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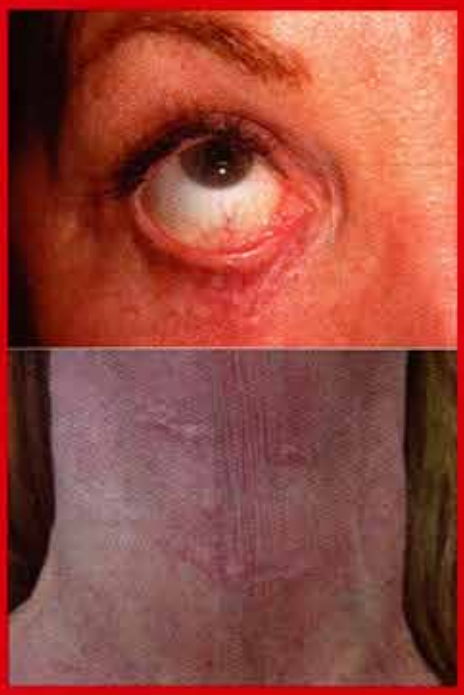
In Vivo Effects of Low Level Laser Therapy on Inducible Nitric Oxide Synthase



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In Vivo Effects of Low Level Laser Therapy on Inducible Nitric Oxide Synthase

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Background and Objective: Low level laser therapy (LLLT) has been demonstrated to modulate inflammatory processes with evidence suggesting that treatment protocol, such as wavelength, total energy, and number of treatments determine the clinical efficacy. In this study, the effects of LLLT mediated by different wavelengths and continuous versus pulsed delivery mode were quantified in a transgenic murine model with the luciferase gene under control of the inducible nitric oxide synthase (iNOS) expression.

Study Design/Materials and Methods: LLLT modulated iNOS gene expressed in the acute Zymosan-induced inflammation model is quantified using transgenic mice (FVB/N-Tg(iNOS-luc)). Here an energy density of 5 J cm^{-2} at either 635, 660, 690, and 905 nm in continuous wave mode and at 905 nm for short pulse delivery were evaluated. Age of the animals was determined as additional modulating the inflammatory response and the LLLT efficacy for some treatment protocols.

Results: Animals younger than 15 weeks showed mostly reduction of iNOS expression, while older animals showed increased iNOS expression for some LLLT protocols. Intensity and time course of inducible nitric oxide expression was found to not only depend on wavelength, but also on the mode of delivery, continuous, or pulsed irradiation.

Conclusion: LLLT exhibit different effects in induced inflammatory process according to different wavelengths and wave mode. Upregulation of iNOS gene following 905 nm pulsed wave suggests a different mechanism in activating the inflammatory pathway response when compared to the continuous wave. Lasers Surg. Med. 41:227–231, 2009. © 2009 Wiley-Liss, Inc.

Key words: bioluminescence imaging; biostimulation; inflammation; pulsed laser; Zymosan A

INTRODUCTION

Low level laser therapy (LLLT) has been demonstrated to promote photobiomodulation, including stimulation and inhibition effects and is used clinically for various conditions, including treatment of wounds, chronic pain, inflammation, and infections [1–6]. One of the proposed mechanisms of laser photobiomodulation involves the absorption of photons by intracellular chromophores and the production of reactive oxygen species (ROS), which in concentrations below the cytotoxic level has positive stimulatory effects on the cell [7].

For the continuous mode of light delivery, the wavelength-dependent ability to alter cellular mechanism in the absence of significant heating has been demonstrated through action spectra, suggesting a direct photochemical basis for the LLLT efficacy. A variety of potential photochemical targets have been suggested to give rise to the action spectra such as cytochromes within the red part of the optical spectrum, and temporary increase in the cell membranes to calcium ions [8]. Thus, the red and infrared portion of the optical spectrum opens possible therapeutic modulations in living tissues so their effects are dependent on the physiological state of the tissue at the moment of irradiation [9]. Studies show that stimulation and inhibition due to light irradiation can occur via the same photoacceptors, and therefore as the light dose increases, the photoacceptors are damaged and the effect decreases, showing a bi-phasic LLLT responses as function of irradiance (W cm^{-2}) [10]. Similar studies have been executed at 50% duty cycle in frequency modulation up to the kHz range [11]. Pulse laser radiation with low duty cycle [12] suggested transient heating possibly inhibiting NADPH oxidase.

