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Transdentinal Cell Photobiomodulation Using Different Wavelengths

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Clinical Relevance

Determining the optimal irradiation parameters of odontoblast-like cell stimulation using dentinal barrier as a function of the wavelength is the first step toward establishing the ideal window for biostimulation of pulp tissue previously injured by caries lesion progression and cavity preparation.

SUMMARY

Objective: The aim of this study was to investigate the effects of transdentinal irradiation with different light-emitting diode (LED) parameters on odontoblast-like cells (MDPC-23).

Methods and Materials: Human dentin discs (0.2 mm thick) were obtained, and cells were seeded on their pulp surfaces with complete culture medium (Dulbecco modified Eagle medium). Discs were irradiated from the occlusal surfaces with LED at different wavelengths (450, 630, and 840 nm) and energy densities (0, 4, and 25 J/cm^2). Cell viability (methyltetrazolium assay), alkaline phosphatase activity (ALP), total protein synthesis (TP), and cell morphology (scanning electron microscopy) were evaluated. Gene expression of collagen type I (Col-I) was analyzed by quantitative polymerase chain reaction (PCR). Data were

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analyzed by the Mann-Whitney test with a 5% significance level.

Results: Higher cell viability (21.8%) occurred when the cells were irradiated with 630 nm LED at 25 J/cm^2. Concerning TP, no statistically significant difference was observed between irradiated and control groups. A significant increase in ALP activity was observed for all tested LED parameters, except for 450 nm at 4 J/cm^2. Quantitative PCR showed a higher expression of Col-I by the cells subjected to infrared LED irradiation at 4 J/cm^2. More attached cells were observed on dentin discs subjected to irradiation at 25 J/cm^2 than at 4 J/cm^2.

Conclusion: The infrared LED irradiation at an energy density of 4 J/cm^2 and red LED at an energy density of 25 J/cm^2 were the most effective parameters for transdentinal photobiomodulation of cultured odontoblast-like cells.

INTRODUCTION

Inflammatory pulp reaction is commonly observed subjacent to active decay. The intensity of tissue inflammation may increase during mechanical cavity preparation and following cavity restoration with dental materials with nonbiocompatible components. About and others[1] reported that the sum of damages experienced by the pulp tissue can result in pain and exacerbate local inflammatory reactions. Therefore, several products and techniques have been proposed to prevent or relieve pulp sensitivity and biostimulate the healing of this specific connective tissue. Phototherapy has appeared as a promising treatment for this purpose.[2]

Recent studies have demonstrated that light-emitting diode (LED) irradiation is capable of stimulating cells to synthesize collagen-rich matrix and proteins that play a role in its mineralization.[3-5] Additionally, several *in vitro* experiments with LED at different wavelengths have demonstrated a significant increase in the proliferation of fibroblasts, osteoblasts, muscle cells in rats, human epithelial cells, and mesenchymal stem cells.[4-7] Other positive effects caused by light, such as reduced dentin sensitivity, formation of mineralized tissue stimulus, improvement in rheumatoid arthritis, and mucositis healing, have also been reported.[8-12]

Despite these interesting scientific data, little is known about the transdentinal effects of LED irradiation on pulp cells. In current studies, it was demonstrated that the power density that reaches cells is much lower than the one applied to dentin, mainly because of light scattering.[13,14] For 0.2 mm, specifically, the mean of power loss in dentin discs is approximately 40.0%.[14] Some recent studies have shown that phototherapy promotes biomodulation when applied directly to cells with an odontoblast phenotype, increasing the synthesis and expression of dentin matrix proteins.[15] Therefore, it could be speculated that, in clinical situations, the transdentinal LED biomodulation of odontoblasts subjacent to the dental cavity may cause deposition of tertiary dentin, protecting the pulp tissue against further assaults from different sources.[16] It is known that transdentinal irradiation causes an effective decrease of energy density.[13,14] Although the energy density applied on the external surface is known, the energy density that actually reaches the cell layer is much lower due to light scattering. One purpose of this study was to determine that, even with this loss, LED irradiation is capable of causing biostimulatory effect on odontoblast-like cells.

