# Red laser attenuation in biological tissues: study of the inflammatory process and pigmentation influence

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## ABSTRACT

Several studies indicate that low level laser therapy (LLLT) accelerates the healing process, however, for a determined pathology, dosimetry remains difficult to be established. To understand the tissue optical properties under different conditions is extremely relevant since the dose delivered to the target tissue is known to be critical. The skin pigmentation influence on the laser attenuation is not yet well established on different mice lineages or human ethnical groups, making the dose problematic. Along the same line, inflammatory processes may cause similar problems since the tissues in this condition change their optical properties due to inflammatory cell accumulation. This work evaluated the attenuation pattern of a HeNe laser ($\lambda$=632.8 nm) using *ex vivo* skin samples from Balb/C and C57BL/6 mice under inflammatory stages induced in their paw by local carrageenan inoculation. The samples were placed between two microscope slides, and a CCD camera was placed orthogonal to the beam path. The intensity distribution of the scattered light was photographed in grayscale and analyzed by ImageJ software. Our findings suggest that even slight differences of the epithelial pigmentation could result in a relevant dose loss delivered to the deeper tissues. The increase of the inflammatory cell density in the connective tissue indicated a highly scattering area also resulting in a dose loss for the deeper tissues when compared to control group.

**Keywords:** light attenuation; red laser; inflammation; oedema; skin pigmentation

## 1. INTRODUCTION

Low level laser therapy (LLLT) was introduced as therapeutic modality due to the required low energy densities and to the high penetration of red and infrared lasers in biological tissue.

The positive clinical results of LLLT are attributed to a sequence of cellular and molecular events. The determinant factors in photochemical, photophysical or photobiological response are the wavelength, energy density, power density, chromophore concentration and the optical properties of the treated tissue (mainly absorption and scattering), as well as its physiologic state. The primary mechanisms are explained by absorption of respiratory chain's components of light quantum, signaling a cascade of events, which leads to the final effect[1], for example, to wound healing[2] and prevent surgical complications[3,4] reducing the hospital stay needed also reducing the chances of opportunistic infirmary infections.

Although several studies have been published, light dosimetry is not largely explored in literature. In fact, a few studies report optical properties of pathological tissues[5-7].

This study was developed to determine the relative attenuation coefficient of the light intensity in skin of black and albino mice as well as we measured light attenuation following inflammatory process. The technique of imaging the light distribution allows us to obtain a qualitative attenuation pattern for the light intensity that, once known, allows the understanding of several effects of light tissue interaction and consequently, the possibility of optimization of light parameters for LLLT.

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## 2. MATERIALS AND METHODS

### 2.1 Mice

This study was carried out following the ethical principles elaborated by the Brazilian National Council of Animal Experimentation, an institution linked to the International Council of Laboratory Animals Science (ICLAS), based on international recommendations that seek the enhancement of behavior of animal experimentation, based in three fundamental principles: sensibility, good sense and good science. It is also approved by the Ethical Committee on Animal Research of the Institute for Energetic and Nuclear Research (IPEN).

We used nine Balb/C and nine B57BL/6 adult (between 40 and 60 days old) female mice weighting from 20 g to 30 g, raised under ideal conditions of temperature with water and food *ad libitum*. According to Calabro et al.[8] relevant skin optical differences can be found between gender variations of mice, what means that all animals must belong to the same sex.

### 2.2 Carrageenan and oedema induction

The oedema induction was performed through the inoculation of 50 μL of carrageenan (CGN) at 1% (10 mg of CGN diluted in 1 mL of saline solution 0.9%) (Sigma Chemical Co., St. Louis, MO, USA) in doses of 0.5 mg/paw injected in the basal area of left mice's paw[9], keeping the right paw healthy for the control group. Once the CGN inoculation is a fast and its symptomatology is comparable to an anesthetic application, sedation remained unnecessary.

Carrageenan is a polysaccharide widely used for inflammatory induction on animal experimentation causing the formation of many inflammatory mediators such as histamine, bradykinin, prostaglandin and others[10]. The use of CGN as a irritating agent for oedema induction was first used in the 60's by Winter *et al.*[11] and became one of the most popular pharmacological compound for anti-inflammatory medication tests.

