Lasers Med Sci
DOI 10.1007/s10103-014-1644-y

ORIGINAL ARTICLE

Effect of low-level laser therapy on the gene expression of collagen and vascular endothelial growth factor in a culture of fibroblast cells in mice

C. C. S. Martignago · R. F. Oliveira · D. A. A. Pires-Oliveira · P. D. Oliveira · C. Pacheco Soares · P. S. Monzani · R. C. Poli-Frederico

Received: 10 April 2014 / Accepted: 21 August 2014

Abstract Low-level laser therapy treatment (LLLT) is widely used in rehabilitation clinics with the aim of accelerating the process of tissue repair; however, the molecular bases of the effect of LLLT have not been fully established. The aim of the present study was to evaluate the influence of the exposure of different doses of LLLT on the expression of collagen genes type I alpha 1 (COL1α1) and vascular endothelial growth factor (VEGF) in the fibroblast cells of mice (L929) cultivated in vitro. Fibroblast cells were irradiated with a Gallium-Arsenide laser (904 nm) every 24 h for 2 consecutive days, stored in an oven at 37 °C, with 5 % CO_2 and divided into 3 groups: G1—control group, G2—irradiated at 2 J/cm^2, and G3—irradiated at 3 J/cm^2. After irradiation, the total RNA was extracted and used in the complementary DNA (cDNA) synthesis. The gene expression was analyzed by real-time polymerase chain reaction. The cells irradiated in G2 exhibited a statistically significant growth of 1.78 in the expression of the messenger RNA (mRNA) of the COL1α1 gene (p=0.036) in comparison with G1 and G3. As for the VEGF gene, an increase in expression was observed in the two irradiated groups in comparison with the control group. There was an increase in expression in G2 of 2.054 and G3 of 2.562 (p= 0.037) for this gene. LLLT (904 nm) had an influence on the expression of the genes COL1α1 (2 J/cm^2) and VEGF (2 e 3 J/cm^2) in a culture of the fibroblast cells of mice.

Keywords Type I collagen · Gene expression · Low-level laser therapy · Vascular endothelial growth factor · Fibroblastic cell · Mice

Introduction

Low-level laser therapy (LLLT) is widely used in clinical treatments aimed at lessening pain, reducing inflammatory processes, and also in promoting tissue repair [1]. Studies [2–5] have been conducted with the aim of understanding the wound healing process, its complexity, and its diversity [6]. Recent studies have reported a large number of proteins that perform functions that are important to the process of cell proliferation induced by LLLT [7], prominent amongst which are collagen type 1 (COL1) [1, 8, 9] and vascular endothelial growth factor (VEGF) [6, 10–12], as they are intimately connected to this process.

Laser wavelengths ranging from 524 to 904 nm have been reported to increase the rate of wound healing, increase collagen production, and improve dermal vascularity in a variety of types of animal wound [13–15]. Some in vitro studies were carried out with cell lines derived from human fibroblasts [16, 17]. Although human cell lines have some importance to the field, the line derived from rodents, such as lung fibroblasts, Chinese hamster, Chinese hamster ovary, mouse keratinocytes and fibroblasts, and murine hepatocytes, and primates, such as monkey kidney fibroblasts (Vero line), are the first choice because of safety issues, use and cultivation of well-

C. C. S. Martignago · P. D. Oliveira
Rehabilitation Science, North Paraná University (UNOPAR), Londrina, Paraná, Brazil

R. F. Oliveira · D. A. A. Pires-Oliveira · R. C. Poli-Frederico (✉)
Molecular and Cell Biology, Rehabilitation Science, North Paraná University (UNOPAR), Av. Paris 675, Jd Piza, CEP 86041-140. Cx. P. 401, Londrina, Paraná, Brazil
e-mail: reginafrederico@yahoo.com.br

R. C. Poli-Frederico
e-mail: regina.frederico@kroton.com.br

C. Pacheco Soares
Instituto de Pesquisa e Desenvolvimento, Universidade Vale do Paraíba—UNIVAP, São José dos Campos, SP, Brazil

P. S. Monzani
Universidade Norte do Paraná, Londrina, Paraná, Brazil

Published online: 30 August 2014

established conditions, and their known genetic and physiological patterns [18–20]. Therefore, the majority of in vitro studies have shown that the application of LLLT to a cell culture of fibroblasts and epithelial cells promotes growth in cell proliferation and in the gene expression of COL1 [21–23].

