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ORIGINAL ARTICLE

The effect of low-level laser therapy (660 nm) on the gene expression involved in tissue repair

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Abstract The effect of low-level laser therapy (LLLT) on the healing of skin lesions has been evaluated in many studies; however, the molecular mechanisms involved in the biostimulatory effects resulting from this treatment need to be better understood. The paper aims to analyze the effects of LLLT (660 nm) at doses of 1 and 5 J/cm^2 on cell viability and expression of vascular endothelial growth factor (VEGF) and interleukin (IL6) genes in L929 fibroblast cells. The dose-response curve was performed with the GaInAlAs (660 nm) laser-treated cells at energy rates of 1 and 5 J/cm^2. Cell viability was quantified at 24, 48, and 72 h after irradiation and the effects of TLBP on the cytoskeleton and endoplasmic reticulum were evaluated by fluorescence microscopy and the RT-qPCR method was used for the analysis of gene expression. It was observed that the 72 h group had a statistically significant increase in cell viability compared to the 48 h group ($p < 0.01$) and when compared to the 72 h control ($p = 0.03$). In 72 h, a greater distribution of the cytoskeleton filaments and the more evident endoplasmatic reticulum was verified, indicating an increase in the protein synthesis when compared with the control group. In the expression of the VEGF gene, a significant increase of 1.98 times ($p < 0.05$) in the number of transcripts was observed; whereas for the IL6 gene, a decrease of the transcripts was 4.05 times ($p < 0.05$), both occurring within 72 h after irradiation at 5 J/cm^2. The LLLT (660 nm) at the dose of 5 J/cm^2 should modulate cellular viability, upregulated VEGF, and downregulated IL6 expression of messenger RNA in culture of L929 fibroblast cells.

Keywords Low-level laser therapy · Cell viability · Tissue repair · VEGF · IL6

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Introduction

Low-level laser therapy (LLLT) is a resource that has been widely used in clinical practice to accelerate the repair of both soft and hard tissue, modulating various biological processes in animal models and in human beings [1]. Studies have suggested that photo-stimulation via laser may occur during the inflammatory and proliferative stages of healing [2–4], due to the fact that LLLT promotes cell proliferation through neovascularization and a reduction in the amount of inflammatory infiltrate [3].

Pro-inflammatory cytokines play an important role in repairing wounds, since they likely have an influence on various processes at the site of the wound, including the stimulation of keratinocytes, proliferation of fibroblasts, breakdown of proteins in the extracellular matrix, and the regulation of immune response. Particularly with the repair of wounds, the expression of IL6 was seen to be heavily upregulated during the inflammatory phase of the healing process. Similarly, an upregulation of the expression of growth factors after an injury is frequently

observed, suggesting a need for high levels of growth factors and cytokines during the normal repair process, in a correct spatial and temporal expression of the respective genes [5].

In addition, vascular endothelial growth factor (VEGF) is involved in vasculogenesis and angiogenesis during healing via multiple mechanisms including the deposition of collagen and by epithelialization. It should be stressed that this gene is expressed most in the first day following the skin lesion [6] and is produced by endothelial cells, fibroblasts, cells of the smooth muscle, and the majority of the inflammatory cells [7].

LLLT is a resource that has been commonly used in clinical practice with various types of lesions as it has a biostimulatory ability to repair tissue and is a fast, non-invasive, and effective resource [8]. Researchers have turned their attention to the cell signaling pathways involved in the biological effects of LLLT [9], and are also investigating the best parameters (dosage, energy density, and wavelength) for use in clinical practice. Meireles et al. [10] studied the effects of GaAlAs laser application at two wavelengths on burns induced on the back of rats. Daily laser application using a wavelength of 660 nm and dose of 20 J/cm2 was more effective than a wavelength of 780 nm under the same conditions.

As cytokines and growth factors have played important roles in regulating different stages of the repair process and that the 670-nm laser therapy is effective in increasing the number of fibroblasts [11], this study proposes to evaluate the effects of LLLT on cell proliferation and the expression of genes involved in the processes of inflammation and neovascularization, at a wavelength of 660 nm in doses of 1 and 5 J/cm^2 using the cellular lineage of L929 fibroblasts.