Since there is no previous information about the possibility of transdentinal stimulation of odontoblasts by LED irradiation, the aim of this study was to evaluate whether specific LED parameters at different wavelengths are capable of diffusing through a 0.2-mm-thick dentin barrier to biostimulate cultured odontoblast-like MDPC-23 cells.

METHODS AND MATERIALS

LED Devices and Irradiation Parameters

Irradiation was performed with three devices (LEDTables) containing 24 diodes with wavelengths at 450, 630, or 840 nm. The InGaN diodes (indium, gallium, and nitride) were individually positioned in the LEDTables in such a way that each could homogeneously irradiate the cells attached to the bottom of a well in a 24-well plate. During cell irradiation, the LED device was applied in noncontact mode and perpendicular to the bottom of the well. The distance between the dentin disc and the LED device tip was 2.0 cm.

The energy densities of 4 and 25 J/cm^2 used in this investigation were selected based on previous studies in which the authors irradiated different cell types.[5,15,17] The irradiance emitted by the LEDs was 88 mW/cm^2, and the power loss caused by the plate and the dentin disc was considered, resulting in irradiation times of 1 minute and 20 seconds (4 J/cm^2) and 8 minutes and 40 seconds (25 J/cm^2). For all groups, cells were maintained in contact with

phosphate-buffered saline (PBS) for the irradiation procedure, as previously described.[18]

Temperature Monitoring

PBS temperature variations were evaluated by means of a multimeter (38XR; Metermam, Everett, WA, USA) and a calibrated thermistor (38XR, Metermam) placed at the bottom of the well. This preliminary analysis was performed because PBS heating may cause cell damage.

Obtaining Dentin Discs

This study was approved by the Research Ethics Committee (Protocol 26/09) of the Araraquara School of Dentistry, UNESP, University Estadual Paulista, Brazil. One hundred and eighty-two human dentin discs from sound human molars were obtained, selected by stereomicroscopy as previously described,[13,14] and reduced to 8-mm diameter. The dentin surfaces were rinsed with 0.5 M ethylenediaminetetraacetic acid (pH 7.2) according to a previous study.[19] The discs were then washed with sterile deionized water for 60 seconds and were subjected to measurement of transdentinal LED light transmission.

Measurement of Transdentinal LED Light Attenuation

This test was performed to determine the light attenuation through the disc structure and provide a homogeneous distribution of discs among groups, according to the power loss values of each one. The protocol of transdentinal power measurement was described in detail in a previous study.[13]

MDPC-23 Cell Culture

The MDPC-23 cells were cultivated in Dulbecco modified Eagle medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 2 mmol/L of glutamine (Gibco). The cells were maintained in a humidified incubator with 5% CO_2 and 95% air at 37°C (Isotemp, Fisher Scientific, Bellefonte, PA, USA).

Experimental Conditions

After distribution into the experimental and control groups, the dentin discs were packaged and sterilized by ethylene oxide[19] and, finally, adapted to metal devices designed for this study (Figure 1). The devices and silicon O-rings were autoclaved for 15 minutes at 120°C and 1 kgf of pressure. Each device containing the dentin disc allowed for LED irradiation of the occlusal surface of the disc (facing down), while the pulpal surface on which the cells were seeded remained in contact with the culture medium. The MDPC-23 cells were seeded (3×10^4 cells/disc) on the pulp surfaces of the discs and incubated for 48 hours at 37°C and 5% CO_2.

After this period, the culture medium was replaced by a new culture medium supplemented with 2% fetal bovine serum (FBS),[20,21] and the cells were incubated for an additional 24 hours. Immediately before irradiation, the culture medium was replaced by sterile buffered saline solution (PBS) at room temperature.[18] The 24-well plates were then placed on the LEDTables (Figure 1) for specific periods, according to the energy density. A single LED irradiation was performed to simulate a clinical situation in which a deep cavity is available for restoration. Cells were irradiated in a dark room; thus, the LED irradiation was the only light source that the cells were exposed to.

After irradiation, the PBS was aspirated, and 1 mL of fresh culture medium containing 10% FBS was added to each well. Following incubation for 72 hours, the cell viability (methyltetrazolium [MTT] assay), alkaline phosphatase (ALP) activity, total protein (TP) synthesis, and cell morphology (scanning electron microscopy [SEM]) were evaluated. The expression of collagen type I (Col-I) was analyzed by real-time polymerase chain reaction (RT-PCR). In the control group, the same cell manipulation procedures were performed, but the diodes were not activated.

