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Article

Exploring Light-Based Technology for Wound Healing and Appliance Disinfection

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Our goal was to build, characterize and test a red light-emitting diode (LED) device suitable for wound healing and disinfection of biomedical appliances. We designed and built a unique irradiator metallic box, for which irradiation distribution and spectral irradiance were calculated. In addition, we explored the device's potential in photobiology comparing the healing of irradiated third degree burns with lesions that were left to heal spontaneously in mice. We also compared photodynamic microbial reduction with LED-irradiator and methylene blue *vs.* disinfection with a standard chemical solution, for photochemical applications. Our results showed that the LED-irradiator was able to accelerate the wound healing process compared to control group. In addition, a statistically significant microbial reduction was obtained with photodynamic inactivation compared to chemical decontamination. Thus, the prototype design is suitable for phototherapy studies since it is advantageous for low-level light therapy as well as for antimicrobial photodynamic therapy. In our perspective, this device can potentiate the dissemination of phototherapy studies to determine its suitable application in health sciences.

Keywords: low-level light therapy, methylene blue, microbial reduction, photodynamic therapy, wound healing

Introduction

With the advent of well-controlled and powerful light sources such as lasers and light-emitting diodes (LEDs), a great deal of advances was made in photochemical and photobiological studies.[1]

Currently, low-level light therapy (LLLT) is reported in the literature as an effective method to treat a wide variety of pathological conditions, promoting the modulation of inflammatory processes,[2] reduction of pain in both acute and chronic conditions,[3,4] nerve regeneration,[5] and wound healing acceleration in different etiologies.[6-8] The mechanisms behind LLLT involve light absorption by cellular chromophores, including cytochrome c oxidase,[9] and photoactive porphyrins and flavins.[10,11] In addition, mitochondrion is proposed to be a probable site for the initial effects of light and the involvement of nitric oxide had been postulated.[12] Increasing the activity of the respiratory chain induces augmented adenosine triphosphate (ATP) production, reactive oxygen species modulation, and activation of transcription factors. Sequentially, these effects lead to increased cell proliferation and migration (particularly by fibroblasts and keratinocytes), modulation in levels of cytokines, growth factors and inflammatory mediators, prevention of cell death by anti-apoptotic signaling, and increased tissue oxygenation.[13]

Low-intensity light can also be associated with the administration of non-toxic photosensitizer (PS) to locally promote photochemical reactions that can induce cellular death. Briefly, when the PS absorbs a photon, it is promoted to an excited state and can transfer charges or energy to ground state molecular oxygen, inducing the formation of reactive oxygen species.[14] These photoreactions have been used since early 1900s as a tool to inactivate numerous pathogens and have been established as a therapeutic platform commonly referred to as photodynamic therapy (PDT). Phenothiazine derivatives such as toluidine blue and methylene blue (MB) are among the most studied PS for antimicrobial photodynamic therapy (aPDT) and have been tested over the past decades in association with red

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light to promote bactericidal effect *in vitro* and *in vivo*.[15,16] Combined with light at the correct parameters, the cytotoxic photodynamic dose required for microbial inactivation is in general lower than that required to cause damage to host cells such as keratinocytes and fibroblasts.[17]

LEDs are very versatile light sources that emit a fairly narrow spectral band in comparison with halogen or incandescent lamps. In addition, they present elevated energy efficiency and a long life span, which makes them an attractive alternative for a cost-effective phototherapy. LEDs have been described as a promising light source to be used because they can be assembled in clusters to irradiate large areas,[18] eliminating the need for more powerful and, therefore, more expensive diode lasers.

The year of 2015 was chosen as the "International Year of Light and Light-Based Technologies" by the United Nations. The main goal of the institution is to draw attention to the importance of light as a sustainable solution for challenges in energy, education, agriculture and health.[19] Thus, our objective in this report was to propose a unique light source dedicated to the development of processes in the fields of photobiology, represented by LLLT, and photochemistry, expressed by antimicrobial PDT. The LED-based device was optically characterized and allows *in vivo* and *in vitro* studies with precise parameters. Thus, the applicability of the apparatus was tested by performing a whole-body irradiation on mice to treat third-degree skin burns and we also evaluated the antimicrobial potential using photoactivated MB on *ex vivo* multispecies oral biofilms. To the best of our knowledge, there are not any studies in the literature that used LED-based irradiator useful to irradiate total body of small animals as well as to sterilize biomedical appliances.

