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ORIGINAL ARTICLE

Metabolic activity of odontoblast-like cells irradiated with blue LED (455 nm)

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Abstract Blue light emitting diodes (LEDs) are frequently used in dentistry for light activation of resin-based materials; however, their photobiostimulatory effects have not yet been fully investigated. This study aimed to investigate the effect of blue LED (455 nm) on the metabolism of odontoblast-like cells MDPC-23. Energy doses of 2 and 4 J/cm^2 were used at 20 mW/cm^2 fixed power density. MDPC-23 cells were seeded at 10,000 cells/cm^2 density in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS). After 12 h, the culture medium was replaced with new DMEM supplemented with 0.5 % of FBS, and the cells were incubated for further 12 h. After that, single irradiation was performed to the culture, under selected parameters. Cell viability evaluations (Alamar Blue Assay, $n = 12$), number of viable cells (Trypan Blue Assay, $n = 12$), morphological analysis by scanning electron microscopy (SEM, $n = 2$), gene expression ($n = 6$) of alkaline phosphatase (*Alp*), collagen (*Col-1a1*), and dental matrix protein (*Dmp-1*) (quantitative polymerase chain reaction (qPCR)) were performed 72 h after irradiation. Data were analyzed by Kruskal-Wallis, ANOVA, and Tukey tests ($p < 0.05$). Direct light application at 4 J/cm^2 energy dose had no negative effects on cell viability, while irradiation with 2 J/cm^2 reduced cell metabolism. None of doses affected the number of viable cells compared with the control group. The two energy doses downregulated the expression of *Alp*; however, expression of *Col-1a1* and *Dmp-1* had no alteration. Cells presented change in the cytoskeleton only when irradiated with 2 J/cm^2. In conclusion, the blue LED (455 nm) irradiation, under the evaluated parameters, had no biostimulatory effects on MDPC-23 cells.

Keywords Phototherapy · Odontoblasts · Cell metabolism · Cell proliferation

Introduction

Camphorquinone, which is activated when submitted to visible light at the wavelength of 467 nm, is the photoinitiator most used in biomedical applications [1]. This photoinitiator is one of the constituents of dental materials, such as restorative composites, adhesive systems, resin, and ionomer materials. For this reason, the blue band (440–500 nm) of the visible light spectrum has been used in restorative procedures. It may be emitted by sources such as plasma arc, halogen light, or light emitting diodes (LEDs) [2, 3].

LEDs are small devices with high energy efficiency, which may emit light between the ultra-violet and infrared electromagnetic spectrum [4]. LEDs have been used in low level light therapy [5], particularly in the red and infrared wavelengths [6–8], which have been included in the "optical window" [9]. However, there are few studies regarding the use of LEDs at the blue light wavelength [5, 10, 11], even though this light wavelength is routinely used to photoactivate dental materials. In the literature, it has been observed that energy doses such as 2 or 4 J/cm^2 promoted biostimulatory effects and fitted into the parameters of low level light therapy [8, 9, 12]. However, these values differ

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from the dose of energy used for restorative purposes, which must be around 16 J/cm^2 [13].

It has been demonstrated that low level light therapy has positive biologic effects on cell proliferation [7, 8, 14] favoring tissue repair [6]. Light stimulation promotes alterations in the production of reactive oxygen species, modulation of ATP production, and increase in cell proliferation [8, 15]. However, the exact mechanism of action of the therapy (AlGhamdi 6), and its molecular effects [15] which are dependent on the physical parameters used, such as wavelength, power density, and energy dose [9, 16, 17], have not yet been completely elucidated.

Since there is no consensus with regard to the results obtained with cellular photobiostimulation by light in the blue spectrum [5, 10, 11, 15, 18] and considering that this wavelength is the most used for restorative purposes in dentistry, the aim of the present study was to determine the effects of irradiation with blue LED on odontoblast-like cells.

Material and methods

Cell culture and irradiation protocol

Odontoblast-like cells MDPC-23 cells were used and seeded at a density of 10,000 cells/cm^2, in 24-well plates, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of fetal bovine serum (FBS) [14]. The samples were maintained in an environment with 5 % of CO_2, at 37 ° C, for 12 h. After this, fresh DMEM containing 0.5 % of FBS was used with the purpose of promoting cellular nutritional deficit.

The irradiation procedures were performed after 12 h of cellular nutritional deficit. A device named LEDTable (Centro de Pesquisa em Óptica e Fotônica, São Carlos, Brazil) [19] was used to provide LED irradiation at the blue band of the visible light wavelength (455 ± 10 nm). This device has 24 light emitting diodes, which are activated simultaneously. Collimators within LEDs allow homogeneous and individualized light exposition to each well of the culture plate. Thus, possible effects of scattering and reflection of light were minimized. Additionally, the loss of power caused by the distance between the diodes and acrylic on the bottom of culture plates (11 mm) was previously calculated. Temperature variation, considering the physical irradiation parameters used in the study, was assessed using a calibrated multimeter and thermistor, both fixed to the base of the culture plate wells. The blue light wavelength (455 nm) was found to produce a 2 °C increase within 10 min irradiation which is below the 3.4 °C threshold necessary to cause harmful effects to MDPC-23 culture cells [20].