Cell Viability (MTT assay)

Cell viability (n=8) was evaluated using the MTT assay (Sigma-Aldrich), which determines the activity of SDH enzyme produced by mitochondria in cells. The dentin discs were carefully removed from the devices and individually placed in wells of 24-well plates. The MTT assay was performed as described in previous studies.[15,21]

TP Production and ALP Activity

Eight samples were selected to evaluate TP production and ALP activity.

TP Production—The production of TP was measured for each experimental and control group according to the protocol described in a previous study by the Lowry method.[15]

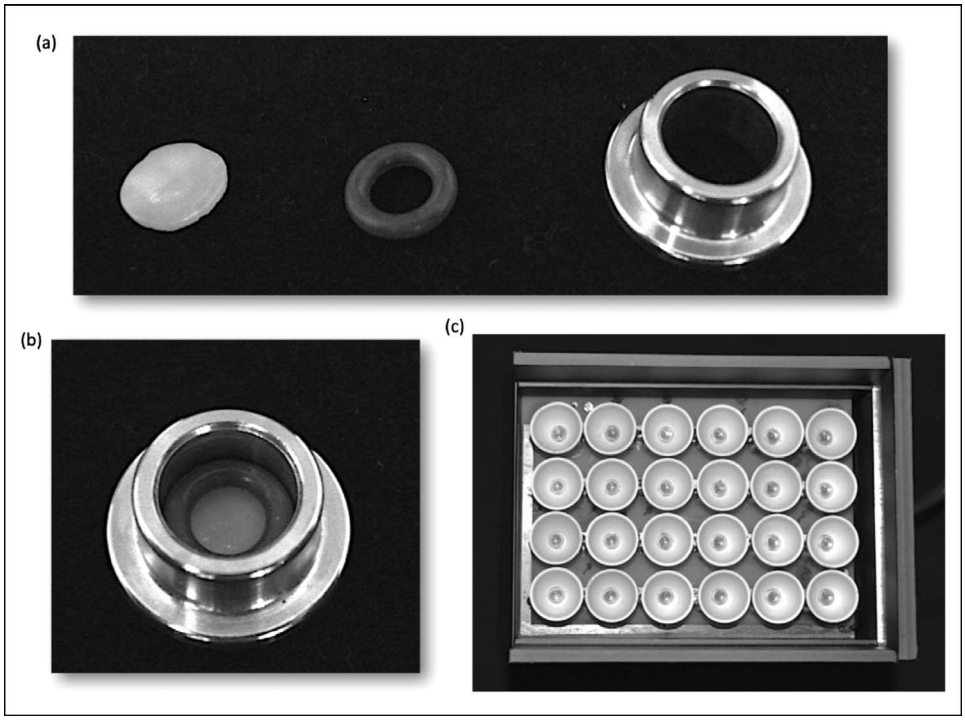

Figure 1. *Illustration of the dentin disc/device set and irradiation apparatus (LEDTable). (a): Dentin disc, silicon O-ring, and the irradiation apparatus, separately. (b): The dentin disc/device set. (c): Top view of LEDTable with 24 diodes and their collimators used to irradiate the dentin discs.*

ALP Activity—ALP activity was evaluated according to the protocol of the Alkaline Phosphatase Kit-colorimetric endpoint assay (Labtest Diagnóstico SA, Lagoa Santa, MG, Brazil). This assay utilizes thymolphthalein monophosphate, a substrate of phosphoric acid ester. ALP hydrolyzes the thymolphthalein monophosphate, releasing thymolphthalein. The enzymatic activity was measured as previously described.[15]

Analysis of Cell Morphology by SEM

For each experimental and control group, samples were prepared for cell morphology analysis by SEM (JEOL-JMS-T33A Scanning Microscope; JEOL USA Inc, Peabody, MA, USA). The laboratory protocol employed in this study is well established and has been widely used.[15,19]

Col-I Expression—RT-PCR

RNA extraction was performed by the Trizol method, which was detailed by Basso and others.[22] The cDNA was obtained by means of the High Capacity cDNA Reverse Transcriptions Kit (Applied Biosystems, Foster City, CA, USA) as previously described.