Experimental

Irradiator construction and characterization

In this work, we used radiometric quantities recommended by the International Commission on Illumination.[20]

A metallic chamber measuring 9.95 × 10 × 10 cm^3 (length, width, height) was assembled with polished aluminum to maximize light reflection and, consequently, the efficiency of the system. This apparatus consisted of an array of 3 red LEDs (Philips Lumileds, Luxeon Rebel LXML-PD01-0040, San Jose, CA, USA) distributed at the device bottom equidistant from each other (Figure 1). LED system was mounted on a circuit board and the power source was built to allow irradiation time settings and, consequently, modulate the total energy to be delivered. To position the samples in a maximized light irradiance area, a platform made of transparent polymethyl methacrylate was placed and sealed 10 cm above the LED array.

Figure 1. Top view of the LED-based irradiator. Red LEDs were placed on (a), (b) and (c).

Since the irradiance was not constant along the irradiation platform, sixteen measurements were made at points equally spaced by 2 cm from each other, on the irradiation platform (10 cm above the LEDs). An Ocean Optics USB 2000 spectrometer coupled to a cosine corrected probe CC-3-UV (Ocean Optics Inc., Dunedin, FL, USA) was used for spectral irradiance measurements. The spectroradiometer was calibrated using a standard irradiance light source (OL 200, Optronic Laboratories, Orlando, FL, USA). The light passed through the probe and it was guided by an optical fiber until an element that diffracted the light, therefore providing information about the wavelength emitted and band width. The irradiance was computed from the measured spectral irradiance[20] at the sixteen points and the mean irradiance is the average of the irradiance at the plane of irradiation.

In vivo LLLT assay

Six female adult Swiss mice with approximately 30 g of body mass were used in the trial. During the experimental period, all animals were housed in individual isolators in a 12 h light/dark cycle, fed with granulated food and water *ad libitum*. The animals were anesthetized by intraperitoneal injection of ketamine (90 mg kg^{-1}) and xylazine (10 mg kg^{-1}) before all experimental procedures. All procedures, care, and handling of the animals were carried out according to the Ethical Principles of Animal Experimentation formulated by the Brazilian College for Animal Experimentation and the protocol was approved

by the Ethics Committee on Research and Animal Care of IPEN/SP (No. 047/09).

Mice had their back fur removed by an electric shaver and the skin was cleaned with a povidone-iodine solution. Seven mm lesions were cryogenerated on the shaved back of mice using a cylindrical brass rod cooled to 77 K in liquid nitrogen. The contact was made in two sequences of 10 s each with an interval of 5 min between applications. This protocol was repeated for 3 consecutive days to standardize a third degree burn.[21]

Mice received light treatment provided by the LED irradiator (LEDG; n = 3) on days 3, 7, 10 and 14 post-wounding (p.w.). The first irradiation on day 3 was immediately after last burn procedure. For LEDG, anesthetized mice were carefully positioned on the irradiator platform with their back facing the LED array to receive a whole body illumination. The radiant exposure was of approximately 1.6 J cm^{-2} during 12 min in each session. In the control group (CG; n = 3) animals were anesthetized and burned, but not irradiated. Burn diameter was measured daily using a caliper rule during all experimental procedure until complete closure of the wound. For a global evaluation of the wound healing process, we calculated the areas under the curves (AUC) of the wound size in function of the time (from t = 0 until total wound closure) for each animal in each group, which represents the overall healing rate of mouse wounds. The AUC data were calculated by numeric integration,[22-24] using Microcal Origin 8.0 software (Northampton, MA, USA).

In vitro antimicrobial photodynamic assay

For this test, the microbial biofilm was created *in situ*. Twenty volunteers used a Hawley's removable orthodontic appliance during eight weeks to allow multispecies biofilm to grow naturally over the acrylic surface. The volunteers were instructed to use the appliance 24 h a day, only removing the appliance during the meals. When removed, the appliances were mechanically cleaned using a toothbrush associated with dentifrice. The protocol was approved by the Ethics Committee of the Centro de Pesquisas Odontológicas São Leopoldo Mandic SS (No. 917.299), and all procedures were conducted according to the principles of the Declaration of Helsinki.

At the end of the last experimental week, appliances were collected just before lunch, allowing biofilm maturation for at least 4 h, kept individually in a sterile recipient containing saline solution and immediately transported to the microbiology laboratory for further experimental procedures.

