Lasers in Medical Science (2022) 37:573–580
https://doi.org/10.1007/s10103-021-03299-w

ORIGINAL ARTICLE

Photobiomodulation using LLLT and LED of cells involved in osseointegration and peri-implant soft tissue healing

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Received: 11 September 2020 / Accepted: 22 March 2021 / Published online: 12 April 2021

Abstract

This study evaluated the influence of photobiomodulation (PBM) using low-level laser therapy (PBM/LLLT) or light-emitting diode (PBM/LED) therapy on peri-implant tissue healing. A laboratory model was used to assess the adhesion and metabolism of osteoblasts (SaOs-2), human gingival fibroblasts (HGF), and normal oral keratinocytes (NOK) seeded on a titanium (Ti) surface. After seeding the cells on disks of Ti placed in wells of 24-well plates, three irradiations were performed every 24 h at energy density of 3 J/cm^2. For PBM/LLLT, a LaserTABLE device was used with a wavelength of 780 nm and 25 mW, while for PBM/LED irradiation, a LEDTABLE device was used at 810 nm, 20 mW, at a density of 3 J/cm^2. After irradiations, the number of cells (NC) attached and spread on the Ti surface, cell viability (CV), total protein (TP), and collagen (Col) synthesis were assessed. Alkaline phosphate activity (ALP) was evaluated only for SaOs-2. Data were submitted to ANOVA complemented by Turkey statistical tests at a 5% significance level. PBM significantly increased adherence of NOK to the Ti surface, while no significant effect was observed for SaOs-2 and HGF. PBM positively affected CV, as well as Col and TP synthesis, in distinct patterns according to the cell line. Increased ALP activity was observed only in those cells exposed to PBM/LLLT. Considering cell specificity, this investigation reports that photobiomodulation with low-power laser and LED at determined parameters enhances cellular functions related to peri-implant tissue healing in a laboratory model.

Keywords Cell culture · Implantology · Light-emitting diodes · Low-level laser therapy · Titanium

Introduction

Photobiomodulation (PBM) using low-level laser therapy (LLLT) and light-emitting diodes (LED) has gained attention for treatment of several human conditions [1]. This therapeutic protocol is based on the absorption of light within a specific wavelength spectrum by organic molecules known as photoacceptors, which convert this luminous energy into biochemical effects such as increasing ATP synthesis, cell metabolism, and gene expression of proteins [2]. Mitochondrial enzymes, such as cytochrome C oxidase and other protein complexes involved in electron transport chain, resemble the major class of these components and are mainly stimulated by red and near-infrared light; therefore, this limited light spectrum has successfully been used to test the effectiveness of photobiomodulation in cells and tissues [1, 3–5].

Several studies demonstrate that PBM using low-level laser or LED devices applied to cells and tissues enhances cell migration and proliferation, as well as the expression of genes and proteins related to down-modulation of inflammatory response and tissue healing [6–10]. However, literature still lacks scientific data concerning the ideal PBM parameters for modulating oral tissues and cells.

For oral implantology, a long-term successful clinical outcome is achieved by two factors. The first is the osseointegration of the implant; this is where bone cells adhere to the implant's titanium surface and synthesize a collagen-rich matrix, which is further mineralized by calcium deposition [11, 12]. The second is an effective peri-implant sealing, which is characterized by healing of the oral mucosa tissue

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surrounding the abutment [11, 12]. The processes governing tissue healing in peri-implant sealing are complex. Gingival epithelial tissue, resembled by oral keratinocytes and the subjacent connective tissue that is in contact with the abutment surface, is primarily responsible for achieving peri-implant sealing which is mediated by collagen fibrils and local fibroblasts [11–13]. Therefore, a number of studies have evaluated the efficacy of different therapies upon the metabolism and adhesion of bone [14–16] and oral mucosa cells to a titanium surface [17].

