

Proliferation, Migration, and Expression of Oral-Mucosal-Healing-Related Genes by Oral Fibroblasts Receiving Low-Level Laser Therapy After Inflammatory Cytokines Challenge

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Background and Objectives: Increased expression of inflammatory cytokines in the oral cavity has been related to the etiopathogenesis of oral mucositis and to delayed oral mucosal repair. Low-level laser therapy (LLLT) stimulates proliferation and migration of gingival fibroblasts, but the effects of specific inflammatory cytokines on oral mucosal cells and the modulation of these effects by LLLT have not been fully investigated. Therefore, this study investigated the effects of LLLT on oral fibroblasts after being challenged by oral-mucositis-related inflammatory cytokines.

Methods: Human gingival fibroblasts were seeded in plain culture medium (DMEM) containing 10% fetal bovine serum (FBS) for 24 hours. Then, cells were kept in contact with inflammatory cytokines (TNF- α , IL-1 β , IL-6, and IL-8) in serum-free DMEM for 24 hours. After this period, cells were subjected to LLLT with a diode laser device (LaserTABLE, InGaAsP, 780 nm, 25 mW) delivering energy doses from 0.5 to 3 J/cm². Irradiation was repeated for 3 consecutive days. Twenty-four hours after the last irradiation, cell migration (wound-healing and transwell migration assays), cell proliferation (BrdU), gene expression of COL-I and growth factors (real-time PCR), and synthesis of COL-I (Sirius Red assay) and VEGF (ELISA) were assessed. Data were subjected to two-way ANOVA and Tukey's tests or Kruskal–Wallis and Mann–Whitney tests ($P < 0.05$).

Results: The inflammatory cytokines decreased the migration capacity of gingival fibroblasts. However, a statistically significant difference was observed only for IL-6, detected by transwell assay, where 30% less cells migrated through the pores ($P < 0.05$) and IL-8, with an increased wound area (116%; $P < 0.05$), detected by the wound healing method. Cell proliferation was not affected by contact with cytokines, while growth factors and COL-I expression (approximately 80%; $P < 0.05$), as well as VEGF synthesis (approximately 20%; $P < 0.05$), were decreased after contact to all tested cytokines. The opposite was seen for total collagen synthesis. LLLT promoted an acceleration of fibroblast migration (30%; $P < 0.05$) and proliferation (112%; $P < 0.05$) when delivering 0.5 J/cm² to the cells previously in contact with the inflammatory cytokines.

Gene expression of VEGF (approximately 30%; $P < 0.05$), and EGF (17%; $P < 0.05$), was stimulated by LLLT after contact with TNF- α and IL-6.

Conclusion: LLLT can counteract the negative effects of high concentrations of inflammatory cytokines, especially IL-6 and IL-8 on gingival fibroblast functions directly related to the wound-healing process. *Lasers Surg. Med.* 48:1006–1014, 2016. © 2016 Wiley Periodicals, Inc.

Key words: cell proliferation; gene expression; mucositis; wound healing

INTRODUCTION

The increased expression of specific inflammatory cytokines, such as TNF- α , IL-1 β , IL-6, and IL-8 in the oral cavity plays an important role in the etiopathogenesis of oral mucositis [1–3] and delayed oral mucosal healing [4,5]. Such overexpression may lead to cytopathic effects on oral mucosal cells, such as decreased proliferation and migration, and decreased expression of growth factors and other molecules related to oral mucosal homeostasis and healing [6–8]. It has also been demonstrated that the increased expression of inflammatory cytokines in response to LPS stimulus decreases the viability and proliferation of cultured cells [4,5]. However, the direct effects of inflammatory cytokines related to the

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development and severity of oral mucositis have not been completely elucidated.

Low-level laser therapy (LLLT) can promote biostimulatory effects on different cell types, including oral mucosal cells, such as increased cell migration and proliferation, as well as increased expression of growth factors and the activation of cellular pathways involved in cell proliferation and survival [9–12]. In addition, LLLT can also biomodulate the expression of inflammatory cytokines by gingival fibroblasts [13] and has been an effective therapy for several oral conditions, including infectious lesions, like herpetic manifestations or ulcerative disorders. Among the ulcerative disorders, LLLT has been used to treat aphthous lesions, prosthetic mucositis, oral graft-versus-host disease, and antineoplastic-related oral mucositis, to improve and accelerate tissue healing [14–16].

It has been demonstrated that the use of LLLT is clinically effective in oral mucositis patients [14,16] and there are already guidelines proposed by the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO) [17]. However, the cellular and molecular events triggered by this therapy were not completely elucidated, especially about the modulation of events that may be related to the incidence and severity of oral mucositis. It is important to know how LLLT modulates cell proliferation and migration, as well as the expression of growth factors and other proteins related to oral mucosal healing. That knowledge might help improve the benefits of this therapy. Therefore, this study evaluated the effects of LLLT on human gingival fibroblasts previously exposed to mucositis-related inflammatory cytokines. Cellular and molecular events related to oral mucosal healing capacity, such as cell migration and proliferation and the expression and synthesis of growth factors and collagen type I, were investigated.

MATERIALS AND METHODS

Cell Culture

Gingival fibroblasts were obtained from tissue biopsies performed in three healthy patients during a tooth extraction procedure (mean age = 22 years). We obtained signed consent forms, which were previously approved by the Ethics Committee of Araraquara School of Dentistry—UNESP (#14342113.7.0000.5416).

The gingival tissues were immediately immersed in culture medium containing antibiotic/antimycotic solution (Gibco, Carlsbad, CA) and taken to the Experimental Pathology and Biomaterials Laboratory, where, the fibroblasts were isolated by enzymatic digestion. Briefly, the gingival tissues were placed in a cell culture plate containing serum-free culture medium (Dulbecco's Modified Eagle Medium—DMEM, Gibco) and 3 mg/ml of collagenase type I. Then, they were incubated at 37°C in a humidified incubator at 5% CO₂ for 24 hours. After that, the culture medium containing tissue fractions and detached cells was transferred to a tube and centrifuged at 4,000g for 2 minutes. Cells were resuspended in culture medium and transferred to a cell culture flask containing

DMEM supplemented with 10% fetal bovine serum (FBS, Gibco). Cells were incubated at 37°C and 5% CO₂, and culture medium was replaced every 72 hours. Subcultures were performed with 0.25% trypsin (Gibco) until the necessary cell number was acquired.