Material and methods

Fibroblastic cells L929, *Mus musculus* (Mouse, Balb/C, ATCC CCL-1 NCTC) were obtained from Adolfo Lutz Institute (São Paulo, Brazil).The study was approved by the Ethics Committee of University of Northern Paraná (Unopar) under the Protocol #462,478/2013.

Cell culture

Fibroblasts cells were cultured in bottles of 25 cm^3 (TPP®, Switzerland, Europe) with MEM (minimum essential medium) (Gibco® Invitrogen Corporation, Grand Island, USA) supplemented with 5% of FBS (Fetal Bovine Serum) (Cultilab, Brazil) and 1% of antibiotic and antimycotic kept in a CO_2stove in an atmosphere of 5% at 37 °C. The cells used were subcultured whenever they reached a confluence of 80–100%. Connective tissue cells of mice were used in this experiment according to ISO 10993-5 standard, which recommends the usage of this cell line in vitro for cytotoxicity assays.

Irradiation

The AlGaInP—Diode continuum laser (KLD Biosistemas®) was used with the following specifications: wavelength of 660 nm, power density of 35 mW, average power output of 20 mW, beam spot size 0.035 cm^2, and selection of the continuous mode. Laser energy was delivered to each well through an optical fiber of 0.01 cm^2 area in a half-light environment, with laser kept perpendicular to the bottom side of the plate to assure that irradiation would penetrate into the monolayer cells. The radiation was applied in four points within the area. The device had been previously maintained and calibrated and the real average power was gauged with an Agilent oscilloscope (Agilent Technologies, Inc., Colorado Springs, CO).

The irradiation of cells was performed in 24, 48, and 72 h after plating in TPP® 12-well plates at a 5×10^5 cells/mL density. Three groups were established in order to evaluate the laser biomodulation range: Group 1, control (non-irradiated); Group 2, 1 J/cm^2; and Group 3, 5 J/cm^2. The laser energy was sent to the cell culture by optical fiber with the cross-sectional area of 0.01 cm^2 in continuous way and timely application method with laser beam perpendicularly reaching the plate with the lid closed. The whole experiment was performed in triplicate. After each period, the cultures had cell viability assessed by the microculture tetrazolium (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay.

Microculture tetrazolium assay

The MTT assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells [12]. Twenty-four hours after each irradiation, with determination of optical density by means of a reader using a microplate reader (ELISA reader–SpectraCount–Packards Instrument, Offenburg–Germany) at a wavelength of 546 nm. The %cell viability was calculated using the formula:

$$\%cell\ survival = \{(\mathrm{At}-\mathrm{Ab})/(\mathrm{Ac}-\mathrm{Ab})\}\mathrm{x}100$$

where,

At Absorbance value of test compound
Ab Absorbance value of blank
Ac Absorbance value of control

Fluorescence microscopy

To monitor the effects of irradiation LLLT using fluorescence, the L929 cells were subcultured onto glass coverslips at density of 5×10^5 cells/mLon 12 well TPP®plates (Switzerland, Europe). Twenty-four hours after irradiation, the coverslips were analyzed by fluorescence microscope equipped with photographic system Leica MPS-30 photographic system. The cells were labeled with fluorescent dyes: Rhodamine Phalloidin for cytoskeleton (Molecular Probe Eugene, Oregon, USA), DAPI–Fluoreshild (Sigma-Aldrich®, Steinheim, Germany) for the nuclei, and DiOC6 (3.3′-dihexyloxacarbocyanineiodide) (Molecular Probes Eugene, Oregon, USA) for the endoplasmic reticulum. All coverslips were labeled with DAPI and DiOC6, and the coverslips labeled with Rhodamine Phalloidin required the use of the special fixative, 4% Paraformaldehyde in PBS and 0.1% Triton X100.