The epithelium and subjacent connective tissue, plus the maxillary bone, directly contribute to the functional and esthetic success of oral rehabilitation using intraosseous implants. It is therefore beneficial to evaluate specific therapies capable of up-regulating the metabolism and activities of cells within these soft and hard oral tissues. The effects of PBM on peri-implant tissue healing were already assessed by previous investigations, which demonstrated that this therapeutic protocol can improve the success of oral implants by increasing cell migration and proliferation and increase also local angiogenesis and down-modulating the inflammatory response [14]. In addition, PBM also increases osteoblastic differentiation, which accelerates bone deposition around implants [14, 15].

Here, a comparative evaluation of effects of PBM was demonstrated, using specific parameters of low-level laser therapy (PBM/LLLT) and light-emitting diodes (PBM/LED), to irradiate oral keratinocytes, gingival fibroblasts, and osteoblasts seeded onto a titanium surface.

Materials and methods

Cell lines and cell culture

This study was carried out using a human oral keratinocyte lineage (NOK-Si–CVCL # BW57), a primary cell culture of human gingival fibroblasts (HGF-CAAE #55629215.7.0000.5416) and a human osteoblastic lineage (SaOs-2–ATCC# HTB85). All cells were maintained in 75-cm^2 flasks (Corning, New York, NY, USA) in Dulbecco's Modified Eagle's Medium (DMEM – # - Gibco, Carlsbad, CA, USA) supplemented with antibiotics (PenStrep–Gibco) and 10% of fetal bovine serum (FBS–Gibco). Oral keratinocytes and HGF were sub-cultured using .25% trypsin (Gibco) while osteoblasts were sub-cultured by .25% trypsin/EDTA (Gibco).

Experimental design

For this investigation, 13-mm-diameter machined titanium disks were polished in − 400, − 600, and − 1200 granulation bands and then cleaned with acetone, ethanol, and deionized water [18–20]. Surface roughness was analyzed by confocal microscope (OLYMPUS LEXT OLS4000, Japan) and then disks were sterilized in an autoclave. Prior to cell seeding, disks were individually placed in wells of sterilized 24-well plates (Techno Plastic Products-TPP, Trasadingen, CH, USA). Then, 1 mL of complete DMEM was added to each well, followed by cells for seeding (5×10^4 cells/well).

After 24 h of incubation, the complete DMEM was replaced by 1 mL of FBS-free DMEM and the cells were immediately subjected to PBM with LLLT or LED devices, at uniform parameters presented in Table 1 [22].

PBM was applied by means of two prototypes: LASERTable [8, 10, 19, 23] and LEDTable [24], both of which were specifically designed for in vitro studies. These devices provide full irradiation of each cell culture plate at a standardized distance and irradiation area, which allows for a uniform comparison of both therapies. The cells were irradiated three times at 24 h intervals, which corresponds to 9 J/cm^2.

Twenty-four hours after the last irradiation, all cell types were assessed for adhesion, viability, and protein synthesis. Alkaline phosphatase activity (ALP) and mineral nodule deposition were detected only for osteoblasts. Cells seeded on Ti disks and not submitted to PBM were used as a control group.

Cell morphology

Morphological analysis by fluorescence microscopy was performed for the cells that remained attached to the Ti surface. Cells were fixed in 10% paraformaldehyde for 15 min and were permeabilized in .1% triton x-100 (Sigma-Aldrich, St Louis, MO, USA) for 10 min. Then, samples were incubated with Actin Red-probe (1:200-Molecular Probes, Carlsbad, CA, USA) for 30 min for visualization of cytoskeleton filaments while nuclei were stained with Hoescht DNA-intercalant (Molecular Probes) (1:5000) for 15 min. Samples (n = 4) were then assessed by inverted fluorescence microscope (EVOS Floid Cell Image Station, Thermo Fischer Scientific, Waltham, MA, USA) and photomicrographs were analyzed by ImageJ Software (US National Institutes of Health, Bethesda, MA USA). Five photomicrographs of each sample were analyzed to enable quantitative and qualitative data [19].