Gingival fibroblasts from passages three to five were used for this study. Cells were subjected to culture protocols that were standardized according to each assay. Cells were seeded at different densities (4×10^4 cells/cm² for cell migration, gene expression, and protein synthesis; and 1.5×10^4 /cm² for cell proliferation) in 24-well plates in complete culture medium (DMEM) containing 1% antibiotic/antimycotic solution (penicillin and fungizone, Gibco) and 10% FBS, for 24 hours.

Inflammatory Cytokines

After a 24-hour incubation, the culture medium was replaced by serum-free culture medium containing pre-established concentrations of the selected cytokines: tumor necrosis factor alpha (TNF- α , 100 ng/ml), interleukin 1-beta (IL-1 β , 1 ng/ml), interleukin 6 (IL-6, 10 ng/ml), and interleukin 8 (IL-8, 10 ng/ml) (Sigma-Aldrich, St. Louis, MO). These concentrations were selected according to a pilot study that evaluated the synthesis of nitric oxide (NO) by gingival fibroblasts treated with these cytokines. Inflammatory cytokines were maintained in contact with cultured cells for 24 hours.

Nitric Oxide (NO) Synthesis

To determine the concentration of each cytokine that would better simulate a local inflammatory reaction, we first investigated the synthesis of nitric oxide by fibroblasts. The ideal concentration was considered to be the smallest concentration able to stimulate the synthesis of this important inflammatory mediator [18,19].

This method is based on nitrite accumulation as a result of the Griess reagent diazotization [20]. Cells were seeded in 96-well plates ($n = 6$), at 1×10^4 cells/well in plain DMEM containing 10% FBS, for 24 hours. After this period, cells were challenged with the cytokines at 0, 10, 100, or 1000 ng/ml in serum-free DMEM and were incubated for an additional 24 hours. Then, a 50- μ l aliquot of DMEM was collected and was added to a new plate containing 50 μ l of Griess Reagent (Sigma-Aldrich); samples were incubated for 10 minutes in light privation. Nitric oxide production was determined by absorbance at 450 nm in a spectrophotometer (Synergy H1, Biotek, Winooski, VT).

In Vitro Low-Level Laser Therapy (LLLT)

For *in vitro* LLLT, the culture medium was replaced one more time for serum-free culture medium, and cells were subjected to irradiation by means of a laser device (LaserTABLE, InGaAsP, 780 nm, 25 mW) composed of 12 laser diodes, delivering energy doses of 0 (control), 0.5, 1.5, or 3 J/cm², corresponding to 40, 120, and 240 seconds, respectively. Each diode is positioned in a way that each well of the cell culture plate is individually irradiated

(2 cm²), in a distance of 2.5 cm. These parameters were selected according to previously published results [11–13,21]. The energy dose that demonstrated the better effect on the stimulation of gingival fibroblast migration, detected by the wound-healing assay, was selected for the further protocols. Cells were irradiated once a day, for 3 consecutive days (24-hour interval).

Cell Migration Assessment by Wound-Healing Assay

The wound-healing assay is a well-established and simple method that evaluates the migration capacity of cultured cells after the induction of an *in vitro* injury [22]. For this test, gingival fibroblasts were cultured in 24-well plates at 4×10^4 cells/cm² ($n = 9$) for 24 hours. After this period, we created an *in vitro* wound by scratching the monolayer formed by the attached cells using a 5 ml pipette tip. Then, cells were treated as previously described, and after contact with the inflammatory cytokines and LLLT, cells were subjected to fixation in 70% ethanol for 1 hour at 4°C and stained with 0.1% crystal violet (Sigma–Aldrich) in deionized water, for 15 minutes. After being stained, cells were washed with deionized water twice, and wound areas were analyzed in an optical microscope (Olympus BX51, Olympus, Miami, FL) equipped with a digital camera (Olympus C5060). The total area of the wound (mm²) was measured by means of ImageJ Software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Cell Migration by Transwell Assay

The influence of the inflammatory cytokines and LLLT on migration of gingival fibroblasts was also assessed by the transwell method. For this specific protocol, cells were seeded (4×10^4 cells/cm²) in 8- μ m-micropore transwell inserts placed in a 24-well plate containing 1 ml of complete culture medium (DMEM). After 24 hours, cells were subjected to the treatment protocols previously described.

Immediately after the third irradiation, samples were fixed in 70% ethanol for 1 hour at 4°C and stained with 0.1% crystal violet for 15 minutes, then rinsed. The transwell inserts were then analyzed with an optical microscope (Olympus BX51) equipped with a digital camera (Olympus C5060) to determine the number of cells that migrated through and reached the other side of the insert. Cell number was determined by means of ImageJ Software.

Cell Proliferation

Cell proliferation rates were detected by fluorescence analysis with a BrdU analogue kit (Click-iT EdU, Life-Technologies, Carlsbad, CA) [21]. For this protocol, cells were seeded at 1.5×10^4 cells/cm² in 24-well plates and subjected to the same cell culture conditions and treatment described previously. After the final irradiation, a 100- μ l quantity of the EdU solution (10 μ M) was added to the culture medium in contact with the cultured cells. Since EdU molecules are lipophilic, their permeabilization through the cell membrane was easily achieved. Once

inside the cells, this nitrogen base analogue was incorporated into the DNA of replicating cells. Twenty-four hours after that, cells were fixed in 4% formalin for an overnight period at 4°C and permeabilized with 0.1% triton- \times 100 (Sigma–Aldrich) for 15 minutes, to allow for the internalization of the fluorescent probe in the gingival fibroblasts. EdU molecules attached to the double-stranded DNA sequences of the newly proliferated cells can be recognized. Cell membranes were then immuno-stained with HCAM (homing cell adhesion molecule—CD44) antibody to allow for observation of cell limits, in addition to nuclei stained with Hoescht fluorescent dye. Cells were analyzed by InCell Analyzer cell counter (GE Healthcare Life Sciences, Uppsalla, Sweden) for images acquisition, according to each dye, and images were subsequently analyzed with ImageJ Software for quantitative analysis.