After cDNA synthesis, the effect of LED irradiation was assessed on the expression of Col-I with β-actin as the endogenous control. For each of these genes, specific primers were designed from the mRNA sequence (Table 1).

The reactions were prepared with standardized reagents for RT-PCR SYBR® Green PCR Master Mix (Applied Biosystems) in addition to sets of primers for each gene. Fluorescence readings were performed by Step One Plus (Applied Biosystems) in each amplification cycle and were subsequently analyzed by Step One Software 2.1 (Applied Biosystems). All reactions were subjected to the same analysis conditions and normalized by the signal from the passive reference dye ROX to correct for fluctuations in readings due to changes in volume and evaporation during the reaction. Individual results expressed in CT values were transferred to spreadsheets and grouped according to the experimental groups, normalized according to expression of the endogenous gene selected (β-actin). Then the

Table 1: *Primer Sequences and Applications for Mus musculus Used in This Work*

Primer Sequences	Gene
S: 5′-AGC CAT GTA CGT AGC CAT CC-3′	βAct
AS: 5′-CT CTC AGC TGT GGT GGT GAA-3′	
S: 5′-TGA GGT CCA GGA GGT CCA-3′	Col-I
AS: 5′-AAC TTT GCT TCC CAG ATG TCC-3′	

concentrations of target gene mRNA were evaluated statistically.

Statistical Analysis

The data set for each variable—cell viability, TP production, ALP activity, and Col-I expression—were evaluated concerning their distribution. In compliance with the requirements for the selection of parametric tests, nonparametric Kruskal-Wallis tests were complemented by the Mann-Whitney test, set at a predetermined significance of 5%.

RESULTS

Temperature Monitoring

The 630-nm wavelength (red LED) yielded no temperature increase during 10-minute irradiation, which was the maximum time used for cell irradiation. The 450-nm (blue LED) and 840-nm (infrared) wavelengths caused an increase of only 2°C after 8 minutes of irradiation. Thus, the temperature rise did not cause damage to cells; temperature increase of up to 3.4°C does not cause detrimental effects on MDPC-23 cells.[23]

Cell Viability (MTT Assay), TP, ALP, and Col-I Expression

The data for cell viability, ALP, TP, and collagen type-I expression, according to the energy densities and wavelengths used in this study, are shown in Figure 2. When the LED energy densities were compared for cell viability, it was observed that for the 450-nm wavelength (blue LED), there was no statistical difference between the irradiated groups and the control, which was considered with 100% cell viability ($p>0.05$). For the 630-nm wavelength (red LED), the energy density of 25 J/cm^2 increased the cell viability by 21.8% ($p<0.05$). For the 840-nm wavelength (infrared LED), energy densities of 4 and 25 J/cm^2 reduced the cell viability by 18.6% and 29.1%, respectively; all of them were statistically different from the control group ($p<0.05$). Concerning the LED wavelengths at the energy density of 4 J/cm^2, 450 nm (blue light) caused statistically higher cell viability compared to 840 nm ($p<0.05$). For energy density of 25 J/cm^2, the cells irradiated with red LED (630 nm) presented greater viability compared to the infrared LED (840 nm; $p<0.05$).

Concerning ALP, it was observed that the wavelengths used for all irradiated groups showed higher values of ALP activity when compared to the control group, except for the blue LED at 4 J/cm^2 ($p>0.05$). For the wavelength of 450 nm (blue LED), the group irradiated with 25 J/cm^2 provided statistically better results than the control group ($p<0.05$), with an increase of 113% in ALP activity. For the wavelength of 630 nm (red LED), groups irradiated with 4 and 25 J/cm^2 also showed statistically greater values when compared with the control group ($p<0.05$), with an increase of 46.7% and 81.7% in ALP activity, respectively. For 840 nm wavelength (infrared LED), groups irradiated with 4 and 25 J/cm^2 also had higher levels of ALP ($p<0.05$), and the increase in ALP activity compared to the control group was 220% and 121%, respectively. When the wavelengths were compared, it was observed that there was no difference among the groups for 25 J/cm^2 ($p>0.05$). However, when the irradiation was performed with 4 J/cm^2, increased ALP activity was observed for the wavelength of 840 nm ($p<0.05$).