While previously LLLT or laser biostimulation research was predominantly based on subjective evaluation of clinical or semi-quantitative pre-clinical studies, recently quantifiable pre-clinical models have been exploited to access the molecular basis for LLLT efficacy [13]. In this study, transgenic animals carrying the reporter luciferase were used to quantify iNOS gene expression following induction of acute inflammation and immediate single LLLT irradiation mediated by various wavelengths in continuous and pulsed mode.

Zymosan A has been used in several studies as a model system for inflammation induction in vivo [3,5,6,14,15].

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Zymosan A is a polysaccharide from the cell wall of *Saccharomyces cerevisae* and when injected, activates the inflammatory complementary pathway, inducing macrophages and neutrophils to release mediators such as nitric oxide and cytokine production. The Zymosan A induced inflammation represents an acute arthritis model, not fully representative of chronic arthritis or osteoarthritis. The latter was investigated as indication of LLLT, for example, using pulsed (200 nanoseconds) 904 nm exposure at 2.5 kHz delivering 2 J per treatment for 10 treatments, resulting in reduced pain scores and improved painless walking duration compared to sham treatment [15]. As discussed by Gur et al. [16] the exact mechanism of LLLT success in osteoarthritis is unknown, and photochemical actions at the cellular level may produced the observed physiological effects. In the present study, the wavelength dependency of the iNOS gene expression detected in the knee joints of mice after irradiation was monitored and quantified by means of bioluminescence imaging. The age of the animals was considered a potential confounding factor to the LLLT response.

MATERIALS AND METHODS

Animals

All experiments were carried out in accordance with the guidelines of the Animal Care Committee, Ontario Cancer Institute, Toronto, ON. Homozygous iNOS-luc animals (FVB/N-Tg(iNOS-luc)) were mated with non-transgenic FVB-strain animals. All animals were housed in the facility with water and food supplied ad libitum, and litters were weaned from the mothers at 3 weeks of age. Experiments were performed in mice from the age of 10 to 30 weeks. Animals were allocated randomly into six groups: controls ($n = 9$) were injected with the irritant but did not receive laser irradiation. Experimental groups comprised of 635 nm ($n = 9$), 660 nm ($n = 10$), 690 nm ($n = 8$), 905 nm in continuous wave mode ($n = 8$), and 905 nm in pulse wave mode ($n = 10$). All animals received the Zymosan A injection in the articular cavity of both knee joints to induce inflammation followed 15 minutes later by exposure to the respective LLLT wavelength. For each group, animals were treated randomly in multiple batches. For analysis of ages as possible confounder factor groups were analyzed as young (<15 weeks) and old animals (>15 weeks).

Acute Arthritis Induction

Induction of arthritis in the knee joints of mice was described previously [13–15,17]. Briefly, 10 µl of solution containing 300 µg of Zymosan A (Sigma–Aldrich, St. Louis, MO) in PBS was injected in the knee joints of anesthetized mice (5% isufluorane and maintenance at 2%) with a Hamilton syringe and a 27 g needle. After recovering from anesthesia, animals were returned to the cage under normal room light conditions (fluorescent bulb, 120 Hz, 50% duty cycle white light) until laser treatment. For pain control, buprenorphine (0.05 mg/kg) was injected subcutaneously every 8 hours.

