Dosage Effects of an 810 nm Diode Laser on the Proliferation and Growth Factor Expression of Human Gingival Fibroblasts

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Abstract

Introduction: A substantial amount of evidence supports the positive effect of photobiomodulation on the proliferation and differentiation of various cell types. Several laser wavelengths have been used for wound healing improvement, and their actual outcome depends on the settings utilized during irradiation. However, the heterogeneous wavelengths and laser settings applied in the existing literature make it difficult to draw solid conclusions and comparison of different studies. The aim of the present study is to evaluate and compare the effects of various doses of laser energy, provided by an 810 nm diode, on human gingival fibroblasts in terms of proliferation and expression of growth factors with a pivotal role in wound healing.

Methods: Human gingival fibroblasts were cultured on plastic tissue culture and irradiated with 2, 4, 6 or 12 J/cm². The effects of the low-level laser therapy (LLLT) using an 810 nm diode laser on growth factor expression (EGF, TGF and VEGF) were evaluated by qPCR at 72 hours and 7 days after irradiation. Cell proliferation was evaluated at 24, 48 and 72 hours after LLLT using MTT assay.

Results: Energy density of 12 J/cm² provoked irradiated gingival fibroblasts to demonstrate significantly higher proliferation as well as higher gene expression of Col1, VEGF and EGF. LLLT positive effects were obvious up to 7 days post-irradiation.

Conclusion: LLLT with 810 nm presents beneficial effects on proliferation, collagen production and growth factor expression in human gingival fibroblast cells. The application of 12 J/cm² can be suggested as the optimal energy density for the enhancement of the wound healing process.

Keywords: Photobiomodulation; Low-level laser therapy (LLLT); Gingival fibroblasts; Growth factors, Laser doses.

Introduction

Fibroblasts are the most abundant cell population in the connective tissue, and they play a critical role in wound healing and connective tissue turnover. They produce an extra-cellular matrix; they adhere to it and have the capability to contract simultaneously to it, through the activation of their cytoskeleton. Besides these functions, they mediate the inflammatory response; they are activated by bacterial products and by degranulating blood platelets to attract and interact with immune cells nearby to reach the mutual amplification of their response. Fibroblasts also secrete specific proteolytic enzymes which break down tissue in order to repair or regenerate damage and achieve homeostasis.¹

Gingival fibroblasts are supposedly different from other fibroblasts due to their embryonic ectomesenchymal origin and their location in the oral environment which comes with many challenges they need to compensate for during wound healing.

Lasers are mostly used for the removal of soft and hard tissues while simultaneously providing hemostasis. One of their significant benefits is the fact that they constitute a painless method, sometimes to such an extent that no local anesthesia is required. Another use is the so-called low-level laser therapy (LLLT), in which tissues are irradiated at low output power (0.2-0.5W). In this way, laser radiation stimulates them, promoting more rapid healing while also reducing inflammation and increasing the pain threshold. De Sousa et al demonstrated upregulation in the pain threshold after irradiating mice on the head or lower back to achieve analgesia on the hind foot.² The mechanism of an increase in the pain threshold is attributed to endorphin secretion, a reduction in prostaglandin E2 and cyclooxygenase-2, and an increase in prostaglandin which is a neurotransmitter for pain relief.³

The photobiologic phenomenon, where the electromagnetic radiation of a specific source interacts

with cellular components promoting certain aspects of cell function, is called photobiomodulation.^{4,5}

Like plants that photosynthesize with the sun's radiation, human cells seem to upregulate their functions with laser radiation. The exact mechanism is yet unclear, but there are speculations that the red and near infra-red wavelength spectrum stimulates mitochondria through specific chromophores. Chromophores are parts of molecules that are responsible for the hue characteristic, which means that they absorb the energy of photons from a specific wavelength. In this case, certain mitochondrial molecules absorb energy from the laser. The main target mitochondrial chromophores are cytochrome oxidase C (COX), NADPH oxidase, nitric oxide (NO), and cytochrome aa3 quinol oxidase subunit III.^{6,7}