Collagen is a fibrous, insoluble protein found in the extracellular matrix (ECM) and connective tissue, representing an extracellular structure for all multicellular organisms [24]. At the present time, a total of 27 different types of collagen are known [25], collagen type 1 being the most abundant in bones' tendons, skin, ligaments, corneas, lungs, the vascular system, and many other interstitial tissues [26] and is an extremely important protein in the process of tissue repair. It consists of the peptide chains α1 and α2 which are coded by the type I α1 collagen gene (COL1α1) and type I α2 collagen gene (COL1α2), respectively [27].

One of the processes by which LLLT exercises beneficial effects with tissue repair is the induction of the angiogenesis process [10]. The VEGF gene is related to angiogenic potential, as its action stimulates the proliferation and migration of endothelial cells and the formation of new blood vessels [11].

Cury et al. [10] investigated the effects of LLLT at 660 and 780 nm in fluxes of 30 and 40 J/cm^2 in the gene expression of three significant angiogenic mediators (VEGF, HIF-1, and MMP-2) and found that both wavelengths used were capable of altering the expression of the VEGF gene, culminating in an increase in the quantity of new blood vessels.

Laser therapy is commonly associated with cell stimulation by both visible and near-infrared radiation through the membrane potential or mitochondria. Mognato et al. [28] compared HeLla and TK6 cells irradiated with continuous 808-nm and pulse 905-nm laser doses of 1–60 J/cm2, obtaining better results and dose dependence at 905 nm. Pires-Oliveira et al. [29] demonstrated that irradiation at a wavelength of 904 nm increased cell proliferation at both 50 mJ/cm^2 and 6 J/cm^2 over the control group. Using the same type of pulsed laser on fibroblast cell cultures, Pereira et al. [30] also obtained better cell proliferation results with an energy density of 5 J/cm^2. These studies have shown that LLLT 904 nm, in addition to altering mitochondrial activity, also acts through the photobiomodulation of cell proliferation [29], but little is know about the effect of LLLT on gene expression.

In the present study, the effect of LLLT (904 nm) was investigated on the expression of the genes COL1α1 and VEGF in the fibroblast cells of mice (L929) with the goal of correlating alterations in gene expression to the process of tissue repair.

Materials and methods

The experiment was conducted using fibroblast cells derived from the conjunctive tissue of mice, from the L929 cell line (ATCC CCL-1 CTC), supplied by the Adolfo Lutz Institute in São Paulo, Brazil. The study received the approval of the Ethics Committee of UNOPAR, the University of North Paraná, filed under record no. 462.478.

Cell culture

The cells were cultivated in 25-cm^2 flasks (TPP, Trasadingen, Switzerland) using the MEM culture medium (Minimum Essential Medium, *Gibco™—Invitrogen Corporation, Grand Island, USA*) supplemented with 10 % Fetal Bovine Serum (Gibco®, *by Life Technologies*) and 1 % antibiotic-antimycotic (Gibco®, by Life Technologies), kept in an oven and with an atmosphere of 5 % CO_2, at 37 °C (Thermo Forma Scientific, Waltham, MA). The experiment was performed in accordance with the recommendations advocated by ISO 10993-5, for the use of cell cultures in in vitro toxicity testing.

Laser irradiation

Cell irradiation was performed using a KLD® Gallium-Arsenide (AsGa) diode laser (Biosistemas Equipamentos Eletrônicos Ltda., Brazil), with a wavelength of 904 nm, repetition rate of 10 KHz, output power of 50 mW, pulse width of 100 ns, peak power of 50 W, beam area of 0.01 cm^2, and active cycle of 0.1 %.