Low Level Laser

Single LLLT exposure was delivered 15 minutes after induction of inflammation as described above with a dark cloth with an aperture of 1 cm was placed over the animal. Both knee joints were irradiated sequentially in random order. For 635, 690, and 905 nm continuous wave modes, fiber coupled diode lasers from Photonics Research Ontario (Toronto, ON, Canada) were used. Lasers emitting continuous 660 nm and pulsed 905 nm were provided by Theralase, Inc. (TLC-1000 Therapeutic Medical Laser, Toronto, ON, Canada). Emission power of continuous wave lasers was adjusted with a thermopile power meter (Orphia, Tel Aviv, Israel). For pulse power mode detection, a high-sensitivity fast rise time detector was used (Thorlabs Avalanche Photodiode, Newton, NJ). For all experiments conducted in the continuous wave mode, the selected power was set to give a beam diameter of 1 cm, with irradiance of 25 m W cm^{-2}. With pulsed wave mode 50 W cm^{-2} peak power was delivered during 200 nanoseconds pulses with a repetition frequency of 10 kHz, or equivalent to a duty cycle of 5×10^{-4} resulting also in an average irradiance of 25 mW cm^{-2}. Exposure time was adjusted to achieve a radiant exposure of 5 J cm^{-2} on the skin surface of the knee joints.

Bioluminescence Detection for iNOS Expression

Animals received intraperitoneal injections of 0.125 mL of luciferin (28 mg kg^{-1} in PBS) 5 minutes prior to being imaged in surpine position in the IVIS System (Caliper Life Sciences, Hopkinton, MA). Bioluminescence photons were integrated over 5 minutes and photons per seconds in a constant region of interest (ROI) in the knee area were quantified using the Living Image Software (Caliper Life Sciences). Images were collected at multiple time points ($t = 0, 2, 4, 6, 8$, and 24 hours) after inflammation induction. BLI signals collected as photons per second were normalized to $t = 0$ hours time point (when inflammation was not yet developed). The time-dependent bioluminescence integrals were obtained and analyzed by a non-parametric Kruskal–Wallis test. P values <0.05 were considered statistically significant.

After euthanasia the hindlegs of the mice were harvested and fixed in 10% buffered formalin for 3 days prior to decalcification. The intact knee joints were embedded in paraffin, sectioned sagitally, and stained with haematoxylin–eosin (H&E).

RESULTS

The time-dependent BLI signal for (a) mice younger than 15 weeks old and (b) mice older than 15 weeks is shown in Figure 1. The intrinsic BLI signal in the untreated animals (controls) reduces with increasing age. In young animals, the BLI signal following LLLT of wavelengths delivered in continuous wave mode are comparable or lower than the control animals, whereas for older animals they are higher than the respective controls. Pulsed delivery of