RNA extraction and cDNA synthesis

For the extraction of total RNA, we used PureLink® RNA Mini Kit (Invitrogen, Life Technologies) according to the manufacturer's instructions. After the extraction, the RNA was quantified in spectrophotometer NanoDrop Lite (Thermo Scientific). The cDNA was synthesized from 1 μg from total extracted RNA. The final reaction volume was 20 μL, composed of 10% of reaction buffer (200 mMTris-HCl (pH 8.4), 500 mMKCl-Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.5 mM dNTPs (Invitrogen), 80 pmol of oligo DT12–18 (Invitrogen), 100 pmol of RandomPrimers (Invitrogen), 10 units of SuperScript III (Invitrogen) enzyme, and 2 units of RNaseOUT (Invitrogen)enzyme.

To minimize reverse transcriptase performance variations and the possibility of Monte Carlo impact, three different reactions of cDNA synthesis were performed for each experiment, in which products were incorporated to obtain a single mixture for each sample in its respective cultivation condition.

Real-time PCR

The polymerase chain reaction (PCR) in real-time was carried out in a thermocycler StepOnePlus (Applied Biosystems) under the following conditions: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 60 s at 60 °C proceeded by melting curve in the conditions: 95 °C for 15 s, 60 °C for 60 s, and ended at 95 °C for 15 s. The 20 μL final reaction contained 10 μL of TaqMan® Universal Master Mix II (Applied Biosystems), 1 μL of TaqMan® Gene Expression Assays (Applied Biosystems), 8 μL of ultrapure water, and 1 μL of cDNA.

To determine the relative expression of interest genes in the group with better cell growth in the best time, we used the REST program–Version 2009 (relative expression software tool) (QIAGEN) developed by Pfaffl et al. [13] based on their own mathematical model for relative quantification of data obtained by real-time PCR with efficiency correction. The values obtained in the control situation (no treatment) were used as reference for comparison, and the calculations were normalized from the β-Actin reference gene. Only Cq values (quantification cycle) with a variation of ± 0.5 between reaction triplicates were considered.

Statistical analysis

First, we ran the Shapiro-Wilk test to verify that the data presented Gaussian distribution then two-way Anova with Tukey post-test was held to check the effects of the different intensities and in relation to time. For gene expression analysis, the data were expressed according to the Pfaffl method [13] and calculated using the software Rest–2009, considering standard deviation values for the statistical test "pair wise fixed reallocation." Genes were considered differentially expressed to the control (G1) as alteration was significantly different considering the value of $p \leq 0.001$ or alterations greater than 1.5 times ($p < 0.05$). For all tests, it was adopted a 95% confidence interval and 5% significance level.

Results

A comparative analysis of the values of viable cells using the MTT assay on groups G1 (Control), G2 (1 J/cm^2), and G3 (5 J/cm^2) vis-a-vis the irradiation intervals (24, 48, and 72 h), showed no statistical difference ($p = 0.16$), as displayed in Table 1. However, a statistically significant difference was observed when comparing G2 (1 J/cm^2) and G3 (5 J/cm^2) ($p \leq 0.01$) and at 72 h when comparing G1 and G3 ($p = 0.03$) (Fig. 1). At 24 h, no statistically significant difference was observed ($p > 0.05$) (Table 2).

Considering that the 5 J/cm^2 group exhibited a higher rate of viability, the fluorescence microscopy analysis was only conducted in this group. As can be seen from Fig. 2, with greater exposure to the laser (24, 48, and 72 h), a high distribution of cytoskeleton and endoplasmic reticulum occurred and a more conspicuous nucleus, indicating greater protein synthesis activity when compared to the control group.

As for the analysis of gene expression, it was possible to identify a statistically significant difference, in the expression of the genes studied, between the groups G1, G2, and G3. Irradiated cells showed a 1.98 increase in the transcripts of the VEGF gene and 4.05 decrease in the transcripts of the IL-6 gene in G3 at 72 h, when compared to G1 (Fig. 3).