Gene Expression of Growth Factors and Collagen Type I

Gene expression of vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF/FGF2), and collagen type I (COL-I) was analyzed by Real-Time PCR (qPCR). Cells were seeded at 4×10^4 cells/cm² and treated as previously described. After treatments, molecular contents were collected in a lysis solution (RNAqueous Micro Kit, Ambion, Carlsbad, CA) and stored at –80°C until analysis. Total RNA was isolated by sequential filtrations with the RNAqueous Micro Kit (Ambion) and was quantified by means of the Take 3 device in a spectrophotometer (Synergy 1, Biotek). One microgram of each sample was used for cDNA synthesis in a thermo cyclator (BioRad, Hercules, CA), with the High Capacity cDNA Reverse Transcriptase Kit, following the manufacturer's instructions for concentrations (2 μ l of $10 \times$ buffer, 0.8 μ l of $25 \times$ dNTP mix, 2 μ l of $10 \times$ random primers, 1 μ l of Multiscribe Reverse Transcriptase, and 4.2 μ l of ultrapure water per reaction) and cycling (25°C—10 min, 37°C—120 min, 85°C—5 min, and 4°C until storage). Then, for qPCR, specific Taqman Assays were selected for each target gene (Table 1), and reactions were prepared with standard reagents (Taqman Fast Master Mix). mRNA concentrations were determined by the standard curve method, and expression of each target gene was analyzed according to an endogenous control (β actin).

Total Collagen Synthesis

Synthesis and release of total collagen by the cultured fibroblasts seeded at 4×10^4 cells/cm² and treated as described were measured in the culture medium by the Sirius Red Dye method [23]. Briefly, a 500- μ l quantity of the culture medium kept in contact with the cultured cells was collected and treated with 500 μ l of Sirius Red Dye (Sigma–Aldrich) (0.5 M in picric-acid-saturated solution) for 60 minutes under agitation (400 rpm, room temperature) (Thermomixer—Eppendorf, Hamburg, Germany). Then, samples were centrifuged at 12,000 rcf for 10 minutes at 4°C (Microcentrifugal 5415R, Eppendorf),

TABLE 1. Primer Sequences Selected For Gene Expression Analysis

Target gene	Primer sequences
h β Actin	4333762T (Taqman Assay)
hVEGF	Forward—5'-GCACCCATGGCAGAAGG-3' Reverse—5'-CTCGATTGGATGGCAGTAGCT-3' Probe—5'-ACGAAGTGGTGAAGTTCATGGATGTCTATCA-3'
hFGF2	Forward—5'-ACCCCGACGGCCGA-3' Reverse—5'-TCTTCTGCTTGAAGTTGTAGCTTGA-3' Probe—5'-TCCGGGAGAAGAGCGACCCTCAC-3'
hEGF	Forward—5'-CTT TGC CTT GCT CTG TCA CAG T-3' Reverse—5'-AAT ACC TGA CAC CCT TAT GAC AAA TTT-3' Probe—5'-AAG TCA GCC AGA GCA GGG CTG TTA AAC TCT-3'
hCOL	Forward—5'-CAGCCGCTTCACCTACAGC-3' Reverse—5'-TTTTGTATTCAATCACTGTCTTGCC-3' Probe—5'-CCGGTGTGACTCGTGCAGCCATC-3'

allowing the collagen molecules attached to the dye to precipitate, forming a pellet. The supernatant solution was discarded, and the pellet was washed in sodium chloride (0.1M), followed by centrifugation (12,000 rcf—10 minutes—4°C). Supernatant solution was discarded, and the pellet was diluted in sodium hydroxide (0.5M). Aliquots of 200 μ l were transferred to a 96-well plate, and total collagen concentration was determined by absorbance at 555 nm on a spectrophotometer (Synergy H1, Biotek). Collagen concentrations were calculated according to a standard curve containing pre-established concentrations of this protein.

Synthesis of Vascular Endothelial Growth Factor (VEGF)

Culture medium was collected and stored at -20°C until analysis. Then, 100- μ l aliquots were used to evaluate VEGF synthesis, which was detected by the Enzyme Linked Immuno Sorbent Assay (ELISA), in a standardized kit (Duoset human VEGF—R&D Systems, Minneapolis, MN) and following the manufacturer's instructions, as follows: 96-well ELISA plates were coated overnight with primary antibody (anti-VEGF—1 μ g/ml). Then, plates were rinsed three times with wash solution and blocked with 1% BSA solution for 1 hour. After plates were washed, 100- μ l aliquots of each sample were added to the plates and incubated for 2 hours at room temperature, followed by three additional washes and incubation with secondary antibody (100 ng/ml) for an additional 2 hours.

After that, plates were subjected to new washing, and a 100- μ l quantity of 1:40 streptavidin solution was added to each sample and incubated for 20 minutes, followed by washing, incubation with reagent solution, and stop solution. Then, the absorbance of each sample was read in a spectrophotometer at 455 nm. The concentration of this growth factor for each sample was determined according to a standard curve containing known VEGF concentrations.