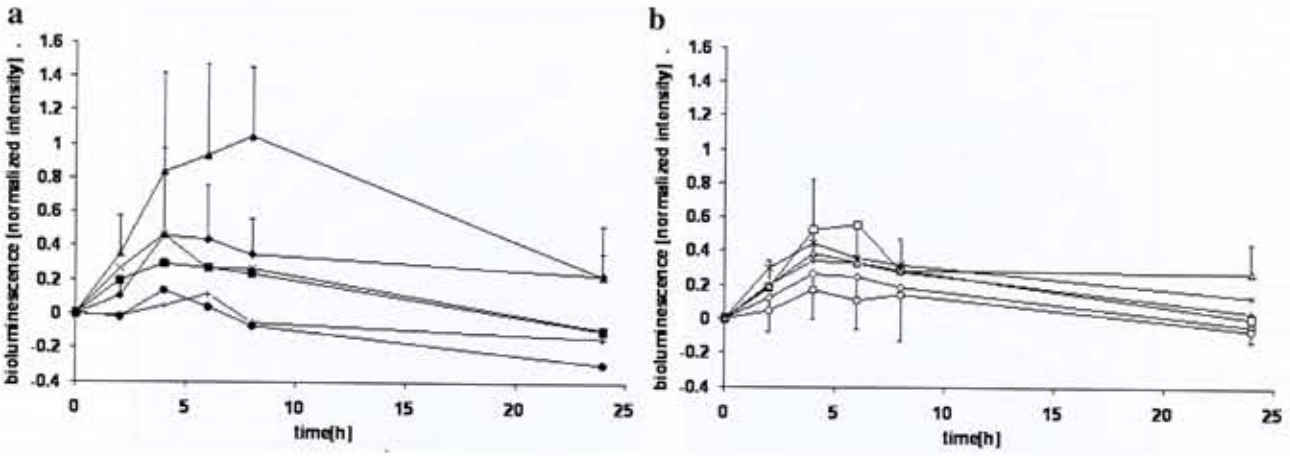

Fig. 1. Time resolved BLI signal for various LLLT protocols. **a**: animals <15 weeks old (solid symbols); **b**: animals >15 weeks old (open symbols). Error bars shown only for 905 nm pulsed mode and non-treated groups for clarity. ♦ = controls, + = 635 nm, ■ = 660 nm, ● = 690 nm, × = 905 nm CW, ▲ = 905 nm pulsed wave (PW). For clarity only representative error bars, for 905 nm PW and controls, are presented.

905 nm radiation showed higher signal in both age groups. Additionally, for the pulsed light mode, the BLI signal peaked at a later time compared to all other groups. LLLT mediated by 690 nm significantly reduced the BLI signal compared to controls for both age groups, indicating an inhibitory effect on iNOS expression.

Plotting the time integrated BLI signals change, resulting of subtracting the time integrated BLI signals form Figure 1, from their respective controls, as function of wavelength resulted in action spectra covering the red and near infrared range of light as shown in Figure 2. Positive values indicate an augmentation of the iNOS BLI signal, and hence of iNOS expression over the first 24 hours post-Zymosan A administration, whereas negative values indicate an inhibition of iNOS expression. Action spectra are available for three age groups, comprised of animals <15 weeks of age and >15 weeks of age but <1 year. Previous data acquired for 635, 785, 808, and 905 nm [13] in continuous wave were normalized and plotted to accommodate results of BLI expression, all pertaining to animals >1 year of age.

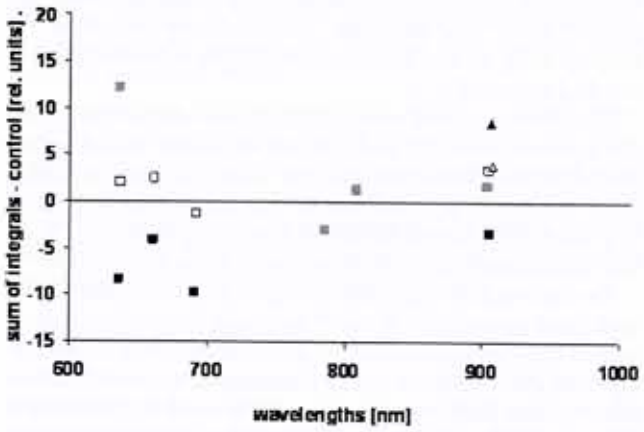

Fig. 2. Action spectra of LLLT on iNOS BLI signal. Solid symbols: animals <15 weeks. Open symbols: animals >15 weeks so younger than 8 months. Gray symbols: data from Ref. [13], with experiments executed in mice older than 1 year (signal corrected for homozygote vs. heterozygote used here). Triangles (△) refer to 905 nm in pulsed wave (PW) mode. Standard deviations are listed in Table I.

Table 1 lists statistical analysis of age related responses for the different wavelengths. There is statistical difference between young and old animals for most groups, excepted for the 660 nm and 905 continuous wave groups.

In all mice, the injection of Zymosan A resulted in an acute inflammation of the joint. Signs of inflammation were the presence of polymorphonuclear cells, fibrin and necrotic cells in the joint space as well as in the synovial membrane, data not shown. However, the degree differed. Severe joint inflammation with the presence of polymorphonuclear cells and necrotic cells as well as synovitis was found in control mice. A moderate amount of inflammation was seen in the joint of mice irradiated with 690 nm conclusive with the interpretation of an iNOS expression inhibition (Fig. 3A).