Table 1 Comparative analysis of cell growth and time in the laser irradiated samples

Intensity	24 h	48 h	72 h
G1 (control)	107.0 ± 0.0	108.0 ± 1.0	106.0 ± 1.0
G2 (1 J/cm^2)	107.0 ± 3.4	99.3 ± 7.2	111.3 ± 13.0
G3 (5 J/cm^2)	114.3 ± 5.5	122.3 ± 6.71	125.3 ± 7.1

Values presented as means and standard deviation (±)

Table 2 Comparative analysis between the groups at the evaluated time intervals

Time (h)	G1 vs. G2	G1 vs.G3	G2 vs. G3
24	1.00	0.87	0.87
48	0.75	0.19	< 0.01*
72	0.97	0.03*	0.21

G1 (Control), G2 (1 J/cm^2), and G3 (5 J/cm^2)

Values represent the two-way ANOVA, Tukey's post-test

*Statistically significant

Discussion

LLLT is used to accelerate tissue repair processes due to its bio-stimulatory effects through at least two parameters: dosage and wavelength [1], and multiple combinations of these parameters make it difficult to standardize for use in clinical practice [2, 14].

Our results showed a significant increase in cell viability in the group irradiated with LLLT (λ 660 nm) in a dose of 5 J/cm^2 at 72 h. Eduardo et al. [15] found similar results conducting an in vitro study irradiating human stem cells (λ 660 nm) with doses of 1, 3, and 5 J/cm^2 for a period of 72 h, and noted an increase in mitochondrial activity in doses of 3 and 5 J/cm^2 at 48 h, though at 72 h the group irradiated at 5 J/cm^2 exhibited a significant interest in this activity.

Marques et al. [16] irradiated fibroblast cells L929 (λ 660 nm) with doses of 0.1, 0.5, 1, 2, 3, 5, 7, 10, 20, and 30 J/cm^2 for a period of 24 h and showed that, between the doses of 0.1 and 3 J/cm^2, there was an inhibitory effect evidenced by a reduction in the density of intact cells and above 5 J/cm^2 an increase in the number of cells due to an intense grouping of mitochondria in the perinuclear region.

According to Zanotti et al. [17], excitatory doses (up to 8 J/cm^2) are recommended when the aim of the intervention is to enhance the sodium/potassium pump, stimulate the production of ATP, reestablish the membrane potential and increase metabolism and cell proliferation. Higher doses (above 8 J/cm^2), on the other hand, inhibit the production of collagen [18]. As for Rocha Júnior et al. [19], they state that LLLT shows evidence of an increase in neovascularization and fibroblast proliferation and a reduction in the quantity of inflammatory infiltrate in surgical wounds.

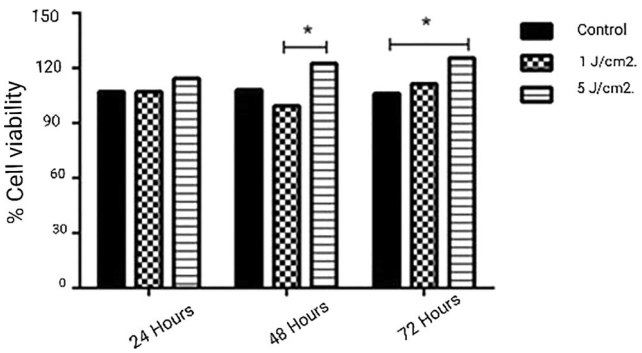

Fig. 1 Comparison of % cell viability between the groups at the evaluated intervals. *p < 0.01 (48 h—1 vs. 5 J/cm^2) and *p = 0.03 (72 h—control vs. 5 J/cm^2)

For a better understanding of the effects of laser irradiation on cell structure, fluorescence microscopy was employed to investigate the cytoskeleton, endoplasmic reticulum, and nucleus, as these are important structures for the cell viability process. At 72 h, a higher distribution of filaments was found in the cytoskeleton, endoplasmic reticulum, and a more conspicuous nucleus, indicating an increase in the protein synthesis when compared to the control group. These results are in agreement with the findings of Carnevalli et al. [20] which found evidence of an increase in the cellular metabolism in the arrangement of the intermediate filaments of the cytoskeleton and the chromosomes, and intense mitochondrial activity.