Purpose

The aim of this in vitro study is to compare the effect of various energy densities of photobiomodulation with an 810 nm diode laser on human gingival fibroblasts in terms of proliferation and certain growth factors, known for their decisive role in the promotion of wound healing and gene expression (VEGF, EGF and TGF- β). Vascular endothelial growth factor (VEGF) promotes revascularization,⁸ epidermal growth factor (EGF) promotes wound healing by stimulating the migratory response of the fibroblasts,⁹ and TGF- β regulates many functions such as proliferation and differentiation of cells as well as wound healing, while also regulating extracellular matrix formation and angiogenesis.^{10,11}

Wound healing is characterized by a temporary matrix. Its primary components are fibrin, fibronectin and platelets, and they are subsequently degraded and participate in the synthesis of the mature collagen matrix, including fibronectin and proteoglycans.¹² Therefore, the evaluation of Col-1a gene expression provides essential information regarding the most effective energy density that will promote wound healing.

Materials and Methods

Cell Culture

Human gingival fibroblasts (hGFs) (CLS, GE) were cultured in the DMEM/F12 medium supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin solution in standard conditions of 37°C, 5% CO₂. The hGF cells were seeded in 12 multi-well plates at a density of 2×10^4 cells/well. 810 nm diode laser irradiation (FOX, A.R.C. Nürnberg, Germany) was performed 24 hours after seeding. In particular, an 810 nm diode low-level laser was used to irradiate the cultures, administering energy densities of 2, 4, 6 or 12 J/cm² (Table 1). Cell proliferation was evaluated 24 hours, 48 hours and 72 hours after the irradiation, whereas gene expression was examined 72 hours and 7 days after the irradiation. The cells seeded on tissue culture plastic surfaces without any laser exposure

served as the control samples (TCP). All the experiments were performed in triplicate in three independent runs, using the cells in the 4th passage.

RNA Extraction and cDNA Synthesis

At specified time points, RNA was isolated from the cells with an UltraClean® Tissue & Cells RNA Isolation Kit (MoBio, US) according to the manufacturer's protocol. Following isolation, the RNA was quantified with a nanodrop photometer (Shimadzu, Japan). RNA samples were preserved at -80°C until further processing. Reverse transcription was performed by using 1 µg of total RNA and ProtoScript II First-Strand cDNA synthesis kit (NEB, US) according to the manufacturer's instructions. cDNA was stored at -20°C until further analysis was performed.

qPCR Analysis

To quantify gene expression, qPCR was performed using SYBR green chemistry and specific primers for each gene. The analysis was performed in a Bio-Rad IQ5 real time PCR cycler (Bio-Rad, US). The expression was normalized to GAPDH, and the analysis was performed using the DDCT method for relative quantification. Amplification curves were obtained from each sample to calculate the cycle threshold (Ct) and melt curves to verify specific product formation (Figure 1). A list of primer sequences used for the quantification of each gene is provided in Table 2.

MTT Assay

At each experimental endpoint, the medium was removed and 200 µL of MTT (Sigma-Aldrich, Ge) solution (5 mg/mL diluted in DMEM without phenol red) and 1.8 mL of DMEM/F12 without phenol red were added to the irradiated cultures and the controls and incubated at 37°C for 4 hours. After discarding the supernatants, the dark blue formazan crystals were dissolved in 0.1N HCl in absolute isopropanol and quantified spectrophotometrically (VersaMax ELISA Microplate Reader, Biocompare, USA)

Table 1: Specific Diode Laser Settings Applied in the Study

Parameter	Value
Type of laser	Diode
Emission mode	Continuous wave
Wavelength	810 nm
Delivery system	Optical fiber 300 µm
Power	500 mW
Distance from the surface	10 mm
Spot diameter at the well	4.6 mm
Spot area at the well	0.16 cm ²
Energy density at the spot area	300 J/cm ²
Energy density at the well	2 or 4 or 6 or 12 J/cm ²