TABLE 1. Comparison of Sum of Integral of BLI as a Function of Ages in Different LLLT Exposures With Respective Mean Values

Groups	Mean value (young)	Mean value (old)	*P*-value
Control	6.97±2.7	2.65±4.4	0.041*
635	−0.22±4.9	5.28±2.3	0.02*
660	2.88±4.9	5.18±5.6	0.290
690	−2.70±4.0	1.44±2.4	0.03*
905 CW	3.66±5.15	6.15±3.1	0.745
905 PW	15.45±5.1	6.61±4.1	0.021*

P values considered statistically significant are represented with asterisk (*).

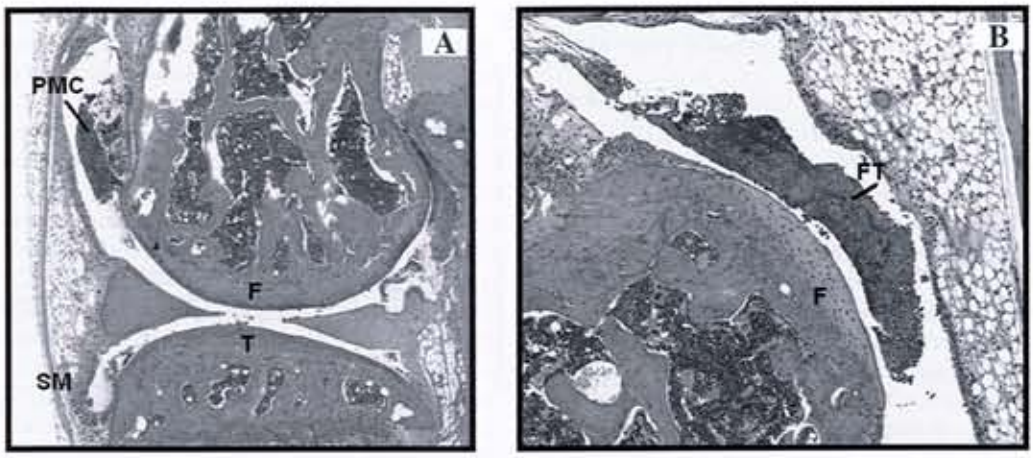

Fig. 3. Histology of knee joints treated with LLLT of 690 nm (**A**) and 905 nm pulsed wave (**B**) after 24 hours. (F, femoral condyle; T, tibia; SM, synovial membrane; PMC, polymorphonuclear cells; FT, fibrous tissue).

Conversely, mice exposed to 905 nm pulsed wave showed the highest amount of polymorphonuclear cells and highest amount of fibrin deposition in the joint space (Fig. 3B). While not quantitative, these histological observations are supporting the BLI results.

DISCUSSION

The effects of therapeutic laser in photobiostimulation and its use in the medical field are intensively discussed, and it is thought that continuous wave irradiation of visible red and near infrared laser light activates a cascade of photochemical reactions in the cells, but the mechanisms are not yet well described. It is generally agreed that a wavelength-dependent capability of photons to alter cellular behavior without macroscopic heating of the tissue stimulates or inhibits cellular activity, which occurs via a photochemical interaction. Photons are absorbed by chromophores within different cell structures, like the mitochondria, nucleus, organelles, and cell membrane, triggering responses that vary according to the wavelength, dose, and intensity of light as demonstrated in experiments observing cell proliferation [9]. Photons absorbed by the mitochondria are believed to transduce photon energy into chemical energy. This cascade of biochemical processes impact the metabolism leading to physiological changes, resulting in improved tissue repair, faster resolution of the inflammatory response, reduction of pain, and wound healing [3] and also clinically demonstrated improvements in osteoarthritis [17]. In this study different wavelengths mediated LLLT effects in the acute inflammation model were compared in addiction to those reported previously. Two wavelengths, 635 and 905 nm, were repeated from a previous study; 660 and 690 nm and the pulsed mode of 905 nm were added, improving the detail of an iNOS related action spectra.