Cell viability

Viability of cells seeded onto a Ti surface and submitted to PBM by LLLT or LED was evaluated by an alamarBlue assay (Invitrogen, Carlsbad, CA, USA). For this protocol, cells were incubated at 37 °C with alamarblue solution at 10% in FBS-free DMEM for 4 h [19]. During this period, mitochondrial enzymes could cleave the resazurin salt in a fluorescent dye (resorufin), which was then detected in a fluorometer at 460/

Table 1 Parameters for in vitro PBM with LLLT and LED according to Jenkins & Carroll [21]

PBM source	LLLT	LED
Device information		
Manufacturer	Optics Group of the Optics and Photonics Research Center, São Carlos Institute of Physics, University of São Paulo (USP), Brazil	Optics Group of the Optics and Photonics Research Center, São Carlos Institute of Physics, University of São Paulo (USP), Brazil
Model identifier	LASERTable	LEDTable
Number of emitters	12 units of laser diode DL-7140-201S (SANYO Electric Co., Ltd., Osaka, Japan)	24 units of infrared LED L1IZ-0850000000000 (Luxeon Star LEDs - Quadica Developments Inc., Alberta, Canada)
Emitter type	InGaAsP LASER diodes	InGaN LED diodes
Spatial distribution of emitters	12 emitters aligned in groups of four in-line diodes	24 emitters aligned in six groups of four diodes associated with colimators
Beam delivery system	Fiberoptic	Fiberoptic
Irradiation parameters		
Center wavelength [nm]	780 nm	850 nm
Spectral bandwidth	780 nm ± 5 nm	850 nm ± 20 nm
Operating mode	Continuous wave	Continuous wave
Frequency	10^{12} Hz to 10^{15} Hz	≥ 100 Hz
Pulse on duration	240 s	300 s
Pulse of duration or duty cycle	240 s	300 s
Energy per pulse	3 J	3 J
Peak radiant power	0.07 W	1.05 W
Average radiant power	0.025 W	0.020 W
Beam profile	Gaussian	Gaussian
Treatment parameters		
Beam spot size at target	0.0002 m^2	0.0002 m^2
Irradiance at target	125 W/m^2	100 W/m^2
Exposure duration	240 s	300 s
Radiant exposure	15000 J/m^2	15000 J/m^2
Radiant energy	3 J	3 J
Number of points irradiated	1	1
Area irradiated	0.0002 m^2 (area of each well of a 24-well cell culture plate)	0.0002 m^2 (area of each well of a 24-well cell culture plate)
Application technique	0.025 m distance between laser diode and well bottom	0.014 m distance between laser diode and well bottom
Number and frequency of treatment sessions	1 irradiation per day, over 3 days	1 irradiation per day, over 3 days
Total radiant energy	9 J	9 J

495 nm (Synergy H1 microplate reader, BioTek Instruments, Winooski, VT, USA).

Total protein synthesis

Total protein synthesis was assessed using the Lowry method and following the detailed protocol described by Basso et al. (2018) [19]. Briefly, after cell lysis with .1% sodium lauryl sulfate (Sigma-Aldrich), samples were incubated with Lowry reagent (.1%) for 40 min and protein conjugate was detected by Folin & Ciocalteu's Phenol reagent (1:5, Sigma-Aldrich) for 20 min. Total protein amount was assessed by spectrophotometry (Synergy H1) at 655 nm. Bovine serum albumin (BSA) was used to obtain a standard curve.

Collagen synthesis

Collagen synthesis was determined by the Sirius Red method. This assay recognizes collagen types I, II, III, and IV, which resemble the collagen tissue of oral mucosa and bone [25]. Therefore, all cells were subjected to this protocol.