In order to evaluate the initial contamination of the appliances, a sterile cotton was swabbed over half of the appliance in both surfaces, i.e., the one in contact with the oral mucosa and the opposite surface facing the oral cavity. The procedure lasted 30 s and was performed by the same practitioner to better standardize the microbial collection. The samples were then transferred to a micro-tube containing 1 mL of sterile saline solution and vortexed during 15 s. One hundred microliter aliquots were added to wells of a 96-well plate for serial dilution and seeded onto brain heart infusion (BHI) agar plates for colony-forming units (CFU) counts according to the method proposed by Jett *et al.*[25] The plates were placed inside a microaerophilic chamber with 5% oxygen, 15% carbon dioxide, and 80% nitrogen and incubated for 72 h at 37 °C.

Ten appliances were selected to evaluate the antimicrobial effect of alkaline peroxide-effervescent tablet (Corega Tabs, GlaxoSmithKline Brasil Ltda., Rio de Janeiro, RJ, Brazil). The appliances were individually immersed in a container with 200 mL of warm water (37 °C) and one effervescent tab was added for 10 min following the manufacturer instruction. The other ten appliances were positioned individually inside the irradiator filled with a 50 μmol L^{-1} MB solution. MB photosensitizer was associated with red LED light because the absorption band of the dye is resonant with the spectral emission of this light source. Prior to irradiation, the samples were kept inside the solution for 2 min, allowing the MB to penetrate and to bind to the microorganisms. After 2 min of pre-irradiation time, the samples were irradiated for 5 min, resulting in a radiant exposure of 0.78 J cm^{-2}.

In order to evaluate the microbial reduction, after each procedure (chemical or aPDT), the orthodontic appliances were then washed with 10 mL of sterile saline solution and a sterile cotton swab was used in the half part of the appliance that was not swabbed before.

Statistical analysis

The average of AUC of both LED and control groups during the whole healing period (from 3 to 16 days p.w.) and the mean values of log CFU mL^{-1} for both chemical and PDT group were compared by unpaired *t*-test. For both assays, the results were considered statistically significant when $p < 0.05$.

Results

For the characterization of the emitted light, we started with the measurement of the spectral irradiance at the sixteen measurement locations on the irradiation

platform. The measured spectral irradiance at an arbitrary measurement point is shown in Figure 2. The spectral irradiances from the sixteen measured points were spectrally integrated, resulting in sixteen irradiance values. Irradiance values obtained spatially interpolated are shown in Figure 3. The minimum and the maximum irradiance over the entire surface of the irradiation plane were 1.1 and 3.6 mW cm^{-2}, respectively. The mean irradiance was computed to be equal to 2.6 mW cm^{-2}.

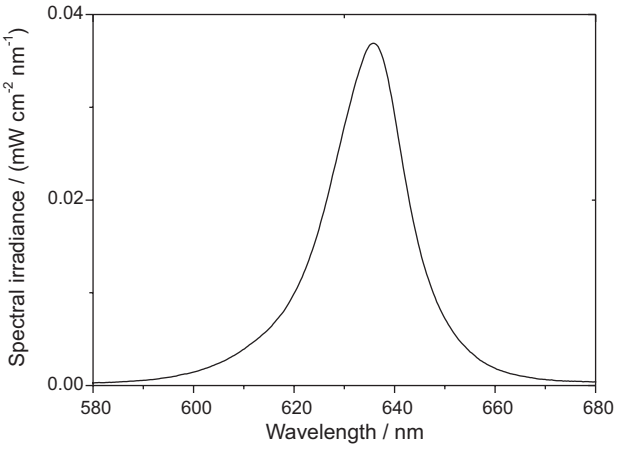

Figure 2. Spectral irradiance of the red LED-based irradiator.

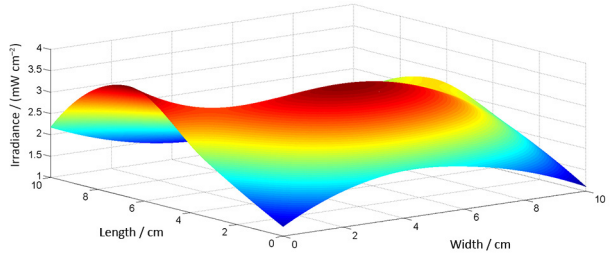

Figure 3. Irradiance distribution of the LED-based irradiator.

The results for the *in vivo* test are presented in Figure 4a as mean values ± standard error of the mean (SEM) of wound area as the percentage of the initial value, during and after the phototherapy procedure. Figure 4b displays the average of the AUC ± SEM. During the entire experimental period, the burn size for LEDG was always lower than that for CG (Figure 4a). In fact, statistically significant differences were observed between treated and control groups ($p = 0.024$), since the mean value of the AUC during overall time course was significantly larger for CG when compared to LEDG (Figure 4b). This finding indicates that the irradiated groups showed a faster healing process compared to control group.