The cells were irradiated 24 h after seeding, for two consecutive days, once every 24 h, on 24-well culture plates with a diameter of 18 mm. For the seeding, 1 mL of cells was used in a concentration of 1×10^6 cells/mL.

The action of the laser's biomodulation was evaluated in three experimental groups: G1—control group, which received no irradiation; G2—irradiated at 2 J/cm^2; and G3—irradiated at 3 J/cm^2. The irradiation was performed shielded from the light in a darkened room, with the laser pointer tip in direct contact with the plate. Each well was divided into two quadrants. For G2, the irradiation took place for a total of 24 s, 12 s for each quadrant, while for G3 it was carried out for 36 s, i.e., 18 s per quadrant. The experiment was conducted in triplicate.

Extraction of RNA

The total RNA was extracted using the *High Pure RNA Isolation* kit (Roche® Roche Applied Science), in accordance with manufacturer's instructions. After extraction, the RNA was quantified with the aid of a Qubit 2.0® Fluorometer (Life Technologies). The RNA extraction was carried out 24 h after the final irradiation, followed by the RT-PCR reaction.

RT-PCR

The synthesis of the complementary DNA (cDNA) was performed using 5 μg of total RNA and the Superscript III reverse transcriptase kit (Invitrogen, Life Technologies) used in accordance with manufacturer's instructions.

The real-time polymerase chain reaction (PCR) was carried out in a *StepOnePlus* thermocycler (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, followed by a melting curve analysis under the following conditions: 95 °C for 15 s, 60 °C for 60 s, and finally, 95 °C for 15 s. The final 15 μL reaction contained 7.5 μL of MasterMix SYBR Green PCR (2×) (Applied Biosystems), 0.75 μL of each primer (100 M) (Table 1), 3 μL DNase- and RNase-free water, and 3 μL of cDNA from the sample previously diluted 10 times. The relative expression of the genes was calculated using the reference gene *β-Actin*, normalized by the gene expression in relation to the control group. The sequence of primers used is specified in Table 1.

Statistical analysis

The data were analyzed in accordance with the Pfaffl method [31] and were calculated with the aid of the Reset 2009 software application [32], taking into consideration standard deviation values and the "pair wise fixed reallocation" randomization test, assuming a significance of $p<0.05$.

Results

The analysis of the results showed a statistically significant difference in G2 (2 J/cm^2) in relation to the levels of COL1α1 messenger RNA (mRNA) expression after irradiation by laser at 904 nm, when compared to G1 (control group). The irradiated cells showed a 1.78× increase in mRNA expression of the COL1α1 gene ($p=0.036$). There were no differences between the control group and G3 ($p=0.138$) in the expression of this gene, indicating that gene expression was not affected by LLLT (Fig. 1).

In the expression of the VEGF gene, a statistically significant difference was observed in the two treated groups compared with the control group. An increase in expression of 2.054 ($p=0.037$) was found in G2 while in G3, the increase in mRNA levels was 2.562 (Fig. 2). No significant differences were observed between G2 and G3 ($p>0.05$).

Table 1 Primer sequences used to analyze the gene expression of irradiated cells

Gene	Primer sequences
COL1α1	F: 5′-ACATGCCGCGACCTCAAGAT-3′
	R: 5′-ATGTCTAGTCCGAATTCCTG-3′
VEGF	F: 5′-TTCTTTTGAGCGATCATCCCGGTCC-3′
	R: 5′-TTGCAGCAACTCCTCCAAACT-3′
β-Actin	F: 5′-AAGTCCCTCACCCTCCCAAAAG-3′
	R: 5′-AAGCAATGCTGCACCTTCCC-3′

Discussion

LLLT is a therapeutic approach with general clinical applications, particularly in the treatment of inflammatory diseases or for healing wounds [10]. The application of LLLT in these treatments is complicated by the diversity of the parameters to be used [33]. Therefore, an adaptation of the parameters that favor and modulate the production of proteins related to the process of tissue rehabilitation will permit an increase in LLLT efficiency in clinical treatments.