Supernatant of each sample was incubated (1:1) with Direct Red reagent at .1% (Sigma-Aldrich) for 1 h under agitation (400 rpm) at room temperature. Then, samples were

centrifuged at 10^4 rpm following washing with hydrochloric acid (HCl-5M). After washing, new centrifugation pellets were dissolved in sodium hydroxide solution (NaOH-.5 M). A 200-μL aliquot of each sample was analyzed using spectrophotometry (Synergy H1) at 555 nm.

ALP activity

ALP is an ectoenzyme involved in the initial phases of bone mineralization. It acts by binding to collagen fibrils and enhancing calcium adherence; it also contributes to the conformational structure of this protein [20, 24]. Therefore, higher ALP activity is an indicator of increased mineralization activity and it can be used as a proxy. The in vitro ALP activity was detected by an end-point assay (Labtest Diagnóstico S.A., Lagoa Santa, MG, BR), as previously described [20].

Statistical data analysis

After normality and homoscedasticity evaluation (Shapiro-Wilk, $p < 0.05$), data were subjected to ANOVA and Tukey tests at a 5% significance level. The adhesion of cells to titanium surfaces was also qualitatively presented.

Results

Similar quantitative and qualitative adhesion of osteoblasts and fibroblasts was observed for all groups (Fig. 1a and b; Tables 2 and 3) ($p > 0.05$). Increased cell population on Ti disks was observed after PBM only for keratinocytes, regardless of the light source used (Fig. 1c; Table 4) ($p < 0.05$).

Cell viability was increased for osteoblasts irradiated with PBM/LLLT and PBM/LED ($p < 0.05$; Table 2) as well for fibroblasts, which showed higher viability rates for PBM/LLLT ($p < 0.05$; Table 3). Viability of keratinocytes was up-regulated by PBM, especially when these cells were irradiated with PBM/LED ($p < 0.05$; Table 4).

Despite that the protein synthesis was enhanced for osteoblasts submitted to PBM/LLLT or PBM/LED irradiation ($p < 0.05$; Table 2), this cell activity was observed only in PBM/LLLT-treated fibroblasts ($p < 0.05$; Table 3). Keratinocytes subjected to PBM exhibited protein synthesis similar to the control group ($p > 0.05$; Table 4).

Collagen synthesis was also increased for PBM-treated osteoblasts and fibroblasts ($p < 0.05$). However, when these cells were irradiated with PBM/LED, they underwent a higher synthesis of collagen than those submitted to PBM/LLLT (Tables 2 and 3). Collagen synthesis by keratinocytes was unaffected by both the PBM protocols used in this study ($p > 0.05$; Table 4).

While the ALP activity was up-regulated in PBM/LLLT-treated osteoblasts, the irradiation of these cells with PBM/LED decreased their ALP activity, even in comparison with the control group ($p < 0.05$; Table 2).

Discussion

In general, both PBM treatment modalities (PBM/LLLT and PBM/LED) evaluated in this study improved all cell parameters related to healing of peri-implant tissues. The migration and adhesion of osteoblasts to the Ti surface, which is the first step that drives the osseointegration process, trigger a cascade of cellular and molecular events related to the synthesis and mineralization of collagen-rich matrix [26–29]. This investigation demonstrated that the adhesion of osteoblasts to a Ti surface was not influenced by PBM. However, both PBM treatment modalities increased cell metabolism, characterized by cell viability, total protein production, collagen synthesis, and ALP activity. Moreover, cell functions were distinctly affected by each PBM source; PBM/LLLT in particular demonstrated better results than PBM/LED, at selected parameters.

In a previous in vitro study, Khadra et al. (2005) [30] reported the positive response of osteoblasts submitted to PBM/LLLT at parameters similar to those evaluated by this investigation. Other studies have also evaluated the effects of PBM using LLLT or LED on osteoblasts [23, 31]. However, comparison of both therapy modalities using similar parameters is scarce [29]. In addition, the poor standardization of PBM protocols applied to the oral implantology field and the lack of detailed information regarding the irradiation parameters inhibit adequate comparison of current scientific data available in the literature [16, 32].