Table 2: The List of Primer Sequences Used for the Experimental Setup

Gene	Primer Sequence
EGF	F: CAACCAGTGGCTGGTGAGGA
	R: AGCCCTTACTACTGGATACTGGAA
TGFb	F: CAGCAACAATTCCTGGCGATA
	R: AAGGCGAAAGCCCTCAATCT
VEGF	F: CTGCTGTCTGGGTGCATTG
	R: AGCTGCGCTGATAGACATCC
Col-1a	F: TGCTCGTGAAATGATGGTG
	R: CCTCGCTTCTCTCTCTCC
GAPDH	F: CCTGCACCACCAACTGCTTA
	R: GGCCATCCACAGTCTTCTGAG

at 570 nm and 690 nm. The results are reported as absolute values of absorbance at 570 nm.

Statistical Analysis

Summary statistics are presented using mean values and standard deviations. Inter-group analyses were performed using ANOVA. Pairwise differences were assessed using Dunnett post-hoc tests. Inter-group differences over time were assessed via repeated measures ANOVA followed by paired post-hoc tests using the Bonferroni correction. Statistically significant differences were considered at *P* values less than 0.01, 0.001, and 0.05.

Results

Effect of Different Dosages on Cell Proliferation

The effect of photobiomodulation with different laser energy densities was evaluated at 24, 48 and 72 hours

after irradiation by using the MTT proliferation assay. The results are presented in Figure 2.

24 hours after LLLT, a statistically significant increase of cell proliferation in the group irradiated with 12 J/cm² compared to the control group was observed. Moreover, in comparison to the control group, a difference of weaker statistical significance was also evident in the 4 J/cm² group, in favor of the control group. What is interesting is that the 2 J/cm² group showed a statistically significant higher proliferation compared to the 4 J/cm² group, which also showed a significantly lower proliferation in comparison to the 6 J/cm² group. Overall, in the first 24 hours, the most effective radiation with regards to proliferation was 12 J/cm², whereas 4 J/cm² energy density failed to increase cell proliferation since it was exceeded

Figure 1: Representative Examples of Melt Curves for the COL1 Gene. Melt curve determination demonstrates a specific product formation, indicating the specificity of the qPCR reaction when using SYBR green chemistry.

Figure 2 . Effect of Different Energy Densities on Human Gingival Fibroblasts Proliferation After 24, 48 and 72 Hours. ** *P* < 0.01, *** *P* < 0.001.

by all groups, even the control group.

In the 48-hour time interval, however, the situation changed. The 12 J/cm² group showed significantly lower proliferation compared to the control group and the 6 J/cm² group. The 4 J/cm² group lagged in terms of cell proliferation and the 2 J/cm² group showed significantly higher proliferation than the 12 J/cm² group.

72 hours after irradiation, the differences in proliferation rates became clearer. Although in the 48-hour interval, the 12 J/cm² group showed significantly less proliferation than the non-irradiated group, 24 hours later, the proliferation skyrocketed, with a statistically significant increase compared to the control. The 2 J/cm² group came second, though it did not show a significant increase in proliferation in comparison with the controls. The most interesting event was that the 'long-term' outcome of 4 and 6 J/cm² irradiation probably suppressed cell proliferation, and although that does not have a statistically significant difference with the control group, the difference between those groups and the 12 J/cm² group was statistically significant.

Lastly, when comparing the proliferation of the same groups at different time intervals (Figure 2), a highly significant increase in the control group between 24 and 72 hours was noted. Similarly, all irradiated groups presented a significant increase in the cell population from 24 to 72 hours after irradiation. However, for the 6 J/cm² group, the increase between 24 and 72 hours was less

significant.