In the present study, the laser irradiation (λ 904 nm) of a culture of fibroblast cells of mice promoted an increase in the gene expression of COL1α1 with a dose of 2 J/cm^2. In a study conducted by Frozanfar et al. [9], investigating the influence of LLLT (λ 810 nm) on the expression of the COL1 gene, after three laser applications at 4 J/cm^2 in a culture of human gingival fibroblast, it was found that multiple applications of laser irradiation had a stimulatory effect on the levels of COL1 mRNA.

The irradiation by Chen et al. [1] of fibroblast cells derived from the Achilles tendon of pigs, using LLLT (λ 820 and 635 nm) in doses of 1, 2, and 3 J/cm^2, gave rise to an increase in the expression of mRNA of the COL1 gene 24 h after a single exposure to irradiation with all doses employed.

Basso et al. [8] evaluated the effect of LLLT (λ 780 nm) on the cells of human keratinocytes using different doses after 3 laser applications at intervals of 24 h between exposure and observed an increase in the expression of the mRNA of the COL1 gene in the groups irradiated at 0.5, 1.5, 3, and 5 J/cm^2.

Our results confirm the data found in the cell cultures of human fibroblasts, those of pigs, and in human keratinocytes, since after two laser applications (λ 904 nm) at a dose of 2 J/cm^2 in the fibroblast cells of mice, a biomodulation effect increasing the expression of the COL1α1 gene was observed.

In addition, we observed no difference in the expression of the COL1α1 gene for a dose of 3 J/cm^2. This result suggests an inhibitory effect on the expression of this gene given the dose used. Hakki and Bozkurt [34] showed that irradiation of human gingival fibroblast at a wavelength of 904 nm (6 J/cm2) significantly reduced the expression of collagen type I in

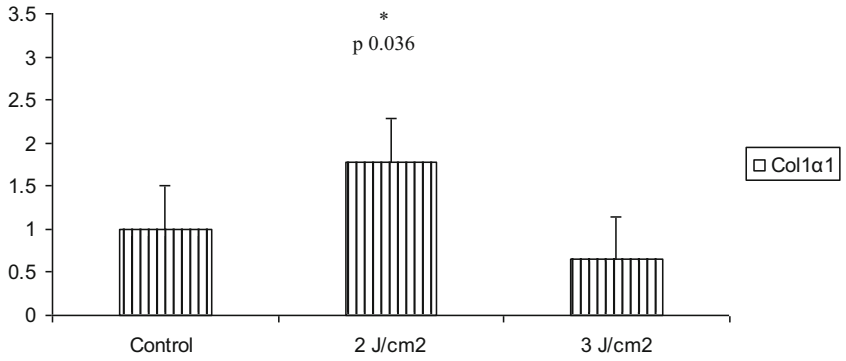

Fig. 1 Relative expression of the COL1α1 gene

the periodontal pocket application when compared to the non-irradiated control, biostimulation, and infected pocket groups. Moreover, studies by Houred et al. [35] confirmed a down-regulation of ECM components, especially COL1α1, COL1α2, COL3α1, COL5α1, and COL5α2 in response to irradiation of WS1 cell as 660 nm and diode laser at a fluence of 5 J/cm^2.

The results demonstrated a biomodulation effect on the expression of the VEGF gene, both for the dose of 2 J/cm^2 dose and that of 3 J/cm^2. These data agree with the findings of Cury et al. [10] and Basso et al. [8], suggesting that this gene has a relationship with the process of neovascularization after LLLT.

Cury et al. [10] irradiated animals with LLLT (λ 660 and 780 nm) using doses of 30 and 40 J/cm^2, for 4 consecutive days following surgery, and the results demonstrated a significant increase in the expression of the VEGF gene in all of the groups evaluated. Basso et al. [8] found an increase in the expression of the VEGF gene in human keratinocyte cells irradiated with LLLT (λ 780 nm) in different doses (0.5, 1.5, 3, 5, and 7 J/cm^2).