Statistical Analysis

Resulted data were statistically analyzed using SPSS Software (IBM Company, New York, NY). The data from all response variables, except for nitric oxide synthesis stimulated by TNF- α , IL-6, and IL-8, were normally distributed (Shapiro-Wilk, $P > 0.05$), and groups were homoscedastic (Levene, $P > 0.05$). Therefore, data were subjected to two-way ANOVA ("cytokine" and "phototherapy"), complemented by Tukey's tests to compare the means of every two groups. Nitric oxide synthesis data were analyzed by the nonparametric tests of Kruskal-Wallis and Mann-Whitney, for pairwise comparisons. All statistical inferences were considered at the 5% level of significance. All experimental protocols were repeated in two distinct moments.

RESULTS

NO synthesis by human gingival fibroblasts in contact with different concentrations of cytokines is shown in Table 2. The lowest concentrations able to increase NO synthesis compared with the control were 100 ng/ml for TNF- α , 1 ng/ml for IL-1 β , and 10 ng/ml for IL-6 and IL-8.

More extensive wound areas were seen for cytokine-treated cells, indicating an inhibitory effect on cell migration capacity. However, only IL-8 caused a statistically significant increase of this area (Table 3 and Fig. 1). For cells treated solely with LLLT (that is, no contact with any cytokine), the energy dose of 0.5 J/cm² promoted a significant decrease of the wound area, therefore demonstrating a higher cell migration to the wound's central area. When applied after the cytokine challenge, LLLT at 0.5 J/cm² could also stimulate cell migration and decrease the wound area (Table 3 and Fig. 1).

Based on the results from the wound area assay, irradiation with the dose of 0.5 J/cm² was selected for the next assays. The migration capacity of human gingival fibroblasts challenged with inflammatory cytokines and treated with LLLT was also analyzed by the transwell method. As observed for the wound-healing assay, all

TABLE 2. Nitric Oxide (NO) Synthesis by Human Gingival Fibroblasts After 24-Hour Contact With Different Concentrations of TNF- α , IL-1 β , IL-6, or IL-8

Concentration (ng/ml)	Inflammatory cytokines			
	TNF- α ^a	IL-1 β ^b	IL-6 ^a	IL-8 ^a
0 (control)	101.4 (98.6–101.4)	100.5 \pm 2.1	101.4 (98.1–101.4)	101.4 (98.6–101.4)
1	101.4 (98.5–104.1)	113.8 \pm 5.6 ^c	103.9 (102.6–106.6)	103.9 (101.3–110.5)
10	104.2 (101.4–106.8)	113.3 \pm 6.4 ^c	116.5 (110.8–117.4) ^c	114.9 (118.8–118.8) ^c
100	116.6 (113.7–119.8) ^c	112.1 \pm 4.1 ^c	114.7 (110.8–116.3) ^c	114.2 (110.4–114.5) ^c
1000	116.2 (115.1–119.2) ^c	118.8 \pm 6.1 ^c	115.8 (114.2–116.2) ^c	114.6 (109.2–117.3) ^c

^aValues are median (percentil 25-percentil 75), $n = 6$.

^bValues are mean \pm standard deviation, $n = 6$.

^cIndicates statistical difference when compared to the control group (columns), ($P < 0.05$).

cytokines hampered the migration of fibroblasts through the transwell insert. However, this effect was statistically significant only for IL-6. LLLT at 0.5 J/cm² did not increase cell migration when compared with the control group. Conversely, for cells treated with IL-6 and IL-8, a significant increase in this cell function was detected (Table 4 and Fig. 2).

These results were confirmed by the qualitative analysis of this assay, as demonstrated by photomicrographs of the transwell inserts (Fig. 2).

Cytokine treatment did not cause any statistically significant alterations in cell numbers, showing no direct cytotoxicity to oral fibroblasts. For LLLT-treated cells, increased cell numbers were detected after irradiation of cells previously treated with IL-6 and IL-1 β (Table 4).

Cell proliferation was also not affected by the cytokine challenge. However, LLLT promoted a significant increase in solely the cell function and when applied after TNF- α and IL-6 (Table 4).

Gene expression of growth factors was negatively affected by inflammatory cytokines applied to the cultured fibroblasts. IL-6 was related to the decreased expression of all evaluated growth factors (VEGF, EGF, and FGF2), while TNF- α decreased only EGF gene expression. LLLT increased VEGF and EGF expression after TNF- α application; however, this therapy decreased

the expression of EGF and FGF2 after IL-8 treatment (Table 5).

Type I collagen gene expression was decreased after the application of all selected inflammatory cytokines, while LLLT at 0.5 J/cm² increased this expression after IL-1 β and IL-6 contact (Table 5). As observed for VEGF gene expression, the synthesis of this growth factor was not altered by the cytokine challenge, the LLLT treatment alone, or the combination of both (Table 5). Collagen synthesis by fibroblasts was greatly increased by cytokines TNF- α , IL-1 β , and IL-8, while LLLT did not affect this synthesis (Table 5).

DISCUSSION

Inflammatory mediators, such as cytokines, are directly related to the healing process, since these molecules promote cell recruitment, migration, and proliferation [24,25]. However, increased concentrations of inflammatory cytokines have also been related to wound chronification (i.e., the process by which episodic pain becomes chronic), the incidence and severity of ulcerous lesions, and delayed oral mucosal wound healing [3]. Besides these clinical and experimental observations, the cellular effects of these increased concentrations remain unclear. Therefore, high concentrations of specific cytokines, related to the development

TABLE 3. Wound Area (% of Control Group Measured in mm²) After the Contact of Human Fibroblast With Different Cytokines Followed by Irradiation With Laser (LLLT) in the Infrared Wavelength Delivering Energy Doses of 0.5, 1.5, or 3.0 J/cm²

LLLT	Inflammatory cytokines				
	None	TNF- α	IL-1 β	IL-6	IL-8
0*	100.0 \pm 26.7 ^{B a}	169.1 \pm 32.9 ^{AB}	160.1 \pm 43.0 ^{AB}	159.2 \pm 44.0 ^{AB}	216.4 \pm 60.1 ^{Aa}
0.5 J/cm ²	71.5 \pm 15.2 ^{B b}	143.4 \pm 14.9 ^A	146.4 \pm 32.1 ^A	115.5 \pm 16.9 ^A	143.6 \pm 20.0 ^{A b}
1.5 J/cm ²	74.6 \pm 6.0 ^{B ab}	170.6 \pm 29.9 ^A	183.2 \pm 15.4 ^A	163.6 \pm 28.3 ^A	173.1 \pm 36.1 ^{A ab}
3.0 J/cm ²	86.5 \pm 9.8 ^{B ab}	142.0 \pm 35.2 ^{AB}	204.3 \pm 51.1 ^A	152.0 \pm 49.2 ^{AB}	167.2 \pm 35.2 ^{A ab}

Uppercase letters allow comparisons in the rows among the inflammatory cytokines (horizontally).