Table 2 Number of attached cells, cell viability, collagen synthesis, total protein production, and ALP activity for osteoblasts seeded onto titanium disks and submitted to PBM with LLLT or LED. Values indicate mean and standard deviation. Groups identified by different letters indicate significant statistical difference (ANOVA/Tukey, $p < 0.05$)

Cell responses/groups	Attached cells (%)	Cell viability (%)	Collagen synthesis (%)	Total protein synthesis (%)	ALP activity (%)
Control	100 (± 2) A	100 (± 5.8) B	99.83 (± 5.3) B	100 (± 18.6) B	100 (± 16.2) B
LLLT	100.7 (± 10.7) A	111.9 (± 6.5) A	99.73 (± 14.3) B	125.7 (± 11) A	121.3 (± 35.2) A
LED	100 (± 2.9) A	108.5 (± 2.6) A	113.7 (± 19) A	115.8 (± 16.5) A	60.79 (± 12.8) C

Table 3 Number of attached cells, cell viability, collagen synthesis, and total protein production for fibroblasts seeded onto titanium disks and submitted to PBM with LLLT or LED. Values indicate mean and standard deviation. Groups identified by different letters indicate significant statistical difference (ANOVA/Tukey, $p < 0.05$)

Cell responses/groups	Attached cells (%)	Cell viability (%)	Collagen synthesis (%)	Total protein synthesis (%)
Control	100 (± 2) A	100.1 (± 3.8) B	100.2 (± 11.6) B	100 (± 4.5) B
LLLT	97 (± 4.6) A	111.9 (± 6.6) A	108.8 (± 2.5) B	115.1 (± 10.8) A
LED	98 (± 5.7) A	97.1 (± 7.7) B	115.8 (± 10.9) A	99.1 (± 8.4) B

There are a limited number of studies that have used Ti as a substrate to evaluate the effects of PBM on peri-implant healing using human cells [21, 30, 33]. PBM investigations on bone and mucosa healing were performed using polystyrene substrate [34, 35], which is suitable for cell response but does not mimic clinical peri-implant conditions. Ross et al. (2012) [36] reported that cells display distinct behavior according to the substrate on which they are seeded. Therefore, it follows that the selection of adequate substrate for cell culture should be carefully considered for in vitro studies related to implant repair. This investigation used a Ti surface, oral mucosa cells, and osteoblasts to simulate in vivo conditions. The results of this in vitro investigation might predict the in vivo interaction of PBM-treated cells in a clinical setting to amplify osseointegration and soft tissue sealing to titanium—a material that is widely used to fabricate screws and abutments for oral implants.

Pagin et al. (2014) [31] demonstrated that PBM/LLLT enhanced the metabolism of osteoblasts and caused greater cell growth and differentiation in comparison to PBM/LED. The authors emphasized that this result may be related to the stimulation of specific chromophores that accelerate osteoblast differentiation. The stimulation of specific chromophores by distinct light sources may also be a relevant factor on PBM effects for each cell type [2]. Additionally, Hamblin et al. (2018) [5] reported that the sensitivity, phenotype, responsiveness, and homeostasis status of cells may influence differing responses when these cells are submitted PBM. However, while the primary effects of PBM may be to activate a similar pathway for different cell types—characterized by increased ATP synthesis and phosphorylation of proteins related to cell cycle—lasers and LEDs may present peculiarities regarding cell-light interaction [1, 5, 36].