For TP, regarding the energy densities used for this *in vitro* study, it was observed that there was no statistical difference between the irradiated and control groups for all wavelengths ($p>0.05$). But when the wavelengths were compared, there was a statistically significant difference between the 630-nm wavelength (red LED) and the 840-nm wavelength (infrared LED) only for 4 J/cm^2, and the wavelength representing the red region spectra showed the best results ($p<0.05$).

Finally, for Col-I expression, comparing the energy densities of 4 and 25 J/cm^2, it was possible to determine that the 450-nm irradiation resulted in a decrease of 64% and 56% in the Col-I expression, respectively. For the red LED, the group of 4 J/cm^2 was not statistically different from the control group ($p>0.05$). However, the energy density of 25 J/cm^2 showed a decrease of 40% in the expression of Col-I, and this difference was statistically significant when compared to the control group ($p<0.05$). For the infrared LED, an energy density of 4 J/cm^2 caused a 168% increase in the Col-I expression when compared with the control group ($p<0.05$). Moreover, the energy density of 25 J/cm^2 did not differ statistically from the control group ($p>0.05$). When the wavelengths were compared, it was observed that for 4 J/cm^2, there was a statistically significant difference among all evaluated wavelengths ($p<0.05$). However, the 840-nm wavelength (infrared) showed the best results for the expression of Col-I, followed by 630 nm (red) and 450 nm (blue). At 25 J/cm^2 energy density, the 840-nm wavelength showed significantly higher values compared to 450 and 630 nm ($p<0.05$).