In the present study, an increase was also observed in the expression of transcripts of the VEGF gene in G3 at 72 h. Research conducted by Cury et al. [21] with LLLT (λ 660 and 780 nm) on rats with doses of 30 and 40 J/cm^2, demonstrated a significant increase in the expression of the VEGF gene after 4 days of irradiation in all the groups evaluated and with both wavelengths used, regardless of energy densities.

Chiarotto et al. [11] found similar results by irradiating rat wounds with a laser at λ 670 and a dose of 4.93 J/cm^2, verifying that the laser had a significant influence on the expression of the VEGF gene in the angiogenesis and proliferation of new fibroblast cells when compared to a non-irradiated group.

The VEGF gene codifies a protein that regulates angiogenesis in physiological and pathological processes, and irradiation with LLLT induces its expression and the proliferation of vascular endothelial cells activating the kinases regulated by extracellular signal-related kinase/specific protein 1 (ERK/Sp1) indicating that Sp1 plays an important role in the proliferation of endothelial cells [22].

Our results demonstrate an inhibition in the expression of the IL6 gene in the 5 J/cm^2 dose at 72 h. These data are in agreement with the findings of Houreld and Abrahamse [23] who, using the laser at λ 660 nm with a dose of 5 J/cm^2, found a significant inhibition in the expression of IL-6 in the

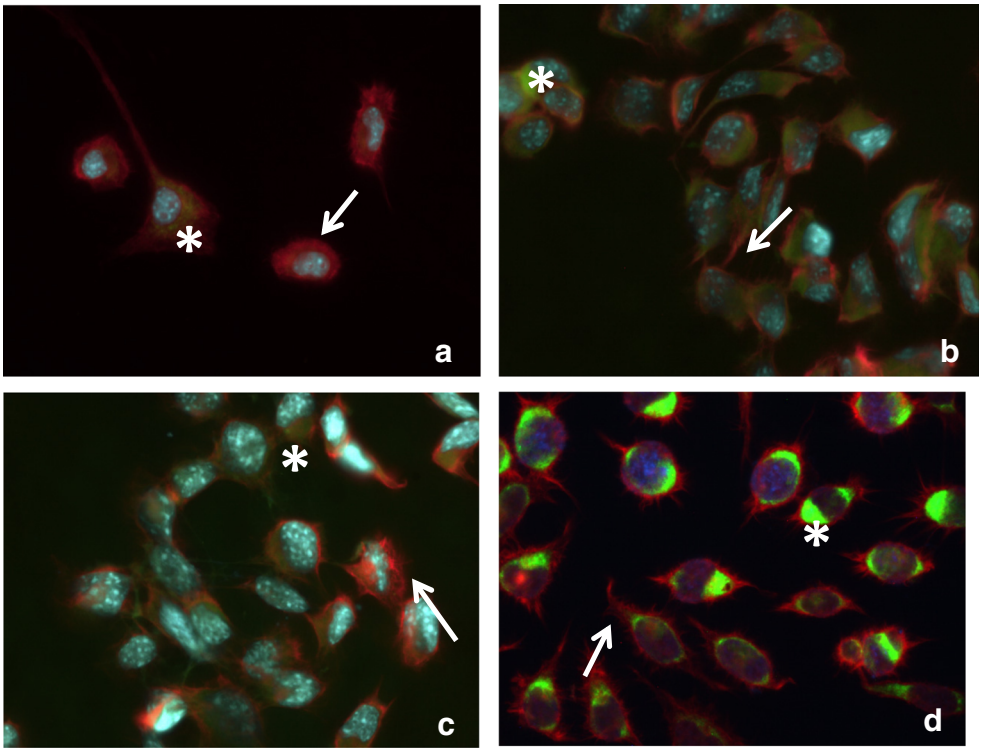

Fig. 2 Fluorescence microscopy. Analysis of the cellular structures stained with Rhodamine Phalloidin (cytoskeleton-asterisk) and DioC6 (Endoplasmic Reticulum: arrow) using overlay technique. A clear biomodulatory effect of the laser was observed in irradiated cells (**b**, **c** and **d**) showing a greater distribution and organization of actin filaments and endoplasmic reticulum when it was compared to the control group (**a**). **a**—non-irradiated group; **b**—5 J/cm^2/24 h group; **c**—5 J/cm^2/48 h group, and **d**—5 J/cm^2/72 h group

fibroblasts of human skin (WS1) when compared to the control group.

