%) between similar moments were found ($0.18 < P < 0.8$).

To identify the influence of the He-Ne dose on the irradiated animals, a statistical test of significance was carried out between computed values of F(%) of the LG and the similar moments of the LCG, and no statistical evidence of differences were achieved ($0.25 < P < 0.8$).

Comparing F(%) of consecutive moments from the LG after He-Ne laser irradiation on days 1, 2, and 3, no evidence of differences was found ($0.16 < P < 0.3$).

DISCUSSION

In this study, lesions were provoked on the skin of rats to follow up, through LDF associated with a normalized perfusion, the effects caused by LILT on microcirculation during skin repair, as well as to observe the possible effects caused by the radiation emitted by the laser Doppler equipment.

Hair-covered skin such as on the back of the rat presents a low basal blood flow, with a small density of arterioles and venules [24]. Therefore, the factors that promote immediately alterations on microcirculation in those areas are mainly temperature and pressure. We did not find evidences in the literature suggesting flux alterations during irradiation, if a higher intensity that leads to a thermal stimulus is not applied. Thus, the measurements were not carried through at the same time of irradiation and sustained perfusion alterations were investigated.

Immediately after the injury, a decrease in the mean perfusion values was observed. This decrease could be associated with a local hypothermia caused by liquid nitrogen, and as it is known often produces necrotizing changes secondarily because of the vascular obstruction and consequent ischemia. Cold damages capillary endothelial cells, and greatly increases their permeability. An increase in vascular permeability that causes extravasation of intravascular blood leads to a diminished number of moving red blood cells.

An elevation of the mean perfusion values on the 7th day was noted. By the 5th day, newly formed blood and lymphatic vessels may be seen in the proliferating connective tissue after a noninfected full-thickness skin lesion. On the 7th day, the wound space would be filled in with a reformed well-vascularized granulation tissue [20].

Hence, the granulation tissue becomes more mature, and progressively less vascularized, the collagen fibers become denser and take on a hyaline appearance [20]. Thus, the mean flux values obtained would become progressively smaller until the initial values would be reached.

The ability of the organism to replace cells varies largely within specimens, and there is a natural variability of microcirculation among animals [17,19]. Another important factor is that according to Karu [25] there is no evidence that all individuals would respond in the same way to radiation, so the flux alterations among animals could be even larger after irradiation. The normalized perfusion parameter F(%), applied in this study, is less sensitive to differences among animals since individual baselines were considered. However, even in this case a raised variation of the perfusion values could be reached during tissue repair.

Although all similar wounds follow roughly the same healing process, which consists of an orderly progression of events that reestablish the integrity of the damaged tissue, the regeneration process is determined by the overall condition of each individual. Thus, analyzes of the maximum perfusion values achieved during the healing response could provide useful data.

The analyses of groups FCG and LCG showed no evidence of differences at any analyzed moment. This data provide a basis for using LDF in this type of study, once its use would not interfere on the research results. Sommer et al. [26] showed that to observe a physiological response, a homogenous distribution of the radiant exposure with the appropriate irradiance must be created during the irradiation of the target area. Both the irradiance and the radiant exposure are important parameters in LILT, but are independent between them. Therefore, the irradiance produced by the LDF equipment provides insufficient radiant exposure in the wound area to trigger biological effects.

The presented results, in comparing groups LG and LCG, would seem to be at variance with the positive findings reported by the use of lasers with the same or different wavelengths on microcirculation effects [2,4,5,15,27]. However, it should be noted that results from such studies are not exclusively positive. Bradley et al. [1] using LDF and lasers with the wavelengths of 660, 820, 1060, and 10,600 nm, did not report significant difference in microcirculation immediately after laser irradiation with 660 and 820 nm, applying doses lower than 100 J/cm^2. Additionally, higher doses than 100 J/cm^2 utilizing 660 nm wavelength did not promote immediate alterations in blood microcirculation, although the authors emphasizes that an angiogenic stimulus during the healing process could occur. Indeed, the current findings suggest such fact, as a high mean flux in the group that received laser treatment was noted on the seventh day. Bisht et al. [14] and Ribeiro et al. [20] reported a higher density of blood vessels in the irradiated groups on day 7 using the same wavelength as in this study.

Laser parameters such as radiant exposure, irradiance, uniform or punctual irradiation, temporal mode of operation (e.g., continuous or pulsed), and the pulse repetition rate, may alter the research results; additionally the wound status could also play an important role in the outcome. The majority of studies on LILT are wound healing and chronic inflammations. Both conditions present decreased oxygen tension and acidosis. In normal regeneration, wound hypoxia is a transient condition, but chronic injuries are characterized by continued aerobic glycolysis and by a redox shift towards a reduced state [28].

Lagan et al. [29] in a clinical study observed that in acute uncomplicated postoperative wounds clinical benefits were not achieved by laser treatment, but with the same laser parameters, good clinical results were observed on the treatment of chronic venous ulceration in a pilot study.

In conclusion, the results showed that the LDF technique, associated with a normalized perfusion parameter, F(%), can be considerate as a reliable method to verify alterations on blood microcirculation under the conditions previously described, and did not promote significant alterations on blood microcirculation during skin repair process. There were no statistical evidences that F(%) is significantly influenced by the radiation of the He-Ne laser in the aforementioned conditions, therefore, further research is required to verify the influence of other sets of laser parameters on blood microcirculation.

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