The results for the *in vitro* antimicrobial assay are presented in Table 1, which describes the microbial burden in orthodontic appliances. Observe that the initial

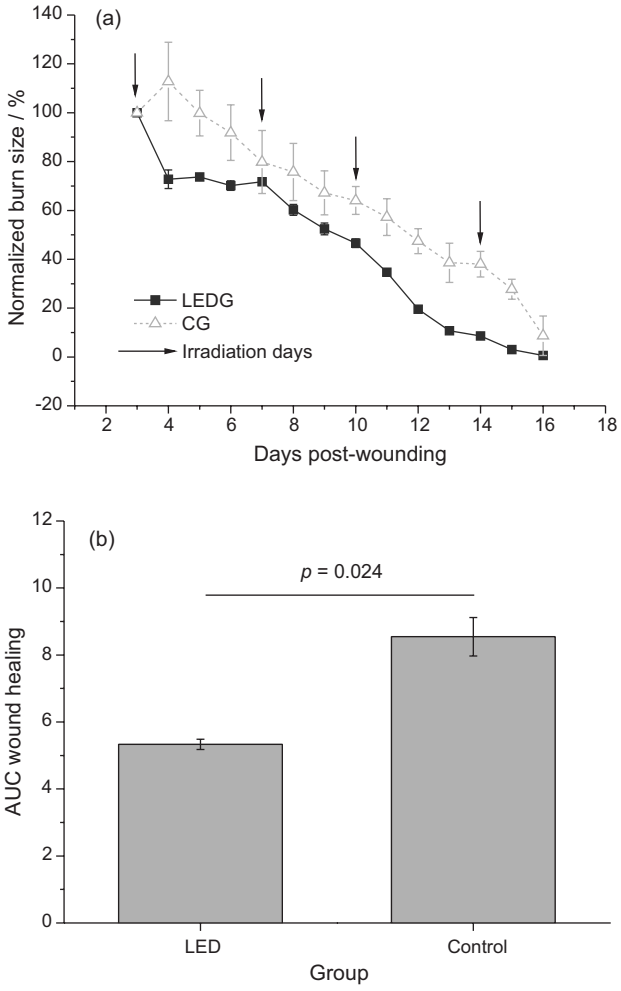

Figure 4. Mean values ± SEM of the percentage of wound closure (a) and area under the curve (AUC) (b). LEDG: LED irradiator group; CG: control group; n = 3 animals *per* group.

contamination varied among individuals with a mean value of 9×10^6 CFU mL^{-1} (range 7×10^5 to 1.2×10^9). This variation was probably caused by differences in the internal anatomy and resident microbiota of each individual oral cavity and also by the degree of cleaning performed by each volunteer. After the use of the effervescent tab, the mean contamination burden was reduced to 2×10^5 CFU mL^{-1} (range 3.6×10^6 to 4×10^4), a mean log reduction of 1.6

Table 1. Mean values ± standard deviation of bacterial concentration (CFU mL^{-1}) and log reduction for each treatment (n = 10)

Treatment	Concentration / (10^6 CFU mL^{-1})	Log reduction
Initial	9.0 ± 6	–
Chemical	0.2 ± 0.1	1.6 ± 0.3
PDT	0.009 ± 0.003	4 ± 0.7[a]

[a]$p < 0.05$ compared to chemical group. The initial counting was prior to the treatments, thus the same for both groups. CFU: colony-forming units; PDT: photodynamic therapy.

or 97%. The mean infectious burden after aPDT was 9×10^3 CFU mL^{-1} (range 2×10^4 to 0), a statistically significant mean log reduction of 4 or 99.99%, when compared to the chemical group.

Discussion

The study of phototherapy is usually multidisciplinary and the involved personnel, as for instance healthcare professionals, often do not have knowledge about distinct characteristics of different light sources. In fact, light-based experiments are performed in a multidisciplinary mode and promote significant advances in the fields of chemistry, physics, biology, and medicine. Nevertheless, the purity and origin of chemical products used in research are usually well controlled while the characterization and precise measurements regarding the light parameters are often disregarded.[26,27] Thus, we presented here an affordable yet well characterized light source that can be used for different purposes. The device was effective either in a photobiological experiment promoting faster wound healing as well as in a photochemical assay to improve disinfection of orthodontic appliances.

Phototherapy can be an interesting alternative for public healthcare systems diminishing costs with a wide variety of pathologies, e.g., diabetic wounds,[28] and therefore, the advances made on basic research can represent a major economic advantage for healthcare systems all over the world.