After inducing an inflammatory process with the injection of carrageenan in rats, Albertini et al. [24] irradiated the tissue using LLLT (λ 660 and 684 nm) with a dose of 7.5 J/cm^2, where both groups exhibited a 30 to 40% reduced expression in the genes TNFα, IL1β, and IL6 3 h after irradiation, when compared to the control group. Similar results were reported by Boshi et al. [25] after the injection of carrageenan and laser irradiation (λ 660 nm) at 1, 2, and 3 h with doses of 0.9, 2.1, and 4.2 J/cm^2, the groups were evaluated 4 h after the final irradiation, where it was observed that the expression of genes TNFα, IL6, MCP1, and IL10 was reduced with doses of 0.9 and 4.2 J/cm^2, though with a smaller reduction in a dose of 2.1 J/cm^2.

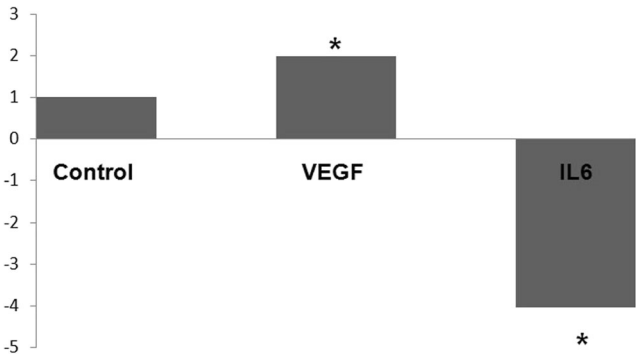

Fig. 3 Relative expression of the VEGF and IL6 genes. Effect of LLLT on VEGF and IL-6 mRNA expression in L929 fibroblasts cells. Expression of VEGF mRNA and IL6 mRNA was analyzed with quantitative real-time RT-PCR, relatively compared to untreated control. The expression levels of the target gene were normalized to the expression level of β-actin. *$p < 0.05$

IL-6 is a pro-inflammatory cytokine that performs a wide range of functions with immune cell effects related to inflammation, protection of the host and tissue lesion [25]. It is one of the earliest and most important mediators of the induction and control of the synthesis and release of acute phase proteins by the hepatocytes during painful stimuli such as traumas, infections, surgical processes and burns. It promotes neutrophil maturation and activation, macrophage maturation and differentiation/maintenance of cytotoxic T-lymphocytes and natural killer cells. It also exercises anti-inflammatory properties during the lesion, by releasing soluble FNT receptors (sFNTRs) [26].

Despite the fact that biostimulation promoted by LLLT is still not demonstrably effective at specific wavelengths, studies and experiments have shown the occurrence of positive, multiple effects such as accelerating tissue proliferation, stimulus to local neovascularization, and formation of a more organized granulation tissue [6, 19] and with the induction of an anti-inflammatory effect [23, 24], corroborating the results in the present study, provides evidence that the expression of

RNAm of genes IL6 and VEGF was modulated by low-level laser therapy (λ 660 nm with a dose of 5 J/cm^2). The results of the present study suggest that LLLT may be an alternative therapy that inhibits the inflammatory process and stimulates the formation of new blood vessels.

These effects are related to the initial phases of tissue repair confirming the photobiomodulation effect and thereby indicating its clinical importance in the treatment of tissue lesions.

Conclusion

The present study concluded that LLLT at a wavelength of 660 nm had a biostimulating effect at 72 h using a dose of 5 J/cm^2, revealing an increase in reticular activity, better organization and distribution of the cytoskeleton in the cytoplasm and greater chromatin decondensation. In addition, these parameters are shown to be more effective in increasing the expression of the Vascular Endothelial Growth Factor (VEGF) gene and reducing the transcripts of the Interleukin 6 gene (IL6).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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