Lowercase letters allow comparisons in the collums among non-irradiated and irradiated cells (vertically). Values indicated with different letters are statistically different (Tukey, $P < 0.05$).

*Control group (non-irradiated cells) = 100%. Numbers are mean \pm standard-deviation, $n = 6$.

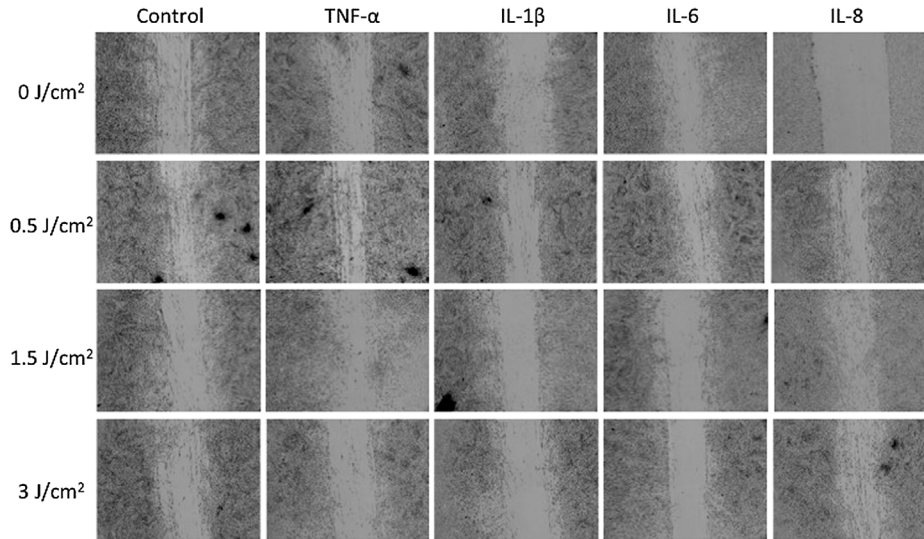


Fig. 1. Photomicrographs of *in vitro* created wounds and migration of cells to the central area of the wound after cytokines challenge and LLLT treatment (24 hours after the last irradiation). The reduction in wound area indicates higher cell migration of gingival fibroblasts.

and severity of oral mucositis [3], were applied to cultured human gingival fibroblasts to simulate cellular responses to an intense inflammatory process.

Overall, all cytokines influenced the cell functions related to the oral mucosal healing capacity. However, the most unfavorable results were seen for IL-6, which decreased both cell migration and gene expression of growth factors.

The cellular capacity of migration is crucial for the healing process [26]. However, cellular migration was the most affected cell function, followed by the expression of VEGF, EGF, and FGF2. Due to that detrimental effect, wound healing was not achieved when fibroblasts were kept in contact with any of the investigated cytokines. For the

healing process to succeed, oral mucosal cells need to migrate for the wound area to promote its closure associated with the secretion of extracellular matrix proteins, such as type I collagen [26].

The wound-healing assay is a simple and well-established method that demonstrates the migration capacity of cultured cells after the creation of an *in vitro* wound [23]. This method was used in the present study to screen for the best LLLT energy dose capable of counteracting the negative effects imposed by contact with inflammatory cytokines. The best energy dose was 0.5 J/cm²; therefore, that dose was selected for further analysis.

Transwell migration also demonstrated decreased migration capacity by gingival fibroblasts treated with

TABLE 4. Cell Migration, Number and Proliferation of Gingival Fibroblasts After Contact With Different Cytokines and Irradiation With Laser in the Infrared Wavelength Delivering Energy Doses of 0.5 J/cm² Compared to Non-Irradiated Cells

Cell event	LLLT	Cytokine				
		None*	TNF-α	IL-1β	IL-6	IL-8
Cell migration (% of control group)	0	100.0 ± 26.3 ^a	92.0 ± 7.8 ^a	85.5 ± 7.7 ^{ab}	67.1 ± 4.3 ^c	80.0 ± 8.7 ^b
	0.5 J/cm ²	117.9 ± 13.5 ^a	112.6 ± 11.6 ^a	118.6 ± 12.9 ^a	120.9 ± 17.8 ^a	131.7 ± 12.9 ^a
Cell number (% of control group)	0	100.2 ± 14.8 ^a	81.6 ± 11.2 ^a	98.0 ± 19.7 ^a	87.7 ± 17.1 ^a	87.2 ± 13.9 ^a
	0.5 J/cm ²	104.8 ± 17.1 ^b	98.0 ± 19.7 ^b	119.3 ± 5.9 ^a	123. ± 16.2 ^a	77.9 ± 15.2 ^c
Cell proliferation (% of control group)	0	100.2 ± 29.5 ^a	127.4 ± 31.8 ^a	99.1 ± 33.6 ^a	70.8 ± 21.2 ^a	70.8 ± 21.2 ^a
	0.5 J/cm ²	212.3 ± 45.0 ^a	191.1 ± 45.0 ^a	155.7 ± 72.0 ^{ab}	148.6 ± 31.8 ^b	127.4 ± 45.0 ^b

Control group (non-irradiated cells) = 100%.

Numbers are mean ± standard-deviation, n = 6.