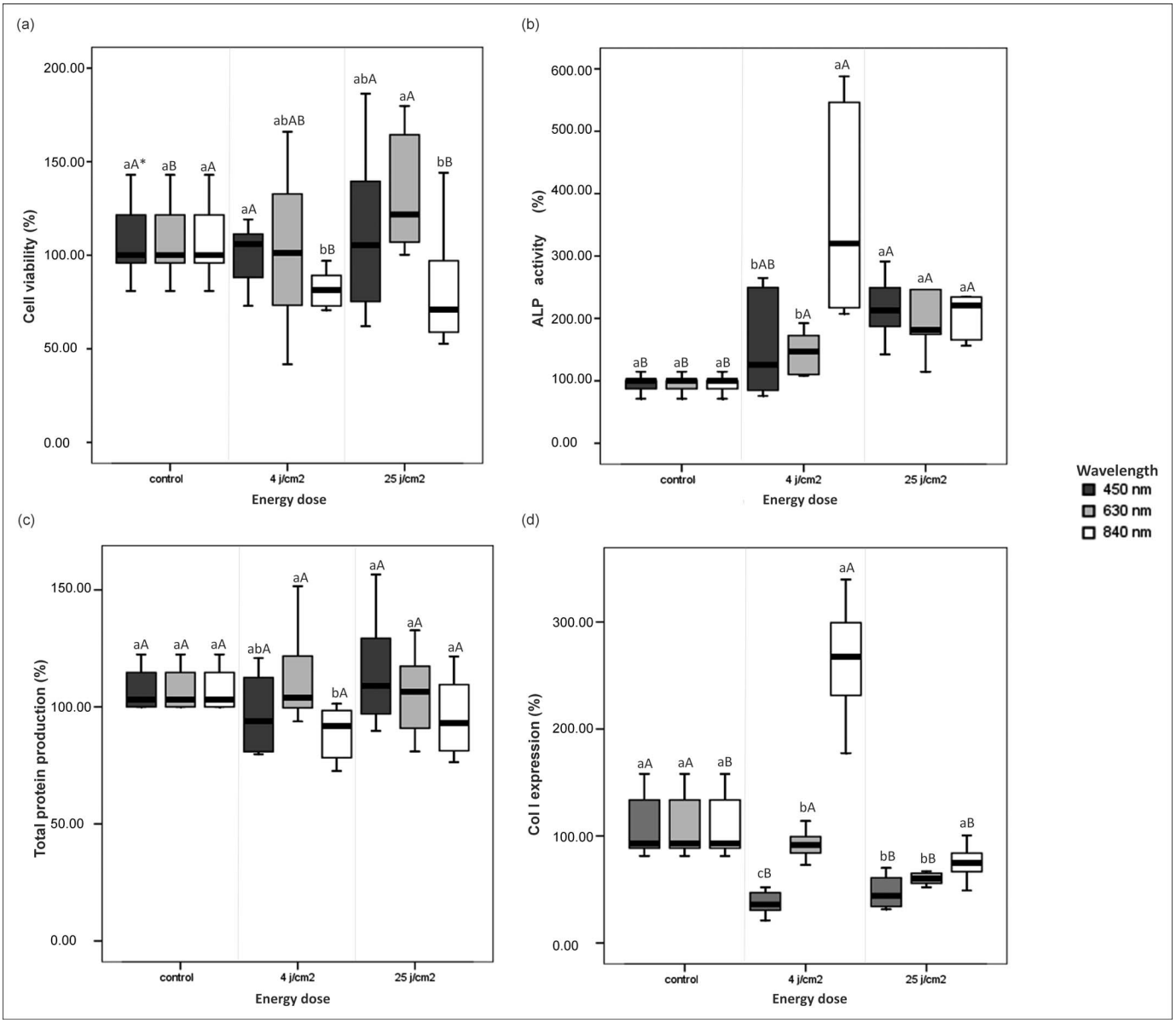

Figure 2. *Box plot indicating data for cell viability, total protein(TP), alkaline phosphatase (ALP) activity, and collagen type I (Col-I) expression. (a): Cell viability (%) detected by the methyltetrazolium (MTT) assay according to energy densities and wavelengths. (b): ALP activity (%) according to the energy densities and wavelength. (c): TP production (%) according to the energy densities and wavelength. (d): Col-I expression (%) according to the energy densities and wavelength (n=8). *Uppercase letters allow for comparison among energy densities, and lowercase letters allow for comparison among wavelengths. Same letters indicate no statistically significant difference (Mann-Whitney, p>0.05).*

Cell Morphology—SEM

Cells subjected to LED irradiation presented number and morphology similar to that of control group cells. More attached cells were observed on dentin discs subjected to irradiation at 25 J/cm^2 than at 4 J/cm^2 (Figure 3).

DISCUSSION

LED therapy has been used in different areas of human health, especially in dermatology[24-26] and neurology.[17] Additionally, this type of light has also been employed for muscle analgesia, anti-inflammatory effect,[7,27,28] and regeneration of injured tissues.[29,30] Specifically, in dentistry, LED has been evaluated as an alternative adjuvant therapy for treatment of mucositis,[11] dentin hypersensitivity,[10,31] and pulp cell stimulation.[32]

Tate and others[9] irradiated sound molars of rats with low-power laser and observed an intense formation of mineralized tissue within 30 days after