Effect of Laser Irradiation on Gene Expression

qPCR results for the expression of genes involved in wound healing processes 72 hours and 7 days after irradiation are presented in Table 3, Figure 3 and Figure 4. In Figure 3, the examined gene expression after 72 hours of irradiation can be observed. EGF showed a statistically significant overexpression for the group of 6 J/cm² versus the other irradiated groups and controls. The same pattern was observed for the TGF factor, although there were no statistically significant differences among the groups. Regarding both VEGF and Col-1a, the group that presented a statistically significant overexpression, $P < 0.001$ and $P < 0.01$ respectively, was the one irradiated with 12 J/cm².

Gene expression of growth factors 72 hours after irradiation is reported in Figure 3. It can be stated that for most irradiated groups, growth factors were expressed at higher levels in comparison with the control. These differences were statistically significant on several occasions. A statistically significant difference in the overexpression of VEGF and Col-1a was presented for the group of 12 J/cm². Moreover, EGF and TGFb were also expressed at a higher extent for the same group compared to the control; however, these differences did not reach any statistical significance.

Figure 4 demonstrates gene expression after 7 days of

Figure 3. Gene Expression Profile 72 Hours After Irradiation With Different Laser Energy Densities for (A) EGF, (B) TGFb, (C) VEGF and (D) Col-1a Genes. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Table 3. Average Values of Gene Expression 72 Hours and 7 Days After Irradiation

Genes	End Points	2 J/cm ²	4 J/cm ²	6 J/cm ²	12 J/cm ²	Control
EGF	72 h	0.49	1.41	5.46	2.75	1
	7 Days	0.49	1.41	1.84	2.93	1
TGFb	72 h	2.54	2.19	2.47	2.09	1
	7 Days	0.06	0.06	0.06	0.11	1
VEGF	72 h	3.57	0.82	2.50	6.33	1
	7 Days	0.35	0.37	0.30	0.40	1
Col-1a	72 h	0.92	0.53	2.85	5.45	1
	7 Days	1.29	2.36	1.76	11.45	1

culture post-irradiation. Except for the 4 J/cm² group, EGF expression remained higher in the irradiated groups compared to the control and these differences reached statistical significance for the 2 J/cm² and 12 J/cm² groups. Interestingly, TGFb and VEGF expression levels decreased for all the irradiated groups 7 days after irradiation. For the 12 J/cm² group, in particular, the expression of Col-1a remained also increased up to 7 days after the initial irradiation, which implies the existence of a long-term effect ($P < 0.001$).

For the analysis of the gene expression, we compared the expression of the genes of interest within the two time points of 72 hours and 7 days to observe potential alterations in expression. The results are presented in Figures 5-8.

Figure 5 shows that EGF expression decreased over time for energy densities of 4 and 6 J/cm² and increased when 2 J/cm² were used. It is obvious that the only energy density provoking a stable significant increase of EGF expression over time and up to 7 days was 12 J/cm².

Figure 6 reports the effect of the laser irradiation with different energy densities on the TGFb gene expression within the two time points. It is noteworthy that all gene expressions statistically significantly decreased ($P < 0.01$) at 7 days post-irradiation for all the applied energy densities.

VEGF gene expression at 72 hours and 7 days is reported in Figure 7. For all energy density groups, VEGF expression decreased at 7 days in comparison to 72 hours after irradiation. The highest statistical significance was

Figure 4. Gene Expression Profile 7 Days After Irradiation With Different Laser Energy Densities for (A) EGF, (B) TGFb, (C) VEGF and (D) Col-1a Genes. ** $P < 0.01$ and *** $P < 0.001$.

Figure 5. Expression of the EGF Gene in Human Gingival Fibroblasts Using LLL of Different Energy Densities (2, 4, 6 or 12 J/cm²) and in Different Time Points (72 hours and 7 days). ** $P < 0.01$ and * $P < 0.05$.