The oedema volume increases until about the forth hour after CGN inoculation, reaching its peak, and decreases until the paw returns to its normal volume[12]. To understand the tissue optical properties during the volume increase, peak and decrease, samples were taken on the moments 2, 4 and 6 hours post CGN inoculation. The animals were divided into three groups containing the same number of members ($N_{Balb/C}$=3 and $N_{C57BL/6}$=3, per group) and euthanized on the moments mentioned above, before the samples acquisition.

Skin samples were carefully taken with surgical scissors to preserve all tissue layers (epidermis, dermis, hypodermis and muscle). To standardize the samples and remove its borders imperfections 4 mm diameter punches were made.

### 2.3 Experimental Setup

The samples were disposed between two microscope slides and frontally irradiated by a 2 mm wide HeNe laser beam (Uniphase®, Manteca, CA, USA. Output power = 7 mW, λ=632.8 nm) (Fig. 1A) attenuated 99.75% by a neutral density filter resulting in a 17.5 μW light beam. A couple charged device (CCD) (Canon G10, 14.7 Mpx), placed 6 cm and orthogonal to the samples (Fig. 1B), captured the scattered light emitted from the samples profile. The first tissue layer on the laser beam path was always the epidermis.

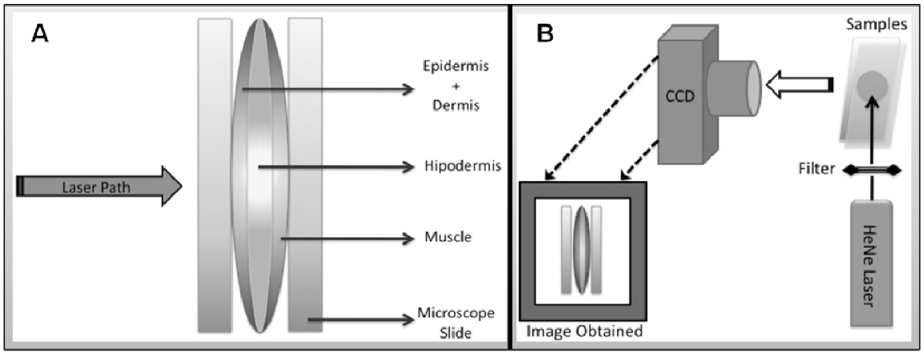


Figure 1. (A) Samples positioned between microscope slides. (B) Experimental setup.

To obtain a pixel-millimeter scale, a caliper rule was photographed at exactly the same distance between the CCD and the samples. It could be concluded that 1mm corresponded to 44(2) pixels.

The captured images were analyzed by the public domain software ImageJ (National Institutes of Health, USA)[13] and two different analyses were made: one-dimensional analysis through the transmission axis was used to verify light intensity in function of tissue depth (mm), and a two-dimensional analysis allowed the analysis of the highly scattering centers inside the samples. The images were taken in a standard gray scale values from 0 for complete dark (threshold) to 255 for the maximum light intensity (saturation) admitted at the CCD calibration. Standardized, this grey values were classified as relative light units (RLU). The association of CCD-ImageJ have a $1x10^{-6}$ RLU resolution.

According to Lambert-Beer's Law light intensity in function of its depth in a homogeneous attenuating media is given by (Eq. 1), where $I_0$ is the initial intensity, $\mu_t$ is the specific media's attenuation coefficient and $z$ the depth value in spatial units. It means that as higher the value of $\mu_t$ is, faster the intensity decreases, resulting in a steeper curve. Also $\mu_t$ is equal to $\mu_a + \mu_s$ (Eq. 2), being $\mu_a$ the absorption coefficient and $\mu_s$ the scattering coefficient. This concept was adopted for both (one and two-dimensional) analysis.

## 3. RESULTS AND DISCUSSION

For the one-dimensional analysis, a rectangular area was selected on the taken image, where its height must be smaller than the laser spot and its width proportional to the samples thickness (Figure 2). In this area, a mean value was calculated for each pixel column to decrease the uncertainty generated by speckle effects and generate a two-dimensional graphic of light intensity (RLU) in function of tissue depth (mm) (Figure 3).