To evaluate the LED-based irradiator on burn healing, we induced the wounds on the center of mice's back. The centralization of the lesion was performed in order to investigate the uniformity of the whole-body irradiation. Literature demonstrates that optical therapy with LEDs and lasers are an alternative approach for wound healing.[29] In fact, the light absorption by components of the respiratory chain induces biochemical and cellular changes that result in improved wound repair. However, to obtain positive results, *in vivo* studies, light parameters such as wavelength, fluence and fluence rate have to be well controlled.

In this study, we used a red LED-based irradiator with a fluence of 1.6 J cm^{-2}. The red region of the electromagnetic spectrum is indicated for wound healing since its absorption by the main tissue chromophores (blood and water) is lower when compared to shorter wavelengths.[13] Thus, the effective light penetration into tissue is maximized. Also, low fluences of light have a far better effect than higher fluences.[13]

Our results are in good agreement with the literature,[8,21,29] since wound healing was faster in the LED group than the control group, in which animals did not receive any treatment and the burns healed spontaneously. In fact, the beneficial effect of the LED device on wound healing can be explained by considering several basic biological mechanisms including the induction of expression cytokines and growth factors known to be important in many phases of the wound healing. Literature reports that red light increases both protein and mRNA levels of interleukins 1 and 8 in keratinocytes,[30] which are cytokines responsible for the initial inflammatory phase of wound healing. In addition, irradiation of fibroblast cells at 660 nm can modulate the expression of genes involved in collagen production.[31] Red light can also increase growth factors responsible for the neovascularization necessary for wound healing and can upregulate TGF-β,[32] which is a growth factor responsible for inducing collagen synthesis from fibroblasts.

In this work, a noteworthy remark is the irradiation of total mice's body. Since the device was useful to promote wound healing, this study opens the possibility of using a whole-body irradiator for burned patients as well as to treat other traumatic injuries. Extensive burns deserve special attention, as they are one of the most common forms of trauma. In fact, third degree burns healing outcome depends on a series of factors as individual health status, affected area and additional contamination of the wound area, for instance. Thus, the construction of an LED-based total body irradiator would be an innovation in the treatment of burning wounds, accelerating the healing process and assisting the stability of the clinical status of patients. In addition, the use of LED devices can enhance the normal healing process, and doing so, it would reduce the possible length of hospital stay, consequently reducing the economic burden of these injuries.[33]

A second goal of this study was to evaluate the potential of the device for antimicrobial photodynamic therapy applications. For this purpose, we used as proof of concept biofilms grown over acrylic orthodontic removable appliances and compared aPDT with a conventional chemical decontamination method, which is considered nowadays a gold standard. aPDT has been successfully used in health sciences to promote microbial reduction in planktonic microorganisms and biofilms,[34,35] and it is a simple and fast method for decontamination of acrylic orthodontic appliances.

The data collected in the initial microbiological sample confirms that the *in situ* method for grown biofilm is reliable and reproducible producing a significant colonization over orthodontic appliances after eight weeks of usage. After chemical or aPDT treatment, microbial burden significantly diminished, with aPDT being more efficient than peroxide exposure. We hypothesize that aPDT surpassed the antioxidant capacity of the microbial cells more intensely

than peroxide. In fact, it is well established that aPDT acts by the formation of oxygen reactive species that generate oxidative stress on microorganisms conducting to cell death. The effects of superoxide and hydrogen peroxide are less severe than those of hydroxyl radical and singlet oxygen, since the formers are much less reactive and can be cleared by endogenous antioxidants. In contrast, no enzyme can detoxify •OH or $^1O_2$, making them more toxic and highly lethal.[36] It is important to highlight that our apparatus emits at 630 ± 10 nm, which could excite monomer and dimer bands of MB, and both dye species are responsible for photodestruction of bacteria.[37] Our data are in agreement with studies in literature that demonstrate microbicidal effect of aPDT comparable to gold standard treatments.[15,35,38] As the device parameters can be adjusted, it could be used to disinfect other oral prosthetic devices as well as medical instruments (scalpel, tweezers, scissors, etc).

New photosensitizers, particularly for PDT, and new light sources are constantly being developed for improvement of the phototherapy in health sciences. In our perspective, light-based technologies can potentiate the dissemination of optical therapies and help understand and determine its suitable applications in healthcare.

Conclusions

Based on our results, the LED-based irradiator achieved all of its goals, being easy to handle, cost-effective and, most of all, effective for wound healing as well as for appliance disinfection.

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