Figure 6. Expression of TGFb Gene in Gingival Fibroblasts Using LLLT of Different Energy Densities (2, 4, 6 or 12 J/cm²) and in Different Time Points (72 hours and 7 days). ** $P < 0.01$.

observed for the 12 J/cm² group, and this is explained by the overexpression of VEGF at 72 hours for this specific group. As presented in Figure 3, the expression of VEGF after irradiation with 12 J/cm² was statistically significantly higher compared to the control group.

Discussion

In this study, we investigated the effect of several laser energy densities (2, 4, 6 or 12 J/cm²) on photobiomodulation of human gingival fibroblasts in terms of proliferation and growth factor expression. To our knowledge, this is the first study to compare the effect of different laser energy densities from an 810 nm diode

on the above-mentioned functions.

From the MTT results in Figure 2, it is evident that at 24 hours after single irradiation with 12 J/cm², a significant increase in the proliferation of cells was observed. This effect was even greater at 72 hours. This is in accordance with other studies that have examined the effects of photobiomodulation on other cell types by using other wavelengths. Góralczyk et al¹³ demonstrated the same phenomenon in endothelial cells using a wavelength of 635nm. Martignago et al¹⁴ demonstrated similar effects of LLLT on mouse fibroblasts. Furthermore, the effects of photobiomodulation have been observed in most mesenchymal cell types.¹⁵⁻¹⁸ For instance, Ginani

Figure 7. Expression of the VEGF Gene in Gingival Fibroblasts Using LLLT of Different Energy Densities (2, 4, 6 or 12 J/cm²) and in Different Time Points (72 hours and 7 days). **** $P < 0.0001$.

Figure 8. Expression of the Col-1a Gene in Gingival Fibroblasts Using LLLT of Different Energy Densities (2, 4, 6 or 12 J/cm²) and in Different Time Points (72 hours and 7 days), *** $P < 0.001$ and ** $P < 0.01$.

et al¹⁵ have reported a positive impact of LLLT on the proliferation of stem cells. Our group has also published the positive influence of LLLT using Nd:YAG (1064 nm) laser on the proliferation of MG-63 osteoblasts *in vitro*.¹⁶

Frozanfar et al verified the positive effect of LLLT on the proliferation of gingival fibroblasts, utilizing an 810 nm diode, with the energy density of 4 J/cm².¹⁷ This seems not to be in accordance with the present study where we noted slight suppression of proliferation when using this specific energy density. However, it should be mentioned that Frozanfar et al irradiated cells for three consecutive days. Furthermore, they did not include but only one energy density in the experiment; thus, from their study, no conclusion can be drawn for an optimal energy density. Our results clearly concluded that the most advantageous energy density to improve the proliferation of gingival fibroblasts up to 72 hours was 12 J/cm².

Additionally, according to the findings of the present study with the 12 J/cm² energy density, there was a marked increase in the Col-1a gene expression 72 hours after LLLT, which not only remained but further increased 7 days after irradiation (Figures 3, 4 and 8).

A similar pattern is presented for the expression of EGF, which is another gene associated with cell proliferation. EGF plays a crucial role in wound healing and its receptor (EGFR) is a regulator of normal and pathological cell function. EGF has also been recently found to regulate cellular senescence through the EGFR-Ras signaling pathway, upon stimulation with certain pro-inflammatory cytokines.¹⁹

In the present study, EGF gene expression was higher in all groups at 72 hours and 7 days after irradiation (Figures 3 and 4). This is in accordance with the results demonstrated by Gkogkos et al⁵ in a model of gingival fibroblasts, with the use of Nd:YAG laser (1064 nm). Furthermore, in a review by Colaco,²⁰ which examined several studies using LLLT, it was stated that EGF was a key gene activated from laser irradiation. What is crucial about our results is that even though proliferation was not enhanced with 4 and 6 J/cm² irradiation, the EGF expression after 72 hours significantly increased for both groups. Consequently, even if these energy densities do not directly support fibroblast proliferation when LLLT is applied in a single session, they could potentially abet the healing process, which is also important.