It has been shown that the success of oral osseointegrated implants also depends on the formation of a peri-implant biological sealing by soft oral tissues [13, 37, 38]. The fast attachment of keratinocytes and HGF to prosthetic surfaces is mandatory for the establishment of such biological sealing, which acts as a physical and biological barrier against periodontal pathogens [13]. In this vein, the adhesion of fibroblasts and epithelial cells to a Ti surface is decisive for the successful outcome of oral osseointegrated implants over time [13]. Therefore, in the present study, the adhesion of PBM-treated human gingival fibroblasts (HGF) and oral keratinocytes (NOK) to the surface of Ti discs was assessed. In general, both modalities of irradiation enhanced keratinocyte population, which was demonstrated by qualitative and quantitative data (Table 4). Besides attaching to the Ti surface, epithelial cells also play a fundamental role in biological sealing, since they release collagenous proteins to strengthen the physical barrier, and immunoglobulins, which also protect the oral mucosa tissue against peri-implant pathogens [13]. Overall, the application of PBM at the selected parameters improved the adhesion and proliferation of keratinocytes to the Ti disks and also their viability when a LED treatment was used. On the other hand, total protein production and collagen synthesis were not influenced by either of the two PBM modalities.

In evaluating the response of fibroblasts to FBM, the application of PBM/LED enhanced the viability of HGF, while PBM/LLLT increased the total protein production by these cells, mainly via the synthesis of collagen. The latter observation was significant because collage is a major extracellular protein of periodontal tissue and plays a role in connective tissue healing [22]. Based on these data, these results provide evidence that the PBM/LLLT parameters assessed in this

Table 4 Number of attached cells, cell viability, collagen synthesis, and total protein production for keratinocytes seeded onto titanium disks and submitted to PBM with LLLT or LED. Values indicate mean and standard deviation. Groups identified by different letters indicate significant statistical difference (ANOVA/Tukey, $p < 0.05$)

Cell responses/groups	Attached cells (%)	Cell viability (%)	Collagen synthesis (%)	Total protein synthesis (%)
Control	100 (± 2.2) B	100.4 (± 10.6) B	100.1 (± 3.4) A	100.1 (± 7.2) A
LLLT	122.3 (± 2.5) A	107.6 (± 2.1) B	100.6 (± 2.4) A	93 (± 10.6) A
LED	122 (± 5.6) A	113.3 (± 3.7) A	92.8 (± 15.4) A	99.1 (± 6.1) A