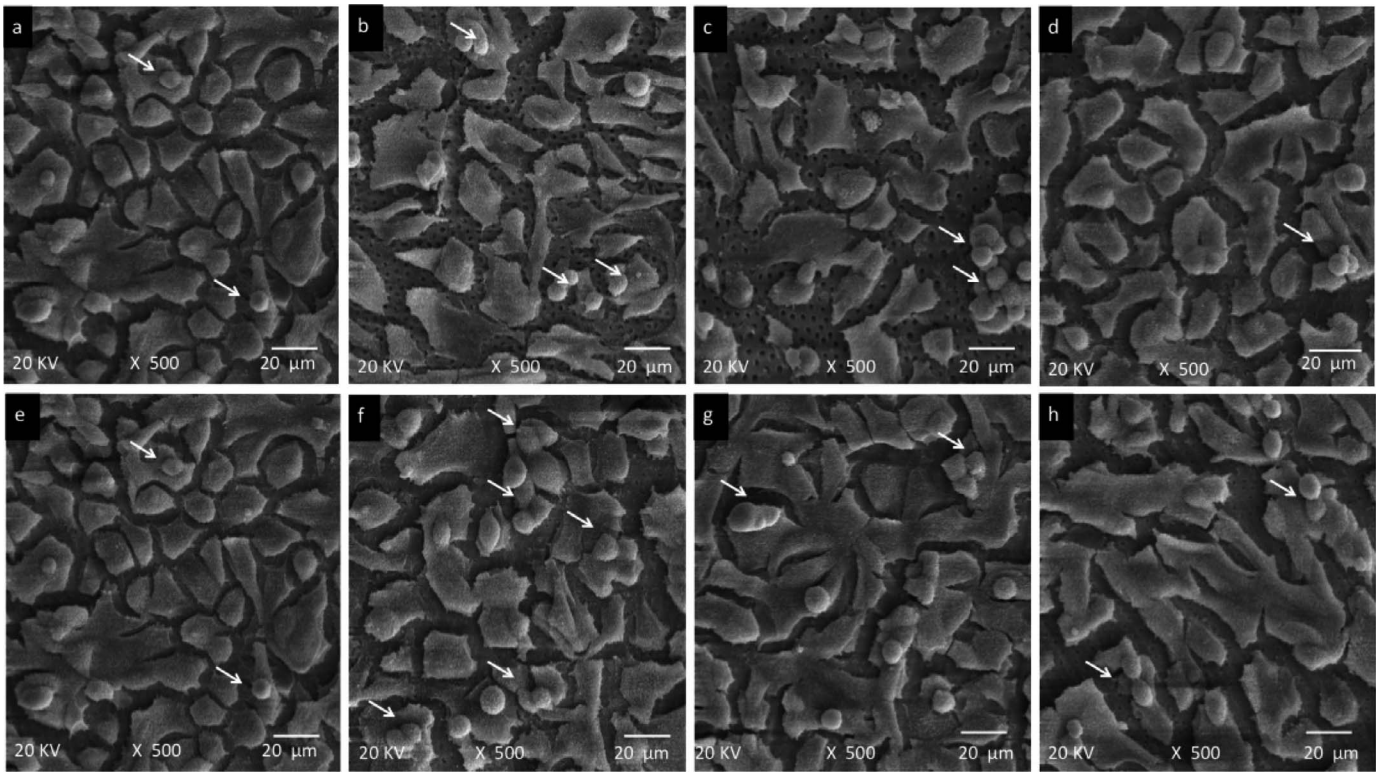

Figure 3. *Panel of SEM micrographs representative of cell morphology for each group. (a-d): Control group, 450 nm, 630 nm, and 840 nm LED, respectively, for 4 J/cm^2. (e-h): Control group, 450 nm, 630 nm, and 840 nm LED, respectively, for 25 J/cm^2. Arrows indicate mitosis. More attached cells were observed on dentin discs subjected to irradiation at 25 J/cm^2 than at 4 J/cm^2. Magnification 500×.*

procedure. However, the light irradiation of dentin-pulp complex able to promote biostimulation of human pulp cells previously subjected to an aggressive stimulus is not well known. To cause any effect on the pulp tissue, light must be transmitted through the hard dental tissues to reach the pulp cells with sufficient energy density to induce biostimulation. Despite the scarce basic research data concerning the optimal parameters of light application for different therapeutic activities, the clinical effects of light have already been assessed and even used as adjuvant therapy.[33] Therefore, this study evaluated the effect of transdentinal LED irradiation on odontoblast-like MDPC-23 cells seeded on dentin discs, using three different wavelengths, for which some positive cellular effects were described in the literature.[5,6,12,34]

In the present study, a significant increase in cell metabolism was observed in the group in which cells were subjected to irradiation with red LED at an energy density of 25 J/cm^2. In contrast, cells irradiated with infrared LED showed decreased metabolism after 72 hours of irradiation. Vinck and others[35] irradiated fibroblasts with red and infrared LED and observed increased cell metabolism 24 hours after irradiation and decreased cell metabolism 72 hours after irradiation. The authors suggested that this reduction in fibroblast metabolism at 72 hours postirradiation may be due to the occurrence of cell confluence after a long incubation, leading to contact-cell inhibition. This inhibitory phenomenon may also explain the negative effects obtained in the present study when the cells were irradiated with infrared wavelengths. The SEM analysis showed that, no matter the energy densities and wavelengths evaluated in this study, the morphology of MDPC-23 cells did not change. Thus, it may be suggested that the infrared LED, at energy densities of 4 and 25 J/cm^2, affected negatively the cell metabolism without causing detectable changes in cell morphology. Other studies have also evaluated the effect of light on cell metabolism, and generally the diverse responses found after irradiation were increased metabolism after 24[35,36] and 72 hours[31,37] and metabolism similar to the control group.[4,38] Importantly, the responses of irradiated cells were always dependent on the type of cell culture and irradiation parameters employed. Thus, cells that showed increased