*Indicates statistical difference between irradiated and non-irradiated groups within the same cytokine. Letters allow comparisons among cytokines in rows; means followed by the same letter do not differ statistically (Tukey, P > 0.05).

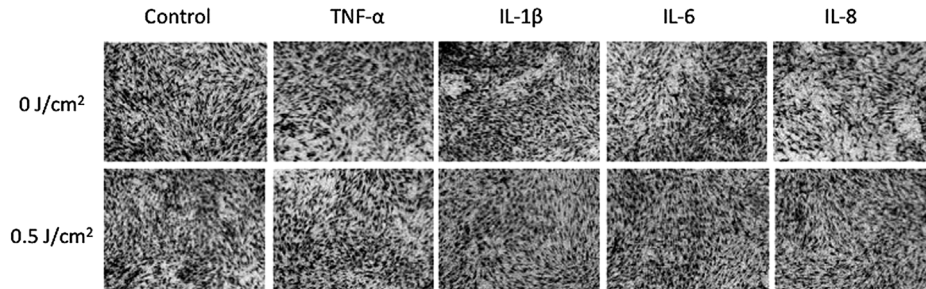


Fig. 2. Light microscopy images of gingival fibroblasts that were able to migrate through the transwell insert after cytokines challenge and LLLT delivering the energy dose of 0.5 J/cm^2 (24 hours after the last irradiation) ($\times 100$ magnification).

inflammatory cytokines. Each methodology used in this study to evaluate cell migration demands that different cellular mechanisms take place. For the wound-healing method, after the creation of the *in vitro* wound, cells need to activate a migratory phenotype and move to the central area of the wound to promote its closure. This methodology is based on the fact that cells can be sensitive to the created wound by the presence or absence of paracrine signals. In addition, for this method, wound closure can also be a result of cell proliferation [22]. Conversely, for the transwell insert, cells must present a different phenotype to be able to migrate through the insert in the presence of a stimulatory or inhibitory

factor. This migration seems to require a more efficient migratory phenotype than that required for the wound-healing method, due to the presence of a permeable barrier, forcing the cells to migrate through the pores, since for the former no obstacle is present and only the cells that could migrate through the pores can be identified [27].

All cytokines hampered cell migration to a certain level, as demonstrated by both protocols. However, negative effects were more evident for IL-6 and IL-8. If we assume that the transwell method can better predict the migratory phenotype (motility) of cells, then we can also assume that IL-6 caused the highest inhibitory effect for gingival fibroblasts.

TABLE 5. Gene Expression of VEGF, COL-I, EGF, and FGF2 and Synthesis of VEGF and Collagen by Gingival Fibroblasts After Contact With Different Cytokines and Irradiation With Laser in the Infrared Wavelength Delivering Energy Doses of 0.5 J/cm^2 Compared to Non-Irradiated Cells

Cell event	LLLT	Cytokine				
		None*	TNF- α	IL-1 β	IL-6	IL-8
VEGF gene expression (% of control group)	0	100.7 \pm 27.9 ^a	80.9 \pm 13.6 ^{ab} *	93.8 \pm 12.7 ^a *	68.0 \pm 13.4 ^b *	79.5 \pm 10.4 ^{ab}
	0.5 J/cm ²	111.4 \pm 9.3 ^b	135.2 \pm 13.9 ^a	130.6 \pm 24.1 ^{ab}	112.1 \pm 8.1 ^b	89.8 \pm 12.1 ^c
COL-I gene expression (% of control group)	0	99.9 \pm 12.6 ^a	11.6 \pm 2.8 ^c *	22.3 \pm 6.0 ^b	14.9 \pm 5.5 ^c *	11.7 \pm 3.5 ^c
	0.5 J/cm ²	96.3 \pm 6.8 ^a	28.6 \pm 5.7 ^b	31.7 \pm 11.4 ^b	32.0 \pm 12.2 ^b	11.7 \pm 1.6 ^c
EGF gene expression (% of control group)	0	100.2 \pm 13.1 ^a	72.6 \pm 9.9 ^b *	77.6 \pm 4.3 ^b	57.4 \pm 13.4 ^c	93.4 \pm 23.0 ^{ab} *
	0.5 J/cm ²	95.6 \pm 11.5 ^b	117.4 \pm 10.0 ^a	98.0 \pm 10.3 ^b	52.9 \pm 9.5 ^d	67.6 \pm 6.3 ^c
FGF2 gene expression (% of control group)	0	100.1 \pm 4.5 ^a	88.1 \pm 8.8 ^b	84.5 \pm 8.6 ^b	68.5 \pm 7.5 ^c	109.0 \pm 5.3 ^a *
	0.5 J/cm ²	108.7 \pm 7.1 ^a	104.1 \pm 5.4 ^{ab}	76.6 \pm 6.3 ^b	65.4 \pm 16.5 ^c	62.7 \pm 13.5 ^c
VEGF synthesis (% of control group)	0	100.0 \pm 41.2 ^a	79.3 \pm 11.7 ^a	89.0 \pm 11.4 ^a	95.1 \pm 9.9 ^a	65.6 \pm 22.1 ^a
	0.5 J/cm ²	106.8 \pm 29.9 ^a	78.1 \pm 13.1 ^a	100.2 \pm 8.0 ^a	102.1 \pm 24.0 ^a	66.5 \pm 19.1 ^a
COL synthesis (% of control group)	0	100.5 \pm 7.5 ^c	1972.4 \pm 719.1 ^a	2406.6 \pm 783.8 ^a	134.0 \pm 42.4 ^c	387.8 \pm 269.5 ^b
	0.5 J/cm ²	108.9 \pm 20.0 ^b	2725.5 \pm 570.2 ^a	2307.9 \pm 867.7 ^a	172.2 \pm 98.6 ^b	249.3 \pm 192.1 ^b

Numbers are mean \pm standard-deviation, $n = 6$.

*For each gene.