Divergent results were found by Rodrigues et al. [12], who demonstrated a reduction in the expression of mRNA in the VEGF gene in the experimental groups irradiated with LLLT (λ 660 nm) with a dose of 10 and 50 J/cm^2, 7 days after surgery. However, on day 14, an increase was found in the expression of mRNA in the VEGF gene in both fluxes, compared with the control group, and on day 21, an increase in the expression of this gene was only detected for the group irradiated at 50 J/cm^2. The authors emphasized that the lack of agreement in relation to the values found in the first evaluation could be related to the inflammatory process suffered by the animals.

Moreover, other studies did not find a statistically significant difference between the control group and the groups irradiated by laser, in relation to the expression of the VEGF gene [6, 36].

On irradiating fibroblast cells with LLLT (904 nm), Pires-Oliveira et al. [29] found that the use of this therapy leads to intense mitochondrial activity, since LLLT modulates biochemical processes related to mitochondrial cell respiration and the synthesis of ATP in the area of the lesion, increasing the expression of genes and stimulating the recruitment of cells, resulting in an accelerated repair process. Previous studies demonstrated that LLLT has a biomodulation effect on the expression of collagen which is important for migration and cell adhesion in the area of the lesion, stimulating the metabolism of the cells of the connective tissue [37], and moreover, LLLT could improve healing through the increase in angiogenesis [10].

Several authors have reported in clinical studies on the benefits of LLLT in tissue repair, but others reported no such effects [38, 39]. The conflicting data in the literature are the result of variations in the treatment factors and limitations with the experiments [5]. Moreover, the mechanisms of cell and molecular action are not clear. Accordingly, the performance

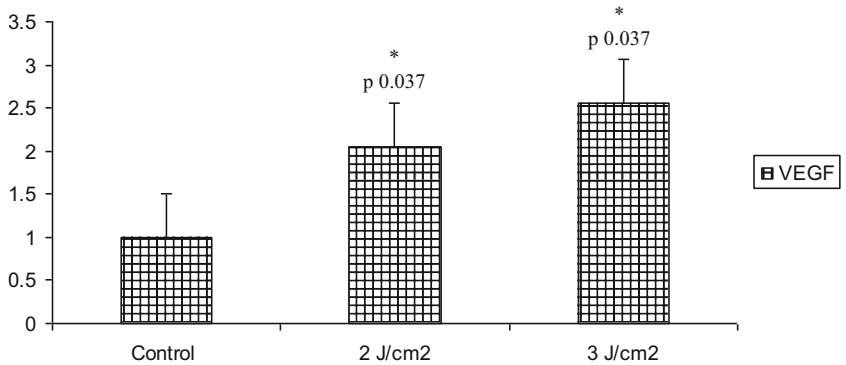

Fig. 2 Relative expression of the VEGF gene

of further studies is justified in order to clarify the effects of the different energy densities and wavelengths on the growth of fibroblast cells and, consequently, on tissue repair.

The result of the present study revealed the occurrence of a significant increase in the expression of the COL1α1 and VEGF genes, which are intimately related to tissue repair events, participating directly in the remodeling of tissue and neovascularization, justifying the use of LLLT in clinical practice.

Conclusion

Based on the results of the present study, it has been shown that LLLT within the parameters presented is capable of stimulating the expression of the COL1α1 and VEGF genes in a culture of the fibroblast cells of mice. Those cells irradiated with 2 J/cm^2 showed an increase in gene expression of both the COL1α1 gene and the VEGF gene, while those irradiated at 3 J/cm^2 only stimulated the gene expression of the mRNA of the VEGF gene. Accordingly, it may be concluded that the 2 J/cm^2 dose is more effective than the 3 J/cm^2 dose for the stimulation of tissue repair, as it produces a biomodulation effect, stimulating the expression of both COL1α1 and VEGF.

Conflict of interest No competing financial interests exist.

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