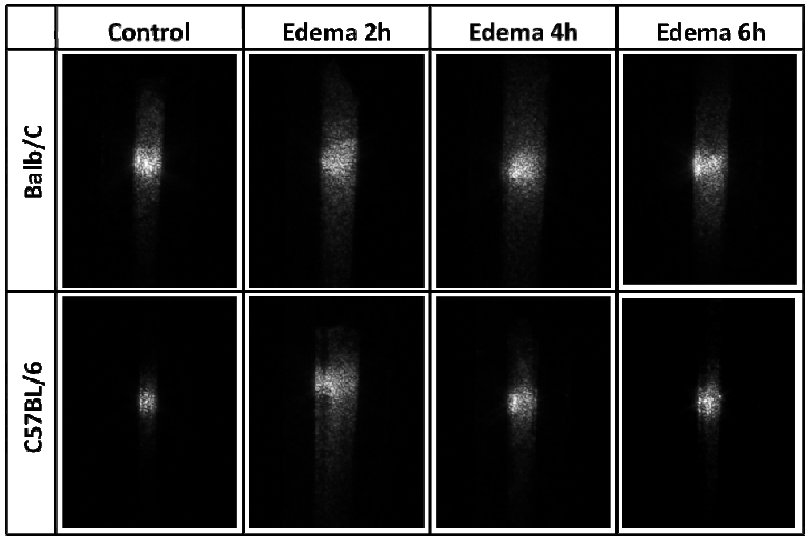


Figure 2. Images obtained by CCD. Beam pathway from left to right.

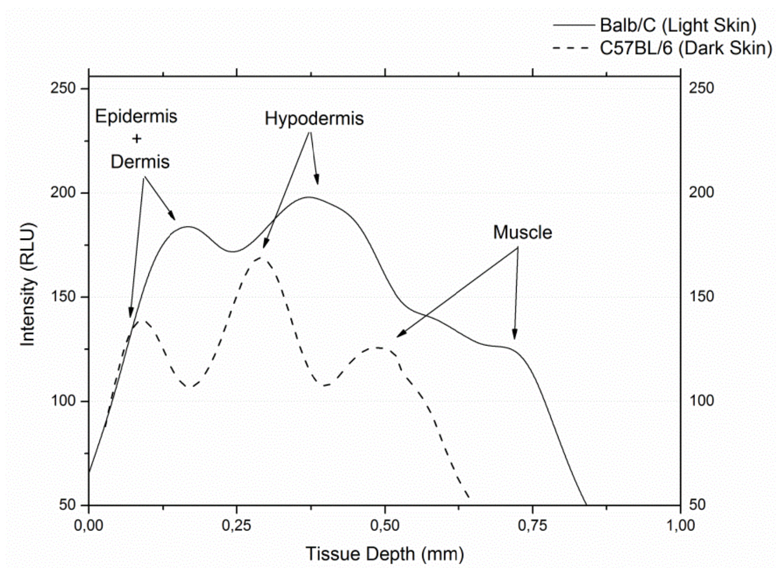


Figure 3. Light intensity (RLU) in function of depth (mm).

Assuming that in the achieved resolution each tissue layer can be considered as a homogeneous media for the control group, intensity peaks on figure 3 can be correlated to tissues by depth comparison with histological images (Fig. 4).

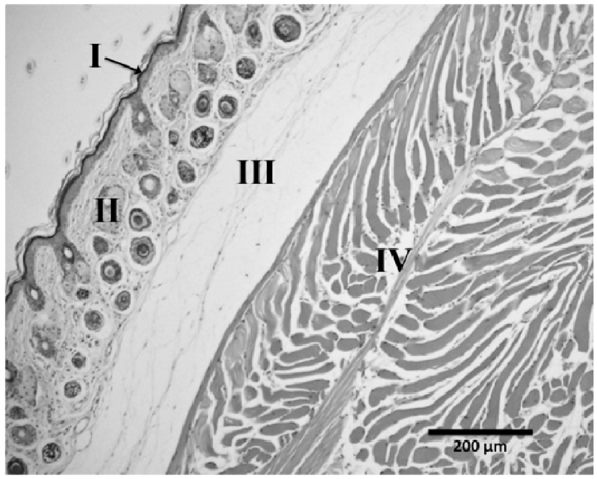


Figure 4: Photomicrograph of mouse skin paw. I. Epidermis; II. Dermis ; III. Hypodermis; IV. Muscle.Scale bar: 200 μm

We can observe in figure 3 that intervals representing muscular and hypodermic layers show a very similar decay slope in both mice lineages indicating a very similar attenuation behavior. However, the first layer (epidermis + dermis) performs a remarkable difference between lineages. The C57BL/6 samples have a higher decay slope, indicating a higher attenuation coefficient for this tissue layer compared to the Balb/C mice. Also, light was recorded in lower intensities through the entire black mouse samples. Once melanin presents a high absorption coefficient for red light[14] this attenuation characteristic is probably given by a higher density of melanin present in the C57BL/6 epidermis. After this first layer, radiation intensity propagates to deeper tissues in lower rates causing problematic issues for dosimetry in phototherapy on animal models when transcutaneous light protocols established in one of these lineages is brought to the other, since the delivered dose is critical for a optimum treatment. In human cases this problem may become more severe once the melanin concentration varies in higher levels between ethnical groups.