metabolism 24 hours after the last irradiation were from a fibroblastic lineage, seeded at a density of 7×10^4 cells/cm^2, incubated for 24 hours, and subjected to three daily irradiations.[35,36] Particularly, this irradiation therapy used by Vinck and others)[36] does not match the protocol tested in the present study since the purpose of this study was to simulate a clinical situation in which a very deep dental cavity needs to be restored. Then, for economic, practical, and time reasons, the clinician has the possibility of irradiating the cavity floor one time only before filling the cavity. Also, since the cells internally lining the dentin tissue are odontoblasts, MDPC-23 cells were used in this study because they have the odontoblast phenotype and have been widely employed in various studies of light biomodulation.[15,21,31,38]

In addition to cell metabolism, ALP activity was evaluated. An increased expression of this enzyme by irradiated cells may indicate an interesting improvement in pulp tissue healing. However, previous studies showed that depending on the irradiation parameters used, different responses may occur in the ALP activity.[31,38] This cell behavior has been confirmed in the present study, where there was an increase in the activity of this protein for almost all irradiated groups. Thus, considering the positive results of ALP activity and correlating it with increased cell metabolism, particularly for the red LED at an energy density of 25 J/cm^2, it may be speculated that this parameter could be studied more deeply in future research both *in vitro* and *in vivo*. Moreover, other studies have shown an increase in ALP expression by mesenchymal cells irradiated with red LED.[4,5] Therefore, besides biomodulation of odontoblast-like cells, LED-specific parameters may also act positively on mesenchymal stem cells, which, in the case of injury by external factors, such as caries, heating, trauma, and so on, can effectively participate in the repair of the pulp-dentin complex.

Regarding Col-I expression, it was observed that irradiation with the infrared wavelength, at an energy density of 4 J/cm^2, increased the expression of this gene by 167% compared with that of the control group. Previous studies that evaluated the expression of Col-I by laser and LED phototherapy, in the red and infrared spectra, reported either similar behavior of irradiated groups compared with the control group[21] or increased expression of this gene after irradiation.[3,4] This result demonstrates that the use of this wavelength in transdentinal irradiation may be effective in forming nonmineralized matrix.

In general, the red light stood out from the other wavelengths, as did the blue light, which caused the lowest cell stimulation. These data can be explained, at least in part, by the occurrence of high scattering when lower wavelengths, like blue light, are applied to tissues, decreasing the energy density that reaches the local cells.[12,14] It is known that infrared light has the lowest scattering; however, it shows lower absorption by cells when compared with the red light.[12,39] Thus, it may be suggested that simultaneous effects of scattering and absorption by cultured pulp cells subjected to transdentinal LED irradiation occurred in the present investigation. It is known that wavelengths above 500 nm are better absorbed by cytochrome *c* oxidase, resulting in a greater synthesis of adenosine triphosphate by the cell with consequent increase of energy by oxidative phosphorylation.[40] On the other hand, flavins are small water-soluble molecules known to initiate free radical reactions when excited by light at wavelengths below 500 nm.[41] This information may explain, at least partially, the different cell responses found in the present study.

The results of this study underscore the importance of determining the optimal parameters for cellular biomodulation by LED phototherapy. For this study, wavelength and energy densities were factors that interfered with the cellular responses to transdentinal irradiation with LEDs, and all three wavelengths were able to cross the dentin barrier and cause some stimulus on pulp cells. Further *in vivo* studies are required to elucidate the effects of LED on the pulp-dentin complex as well as to determine whether LED irradiation may interact with different cells at the same time to trigger distinct pathways of biomodulation and tissue repair.

CONCLUSION

Based on the data obtained in this *in vitro* study, it can be concluded that infrared LED irradiation (840 nm) at an energy density of 4 J/cm^2 and red LED (630 nm) at an energy density of 25 J/cm^2 were the most effective parameters for transdentinal photobiomodulation of cultured odontoblast-like cells.

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Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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