With regard to the gene expression of TGF, our findings did not show any significant difference in 72 hours after irradiation regardless of the energy applied. Interestingly, 7 days post-irradiation, TGF β gene expression in all irradiated groups was significantly lower compared to the control group. The role of TGF β has been extensively explored in the context of wound healing and scar formation. It has been found that following injury and during scar formation, the levels of TGF β can be significantly higher.²¹ TGF β can predominantly act

through the SMAD pathway to mediate these effects.²¹ In another study, Mesquita-Ferrari et al reported lower levels of TGF β and TNF- α expression in the skeletal muscle after LLLT (660 nm).²² Furthermore, TGF β expression is linked with cell differentiation.²³ This can indicate that the irradiated cells commence their differentiation faster than the non-irradiated ones.

Our study also demonstrated that VEGF gene expression was significantly higher in most groups of irradiated cells after 72 hours of treatment (Figure 3), but this was abolished in the 7-day time interval (Figure 4). The expression of VEGF is associated with mitogenic activity in endothelial cells.²⁴ Interestingly, LLLT contributes to the inhibition of apoptosis in umbilical cord mesenchymal stem cells (MSCs) since it upregulates the expression of certain growth factors such as VEGF.²⁵ Moreover, as far as bone-marrow MSCs are concerned, LLLT at 2 or 4 J/cm² significantly increased their proliferation and differentiation into bone cells. However, when the density increased at 12 J/cm², the suppression of proliferation was noted.²⁶ This is very different from what was observed when applying LLLT to gingival fibroblasts, where according to our results, no significant differences were observed in the proliferation for energy densities of 2-6 J/cm². In addition to that, Basso et al. demonstrated the same effects using LLLT (780 nm) in keratinocytes.²⁷ Just like keratinocytes, *in vitro* radiation of fibroblasts with infrared radiation results in the optimal outcome as far as proliferation, migration and viability are concerned.⁹ Therefore, the enhancement of proliferation in combination with the reduction of inflammatory cytokines accelerates the healing process of oral lesions.²⁸ The MAPK/ERK pathway is a possible signaling pathway through which the increase and survival of the cell population are induced.²⁹

In modern literature, a plethora of studies supporting LLLT as an enhancing factor for wound healing can be found. Ustaoglu et al studied the effect of LLLT on donor sites of free gingival grafts. Significantly higher prevalence of full epithelization, as well as a reduction in postoperative bleeding, was noted after just 24 hours. Additionally, the mean value of tissue width increased in the laser group, which was not the case for the control group that was not irradiated.³⁰ In their systematic review, Al-Shibani concluded in favor of the beneficial effect of LLLT on the healing of a donor site after graft harvesting despite the need for further studies.³¹

The use of LLLT in clinical practice must be highlighted for its benefits as an adjunct means of several oral surgical interventions, that promotes rapid and uneventful healing of the soft and hard tissues. The combination of anti-inflammatory properties of LLLT and its positive impact on cell proliferation, differentiation and growth factor expression makes LLLT a very powerful tool in the treatment of periodontitis and peri-implant pathology.

Conclusion

Availing of an 810 nm diode laser enhances the proliferation of human gingival fibroblasts in a dose-dependent manner in vitro. It is remarkable that the energy density of 12 J/cm² significantly increases cell proliferation up to 72 hours after irradiation. Moreover, this energy density provoked the most pronounced EGF levels after 72 hours and 7 days post-irradiation. Furthermore, a similar elevation was also noted for the expression of Col-1a. Therefore, the energy density of 12 J/cm² should be preferred since it contributes to the increase of cell population and enhancement of the wound healing process through collagen synthesis and extracellular matrix deposition.

Additional studies are required to verify these results and to determine the optimal combination of settings that improve wound healing when other wavelengths are used.

Conflict of Interests

The authors declare that they have no competing financial interests or personal relationships that could have influenced the work reported in this paper.

Ethical Considerations

Not applicable.

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