*Indicates statistical difference between irradiated and non-irradiated groups, within the same cytokine. Letters allow comparisons among cytokines in lines; means followed by the same letter do not differ statistically (Tukey, $P > 0.05$).

Despite decreasing the migration capacity, the application of inflammatory cytokines to cultured gingival fibroblasts did not affect cell number or cell proliferation. The influence of IL-1 β and TNF- α on cell proliferation was also previously assessed by Kato et al. (2006) [28], who demonstrated that these cytokines did not have a significant effect on the proliferation of gingival fibroblasts.

The contact of fibroblasts with the cytokines also caused a significant decrease in growth factors and collagen gene expression, especially when IL-6 was used. These results could explain the delayed wound healing observed in clinical conditions where high concentrations of these cytokines were present, such as oral mucositis and other ulcerous lesions [1–3]. During the healing process, moderate levels of inflammatory cytokines have been related to increased cell proliferation and migration, and also associated with increased synthesis of growth factors [25]. As seen in the present study, Kato et al. (2006) [28] also observed decreased gene expression of collagen type I after fibroblasts were kept in contact with high concentrations of TNF- α . That also contributes negatively to the normal healing of the oral mucosa. The results of this study confirmed that it was possible to simulate, *in vitro*, the detrimental effects produced by high concentrations of cytokines *in vivo* and demonstrated that without modulation, inflammatory cytokines can restrain cell metabolism.

Interestingly, the application of cytokines promoted an increase in collagen synthesis, while the gene expression of collagen type I was decreased. It has been demonstrated that the synthesis of collagen by oral mucosal cells is increased during inflammatory diseases, such as fibromatosis [8,28,29]. According to these studies, the presence of higher concentrations of inflammatory cytokines such as IL-1 β and TNF- α can stimulate the synthesis of this extracellular matrix protein, resulting in the formation of fibrous areas. In our study, the results seen for gene expression were specifically associated with the expression of type I collagen, which is related to the oral connective tissue healing process. However, for synthesis analysis, a total collagen production is detected, which means that all collagen types can be identified. The increased synthesis of type III collagen in similar conditions has been demonstrated by Stallmach et al. (1992) [30].

Previous studies demonstrated that LLLT promotes stimulation of different cell types, by activating several pathways and cell functions, such as cell proliferation [9,10]. However, the determination of the effects on this therapy in situations where cells are exposed to a critical condition, such as an intense inflammatory reaction, can contribute to the understanding of how LLLT may participate on the cellular and tissue biomodulation of these conditions. In this study, the LLLT at selected parameters promoted biostimulatory effects on the cytokine-challenged fibroblasts. After the 3-day irradiation protocol, an increased cell migration capacity was observed for 0.5 J/cm², in addition to increased cell proliferation, chief events in the

wound-healing process [24]. When LLLT was applied to cultured gingival fibroblasts, increased proliferation was observed, similar to what Kreisler et al. (2002) [31] observed for proliferation of gingival fibroblasts irradiated with 1.96 J/cm², following similar irradiation protocol as performed during this investigation (three irradiations with 24-hours intervals). The application of LLLT to cultured gingival fibroblasts at this energy dose was also related to an increased expression of EGF for TNF- α -treated cells.

The lack of stimulation of migratory phenotype of gingival fibroblasts after the exposure to LLLT at the other energy doses may be due to an inhibitory effect when this therapy followed the contact of the cells with the cytokines. These irradiation parameters were related to increased cell proliferation and these different cell functions are related to the activation of distinct pathways [9–12]. In addition, a previous study demonstrated that a lower energy dose was capable to biomodulate the expression of inflammatory cytokines, which in the presence of inflammatory stimulus such as in the experimental conditions of this study, may help on the stimulation of proliferative and migratory reactions of cultured cells [13].

The beneficial effects of LLLT on oral mucosal cells have been demonstrated in previous studies [11,12,30]. These effects include increased cell proliferation, migration, and viability. In the present study, the best energy dose capable of producing these effects was 0.5 J/cm², delivered to the cells by an infrared light (780 nm). These results support the fact that the biostimulatory effects of LLLT associated with the previously demonstrated biomodulatory effects [13] of this light therapy can be responsible for the positive effects observed for the LLLT-treated mucositis lesions. By enhancing the proliferation and migration of oral mucosal cells, while also decreasing the levels of the inflammatory cytokines in the oral cavity, the healing of the oral lesions can be accelerated. These effects may occur by activation of different cellular pathways, such as MAPK/ERK, that lead to the induction of cell proliferation and survival [10].

Taken together, these results demonstrated that enhanced inflammatory conditions may clinically lead to delayed wound closure, which can also increase the possibility of wound contamination. Therefore, strategies to control or reduce the local inflammatory reaction are very important to promote a faster tissue regeneration.

However, the presented results represent the effects of inflammatory cytokines and LLLT using specific concentrations and parameters on gingival fibroblasts, and should be carefully evaluated considering the limitations of an *in vitro* investigation, such as cell behavior and the highly controlled interactions of an *in vitro* microenvironment, to which these cells were subjected.

CONCLUSION

Therefore, it can be concluded that the presence of high concentrations of inflammatory cytokines is related to a reduced activity of gingival fibroblasts, which results in

delayed wound closure. In addition, LLLT was able to stimulate cell functions related to oral mucosal healing, even in the presence of high concentrations of inflammatory mediators.