The inflammatory reaction resulting from the injection of irritant is reduced in old compared to very young animals, suggesting age dependent inhibition of the cellular behavior. However, LLLT appears to be able to compensate at least partially for this loss in normal iNOS expression in the older cohorts. It was observed that irradiation with 635 nm showed statistical different results when delivered in young animals (15 weeks old) and older animals (30 weeks old) (Fig. 2). In the previous experiment, when 635 nm laser was irradiated in the knee joints of older animals (over 1 year old), it showed statistical higher relative iNOS expression than non-treated animals of the same age, suggesting that low power laser effects has an inflammatory response that is age-dependent. Similar trends are also noted for the other wavelength tested. The wavelength dependent LLLT response, particular at the short wavelength range, here shown by the action spectrum, is indicative for the presence of photochemical mediated LLLT effects in this model of acute arthritis. The histological observation of increased inflammatory response for 905 nm pulsed wave and decrease inflammatory response for 690 nm (Fig. 3) are consistent with the interpretation of the BLI signal (Fig. 2).

The effect of pulsed versus continuous wave exposure for the same wavelength and radiant exposure cannot be explained with a photochemical light tissue interaction model, since saturation or damage of the photoacceptor would lead to a lower BLI signal for the pulsed light delivery. Hence non-photochemical interactions need to be considered.

For the low duty cycle used in this experiment (0.05%) and high peak power of 50 W cm^{-2}, localized thermal activation within the concept of selective photothermolysis is conceivable [18,19]. Selective photo thermolysis occurs if photon absorption within a target structure is much higher than in the surrounding tissue, but the pulse duration is short compared to the thermal relaxation time of the target structure. Two hundred-nanosecond pulses exceed the thermal relaxation time of structures smaller than 200 nm. Hence, the cell membrane (20 nm) would radiate much energy to the surrounding area not confirming the thermal energy and thus not achieving elevated temperatures, hence presenting an unlikely target structure. The inverse

of the repetition rate of 1/10 kHz = 100 microseconds needs to be equivalent to at least several thermal relaxation time of the target structure, permitting their cooling between pulses to avoid accumulation of heat, which would finally exceed the Arrhenius damage integral [20]. Therefore, the target structure should be smaller than 3–5 μm, but larger than 200 nm. Mitochondria and the rough endoplasmic reticulum have both high relative amount of lipid membrane with peak absorbance at 930 nm, close to the used LLLT wavelength. Their size and the possibility for lipid as the main absorber makes both organelles potential target structures for selective photothermolysis mediated LLLT, wherein the mechanism is possible due to a change in the membranes integrity over time periods of less than a few microseconds but long enough to permit ions or proteins to cross them.

While few studies investigated the effects of pulsed laser, very few proposed a thermal mechanism. El Sayed [2] observed the effects of different pulsing frequencies of LLLT at 820 nm and an energy density of 21.6 J cm^{-2} in vivo in rat skin wound and noted that the total number of mast cells was increased significantly by frequencies ranging from 2.5 to 20,000 Hz (at a 50% duty cycle) when compared to sham irradiated group. Ueda and Shimizu [11] observed that irradiation with low pulse frequencies of LLLT on rat calvarial cells significantly stimulated cellular proliferation in vitro, finding differences in high and low frequencies and also with different energies. The pulsed mode of laser treatment has also been used for treatment of acne vulgaris [21], however the mechanisms of how pulsed mode laser functions was not eluded to. Karu et al. [12] proposes a transient local heating mechanism explaining the inhibition of activity of NADPH-oxidase under pulsed 632.8 nm, primarily stating the importance of dark period between the pulses to avoid detrimental effects.

Of additional clinical importance is the efficacy of the 905 nm NIR light as it experiences a lower attenuation compared to 635 nm red light and hence is better suited for the treatment of large tissue volumes such as knee joints affected by osteoarthritis.

In conclusion, we confirmed prior results of LLLT action spectra for the activation of iNOS transcription and translation in a murine acute inflammation model and could demonstrate that the benefit is age-dependent. Additionally we showed that high peak power LLLT can mediate biomodulation not only via photochemical photon–tissue interaction but also via a photothermal one.

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