For the two-dimensional analysis a wider area is selected and all pixels are analyzed and plotted on a three-dimensional graphic where the depth of the Z axis is reported as isolines. Thus, the light distribution can be analyzed trough the entire sample's profile.

In the beginning of the inflammatory process the damaged cells activates the mononuclear phagocytic system (circulating monocytes and tissue macrophages) initiating a chain reaction by the secretion of cytokines from the family IL-1 and TNF (Tumor Necrosis Factor). These molecules have a pleiotropic action in local and systemical levels. Locally they interact over tissue's matrix or stromal cells, mainly endothelial cells and fibroblasts causing the liberation of a second set of cytokines, including IL-1 and TNF againg, but also IL-6 and IL-8 and its inflammatory proteins (MIP-1) and chemotactics' (MCP) macrophages. This last protein together with IL-1, IL-8 and TGF-β to the inflammatory focus monocytes and neutrophils which secretes a third set of cytokines including TNF and other chemotactics that will feed back the inflammatory process.

The inflammatory cells migration to the dermal stromal region is evidenced on figure 5 where it can be clearly seen a highly light scattering material accumulation on the second and forth hours post CGN inoculation. In fact, oedema's volume tends to increase and reach its peak at the forth hour post CNG inoculation. It means that at this moment occurs the highest defense cells accumulation in the dermal stroma resulting at the highest light attenuation due to a severe light scattering increase (according to equation 2). On the sixth hour non stromal scattering peaks begin to appear suggesting a scattering particles dispersion by this moment. At this point the oedema's volume is in decrease and defense cells are migrating back to the vascular system.

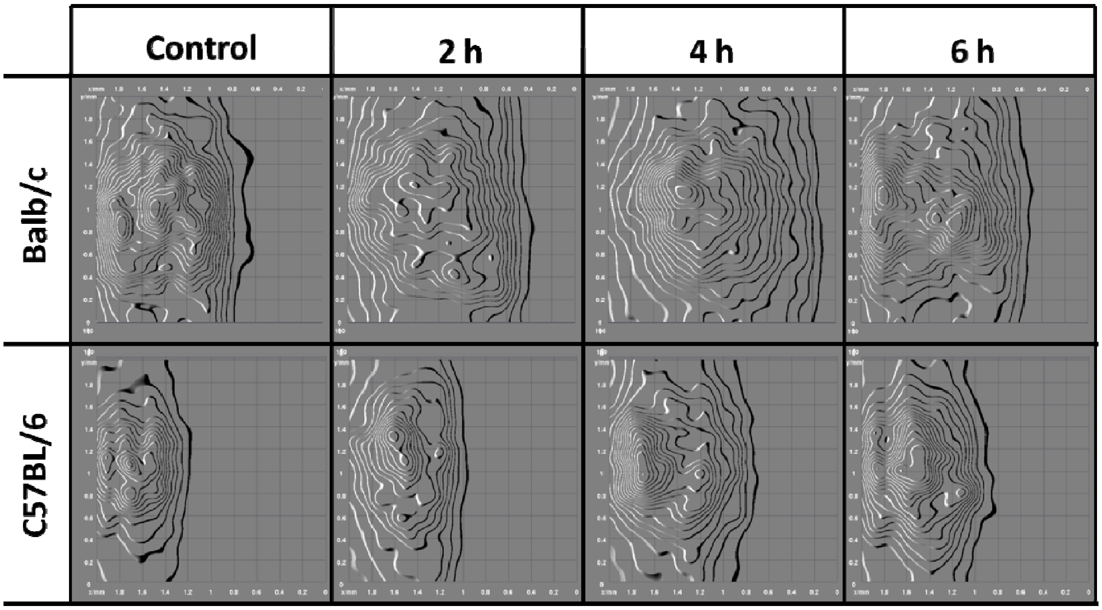


Figure 5. Three-dimensional plots in isolines of light distribution along the samples under inflammatory processes. Space scale (x,y) in millimeters and light intensity (z) in RLU.