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REFERENCES

- Scully C, Epstein J, Sonis S. Oral mucositis: A challenging complication of radiotherapy, chemotherapy, and radiochemotherapy: Part 1, pathogenesis and prophylaxis of mucositis. *Head Neck* 2003;25:1057–1070.
- Sonis ST. Pathobiology of oral mucositis: Novel insights and opportunities. *J Support Oncol* 2007;5:3–11.
- Meirovitz A, Kuten M, Billan S, Abdah-Bortnyak R, Sharon A, Peretz T, Sela M, Schaffer M, Barak C. Cytokines levels, severity of acute mucositis and the need of PEG tube installation during chemo-radiation of head and neck cancer—A prospective study. *Radiat Oncol* 2010;5:16–22.
- Martin P, Leibovich SJ. Inflammatory cells during wound repair: The good, the bad and the ugly. *Trends Cell Biol* 2005;15:599–607.
- Sculean A, Gruber R, Bosshardt DD. Soft tissue wound healing around teeth and dental implants. *J Clin Periodontol* 2014;41:S6–S22.
- Bartold PM, Walsh LJ, Narayanan AS. Molecular and cell biology of gingiva. *Periodontol* 2000;24:28–55.
- Werner S, Krieg T, Smola H. Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol* 2007;127:998–1008.
- Barrientos S, Stokadinovic O, Golinko MS, Brm H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Rep Reg* 2008;16:585–601.
- Gao X, Xing D. Molecular mechanisms of cell proliferation induced by low power laser irradiation. *J Biomed Sci* 2009;16:4–19.
- AlGhamdi KM, Kumar A, Moussa NA. Low-level laser therapy: A useful technique for enhancing the proliferation of various cultured cells. *Lasers Med Sci* 2012;27:237–249.
- Basso FG, Pansani TN, Turrioni APS, Bagnato VS, Hebling J, Souza Costa CA. In vitro wound healing improvement by low-level laser therapy application in cultured gingival fibroblasts. *Int J Dent* 2012;2012:719452:1–6.
- Basso FG, Oliveira CF, Kurachi C, Hebling J, de Souza Costa CA. Biostimulatory effect of low-level laser therapy on keratinocytes *in vitro*. *Lasers Med Sci* 2013;28:367–374.
- Basso FG, Pansani TN, Soares DG, Scheffel DL, Bagnato VS, de Souza Costa CA, Hebling J. Biomodulation of inflammatory cytokines related to oral mucositis by low-level laser therapy. *Photochem Photobiol* 2015;91:952–956.
- Migliorati C, Hewson I, Lalla RV, Antunes HS, Estiló CL, Hodgson B, Lopes NNF, Schubert MM, Bowen J, Elad S. Systematic review of laser and other light therapy for the management of oral mucositis in cancer patients. *Support Care Cancer* 2013;21:333–341.
- Oberoi S, Zamperlini-Netto G, Beyene J, Treister NS, Sung L. Effect of prophylactic low laser therapy on oral mucositis: A systematic review and meta-analysis. *PLoS ONE* 2014;9:e107418.
- Silva GB, Mendonca EF, Bariani C, Antunes HS, Silva MA. The prevention of induced oral mucositis with low-level laser therapy in bone marrow transplantation patients: A randomized clinical trial. *Photomed Laser Surg* 2011;29:27–31.
- Lalla RV, Bowen J, Barasch A, Elting L, Epstein J, Keefe M, McGuire DB, Migliorati C, Nicolatou-Galitis O, Peterson DE, Raber-Durlacher E, Sonis ST, Elad S. MASCC/ISOO clinical practice guidelines for the management of mucositis secondary to cancer therapy. *Cancer* 2014;120:1453–1461.
- Kendal HK, Haase HR, Li H, Xiao Y, Bartold PM. Nitric oxide synthase type-II in synthesized by human gingival tissue and cultured human gingival fibroblasts. *J Periodontol Res* 2000;35:194–200.
- Daghigh F, Borghaei RC, Thornton RD, Bee JH. Human gingival fibroblasts produce nitric oxide in response to pro-inflammatory cytokines. *J Periodontol* 2002;73:392–400.
- Bryan NS, Grisham MB. Methods to detect nitric oxide and its metabolites in biological samples. *Free Rad Biol Med* 2007;43:645–657.
- Pansani TN, Basso FG, Turrioni APS, Kurachi C, Hebling J. Effects of low-level laser therapy on the proliferation and apoptosis of gingival fibroblasts treated with zoledronic acid. *Int J Oral Maxillofac Surg* 2014;43:1030–1034.
- Wiegand Hipler U. Methods for the measurement of cell and tissue compatibility including tissue regeneration process. *GMS Krankenhhyg Interdiszip* 2008;3:1–9.
- Tullberg-Reinert H, Jundt G. In situ measurement of collagen synthesis by human bone cells with a sirius red-based colorimetric microassay: Effects of transforming growth factor beta2 and ascorbic acid 2-phosphate. *Histochem Cell Biol* 1999;112:271–276.
- Guo S, DiPietro LA. Factors affecting wound healing. *J Dent Res* 2010;89:219–229.
- Häkkinen L, Uitto VJ, Larjava H. Cell biology of gingival wound healing. *Periodontol* 2000;24:127–152.
- Entschladen F, Drell IVTL, Lang K, Masur K, Palm D, Bastian P, Niggemann B, Zaenker KS. Analysis methods of human cell migration. *Exp Cell Res* 2005;307:418–426.
- Lianf C, Park AY, Guan J. In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration *in vitro*. *Nat Protocols* 2007;2:329–333.
- Kato T, Okahashi N, Ohno T, Inaba H, Kawai S, Amano A. Effect of phenytoin on collagen accumulation by human gingival fibroblasts exposed to TNF- α *in vitro*. *Oral Diseases* 2006;12:156–162.
- Nakao K, Yoneda K, Osaki T. Enhanced cytokine production and collagen synthesis of gingival fibroblasts from patients with denture fibromatosis. *J Dent Res* 1995;74:1072–1078.
- Stallmach A, Shuppan D, Riese HH, Mathes H, Riecken EO. Increased collagen type III synthesis by fibroblasts isolated from strictures of patients with Crohn's Disease. *Gastroenterol* 1992;102:1920–1929.
- Kreisler M, Christoffers AB, Al-Haj H, Willershausen B, d'Hoedt B. Low level 809-nm diode laser-induced *in vitro* stimulation of the proliferation of human gingival fibroblasts. *Lasers Surg Med* 2002;30:365–369.

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