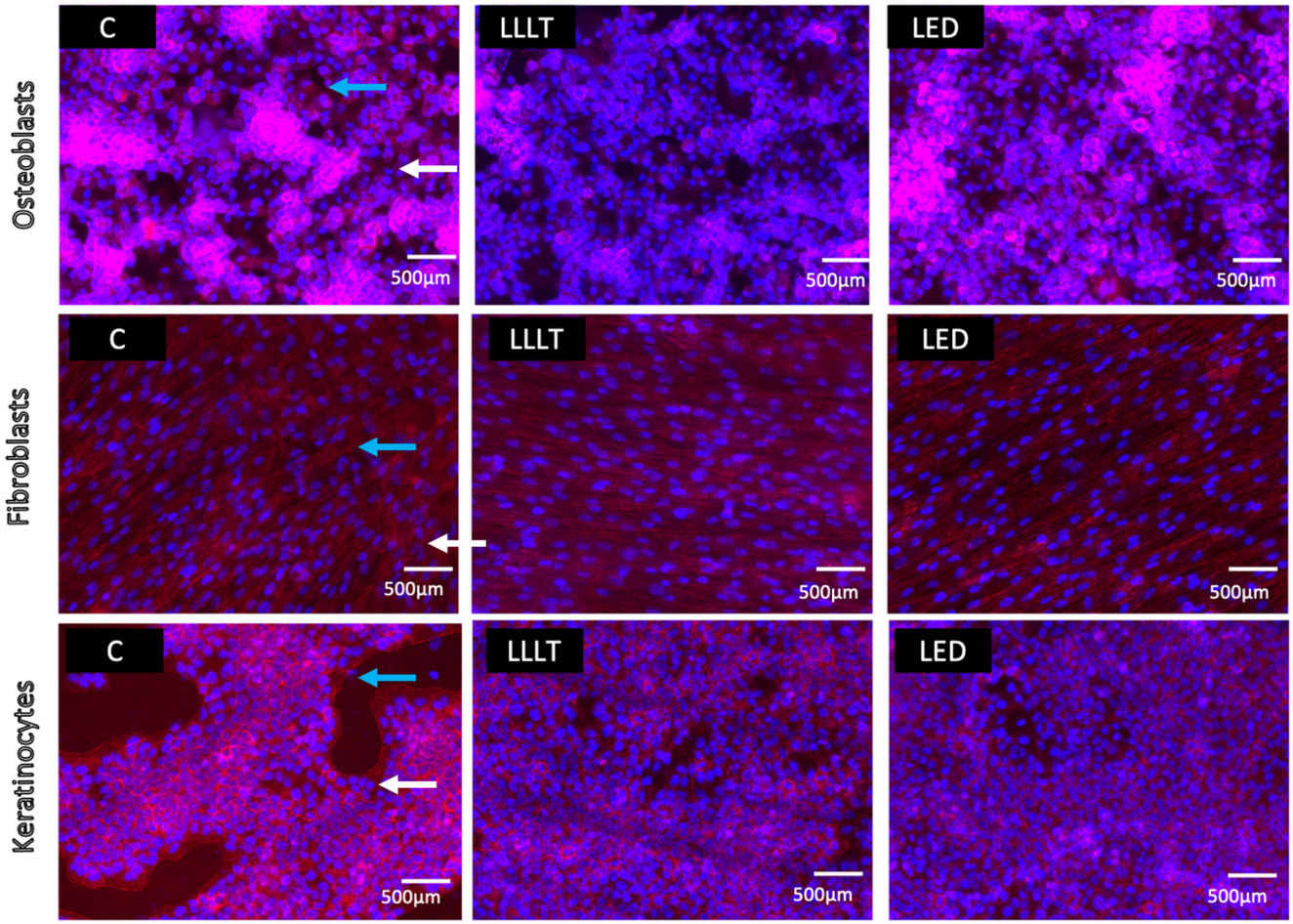

Fig. 1 Representative photomicrographs of osteoblasts (SaOs-2), fibroblasts (HGF), and keratinocytes (NOK) attached to Ti disks (C–control; LLLT; and LED). Cell cytoplasm (blue arrows) is identifiable by actin filaments in red while the nuclei are stained in blue (white arrows)

study may induce the fast deposition of collagenous tissue around a Ti abutment surface and improve the biological sealing at the implantation site. Previous studies also demonstrated the biostimulation of PBM/LLLT-treated fibroblasts [8, 19, 39, 40]. For instance, the data of this in vitro study highlight the distinct sensitivity and responsiveness of oral keratinocytes and fibroblasts, which were directly related to the light source used. Therefore, careful standardization of the PBM protocols is recommended and selection of specific light therapies for the target tissue characteristics and its cell responsiveness, such as those previously reported by Arany et al. (2016) [7] and Engel et al. (2016) [40, 41]. In general, PBM/LED therapy promoted higher positive biological effects on cultured oral keratinocytes and fibroblasts than PBM/LLLT for the PBM parameters assessed in this investigation.

In this study, the authors showed the photobiomodulation of osteoblasts as well as oral fibroblasts and keratinocytes seeded on a Ti surface and then submitted to specific parameters of LED and low-level laser therapies. The methodology used in this investigation was established to mimic in vivo conditions in which specific cells play key roles in the osseointegration and biological soft tissue sealing around dental implants. While recognizing that the results from laboratorial studies cannot be extrapolated directly to clinical situations [41], such as extremely controlled conditions, single-cell source for each cell line, and the absence of interaction among different tissues, the original scientific data reported from our investigation is promising and should drive further in vitro and in vivo studies to improve the research field and clinical outcomes for patients with dental implants.

Conclusion

At selected parameters, and considering cellular and tissue specificities, PBM may be a suitable therapy to promote peri-implant healing.

Funding The National Council for Scientific and Technological Development, CNPq (Grant # 302108/2019-0) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) provided financial support.

Declarations

Conflict of interest The authors declare no competing interests.

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