## 4. CONCLUSION

According to our data slight melanin density differences, such as between Balb/C and C57BL/6 mice lineages, result in a relevant light transmittance decrease. Also, inflammatory processes induce a defense cells migration to the damaged tissue resulting in a very relevant cell density increase (mainly in the stromal area) causing a high increase at tissue's scattering and attenuation coefficients. Consequently, tissue's photon transmission is decreased. Finally, relating both results to the phototherapeutics we concluded that the effectiveness can be affected by those phenomena described above. If the target tissue is located below the dermis tissue layer variables such as pigmentation and inflammatory processes must be considered, otherwise, the absorbed dose at the target tissue may present discrepancies between expected and effectively delivered energy causing unexpected or unsatisfactory results at the end of treatment.

## REFERENCES

[1] Reddy G.K., "Photobiological basis and clinical role of low-intensity lasers in biology and medicine", J. Clin. Laser Med. Surg. 22, 141-150 (2004).

[2] Ribeiro M. S., Silva D. F. T., Araújo C. E. M., Pellegrini C. M. R., Oliveira S. F., Zorn T. M. T., Zezell D. M., "Effects of low-intensity polarized visible laser radiation on skin burns. A light microscopy study", J. Clin. Laser Med. Surg. 22, 59-66 (2004).

[3] Baptista I. M. C., Chavantes M. C., Oliveira A. S., "Laser de baixa potência pode prevenir deiscência incisional em esternotomia pós-cirurgia cardíaca", Rev. Soc. Bras. Laser 3, 10-16 (2006).

[4] Pinto N. C., Chavantes M. C., Stolf N. A. G., "Prevent complications in cardiovascular surgery: a successful method applying low level laser therapy", Lasers Surg. Med. 40, 61 (2008).

[5] Silva D. F. T., Vidal B. C., Zezell D. M.; Zorn T. M. T., Núñez S. C., Ribeiro M. S., "Collagen birefringence in skin repair in response to red polarized-laser therapy", J. Biomed. Opt. 11, 24002-24006 (2006).

[6] Silva, D. F. T., "Caracterização e modelagem dos fenômenos anisotrópicos do colágeno após irradiação com laser polarizado de emissão vermelha", Doctorade Thesis, Universidade de São Paulo - IPEN, (2007).

[7] Schlager A. et al., "Low-power laser light in the healing of burns: a comparison between two different wavelengths (635 nm and 690 nm) and a placebo group", Lasers Surg. Med. 27, 39-42 (2000).

[8] Calabro K., Curtis A., Galarneau J. R., Krucker T., Bigio I. J., "Gender variations in the optical properties of skin in murine animal models", J. Biomed. Opt. 16, 011008.1-011008.8 (2011).
[9] Albertini R., Aimbire F. S. C., Correa F .I., Ribeiro W., Cogo J. C., Antunes E., Teixeira S. A., De Nucci G., Castro-Faria-Neto H. C., Zângaro R. A., Lopes-Martins R. A. B., "Effects of different protocol doses of low power gallium-aluminum-arsenate (Ga-Al-As) laser radiation (650 nm) on carrageenan induced rat paw oedema", J. Photochem. Photobiol. B. v.74, 101-107 (2004).
[10] Di Rosa M., "Pharmacological properties of carrageenan", J. Pharm. Pharmacol. v.24, 89-102 (1972).
[11] Winter C. A., Risley E. A., Nuss G. M., "Carrageenin-induced oedema in the hind paw of the rat as an assay for anti-inflammatory drugs", Proc. Soc. Exp. Biol. 111, 544-547 (1962).
[12] Meneguzzo D.T., "Fototerapia com Laser em baixa intensidade em processo inflamatório agúdo induzido por carragenina em pata de camundongos - estudos de dosimetria", Doctorade Thesis, Universidade de São Paulo - IPEN, (2010).
[13] ImageJ for Windows, version 1.27z: Imaging processing and analysis in Java. Available at: http://rsb.info.nih.gov/ij/features.html
[14] Jacques S., Oregon Medical Laser Center. Available at: http://omlc.bme.ogi.edu/classroom/ece532/class3/muaspectra.html