The parameters for exposure to light consisted of a fixed power density of 20 mW/cm^2, which fitted into the parameters of low level light therapy [9]. Therefore, energy doses of 2 J/cm^2 and 4 J/cm^2 were achieved by irradiating samples for 1 min and 40 s and 3 min and 20 s, respectively. Before irradiation, the culture medium was replaced by 1.0 mL of phosphate buffer saline (PBS 1×), with the purpose of avoiding the absorbance of LED by the culture medium containing phenol-red. After irradiation, the buffer solution was removed, and 1.0 mL of DMEM supplemented with 0.5 % of FBS was added.

The cell viability, the number of viable cells, the analysis of gene expression of *Alp*, *Col-1a1*, and *Dmp-1*, and cell morphology were evaluated 72 h after irradiation. This period of evaluation was previously determined in pilot studies as being the best period to observe the cellular events investigated in this study. For internal validity of the study, a control group was used, in which all the procedures were performed, including mounting the plates on LEDTable, except for activation of the diodes. For each group, including the control, 12 wells of a 24-well cell culture plate were used ($n = 12$).

Cell viability analysis

Cell viability was analyzed by means of the Alamar Blue assay ($n = 12$). In this method, the oxidized form of Alamar Blue (at blue color) is added to the culture, and by means of mitochondrial activity, it is converted into its reduced form, leading to a change in color from blue to pink [21]. Thus, 10 % Alamar blue solution was prepared in DMEM culture medium without FBS, and 500 μL was distributed to each sample. The samples were incubated at 37 °C in an atmosphere of 5 % CO_2. After 4 h, 200 μL of each sample was transferred, in duplicate, to a 96-well plate. Mitochondrial activity was determined by means of detection of fluorescence of the reduced salt, with excitation at 530–560 nm and emission at 590 nm (Synergy H1 Hybrid Reader, Biotek, Vermont, USA). The fluorescence values were normalized by the median of the control group and transformed into percentages.

Number of viable cells

The Trypan Blue assay ($n = 12$) was used for counting the number of viable cells [22]. After 72 h from radiation, the culture medium was removed and 300 μL of 0.25 % Trypsin was inserted (Invitrogen, Carlsbad, CA, USA), for 10 min. After this, 50 μL of the cell suspension was transferred to a 96-well plate and 50 μL of 0.04 % Trypan Blue solution was added (Sigma–Aldrich, St Louis, MO, USA) and incubated for 2 min at room temperature. An aliquot of 10 μL of the solution was inserted into a hemocytometer, used for counting the total number of cells and the number of non-viable cells, under an inverted light microscope (Nikon Eclipse TS 100, Nikon Corporation, Tokyo, Japan). Counts corresponded to $n \times 10^4$ cells/mL of the suspension. Non-viable cells were marked in blue, due to Trypan Blue dye penetration into cells

that presented rupture of the plasma membrane. The number of viable cells was determined by means of subtraction of the number of non-viable cells from the total number of cells. The values found were normalized by the median of the control group, considered 100 % viable cells.

Gene expression assay (RT-qPCR)

The expression of genes ($n = 6$ per gene) that codify alkaline phosphatase (*Alp*), collagen type I (*Col-1a1*), and dentin matrix protein (*Dmp-1*) was evaluated by means of the real-time quantitative polymerase chain reaction (RT-qPCR). Initially RNA extraction was performed with TRIzol (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's indications. The concentration of total RNA in each sample was determined in a biophotometer (RS–232C, Eppendorf, Hamburg, Germany), in using 1:50 dilution, followed by verification of the purity values with regard to the ratio 260/280 nm. After that, 1.0 μg of total RNA was used for cDNA synthesis of each sample, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For each of the genes selected, the specific primers were synthesized (Table 1). The reactions were prepared with standard reagents for real-time qPCR, TaqMan Universal PCR Master Mix (Applied Biosystems) (*Dmp-1*), and Syber Green Universal PCR Master Mix (Applied Biosystems) (*Alp*, *Col1a1* and β*Actin*). The StepOne Plus equipment (Applied Biosystems) was used for readout of the fluorescence, followed by the detection of amplification cycles, which were analyzed by the StepOne Software 2.1 (Applied Biosystems). All the reactions were performed according to the procedure proposed by Basso et al. (2013) [23]. The results for each gene were normalized according to the expression of the selected housekeeping gene (β*Actin*). Analysis of gene expression within groups was made as a percentage of control.

Scanning electron microscopy

The cell morphology was evaluated by scanning electron microscopy, using two samples from each group ($n = 2$). For this, glass slides (13 mm in diameter, Fisher Scientific, Pittsburgh, PA, USA) were sterilized and inserted into 24-well plates immediately before seeding the cells. The culture medium was then removed 72 h after irradiation, and the specimens were fixed and treated according to the method proposed by Oliveira et al. (2011) [14]. The specimens were mounted on metal stubs and stored in a desiccator for 7 days. The samples were sputtered with gold and evaluated by scanning electron microscopy (Inspect Scanning Electron Microscope-S50, FEI, Hillsboro, USA). Images were obtained at 200× and 1000× magnification.

Table 1 Nucleotides sequence of primers used for gene expression analysis

Gene	Sequence/Taqman Assay
β-*actin*	R: 5′ AGCCATGTAAGCCATCC 3′
	F: 5′ CTCTCAGCTGTGGTGGTGAA 3′
Alpl	R: 5′ GCTGATCATTCCCACGTTTT 3′
	F: 5′ CTGGGCCTGGTAGTTGTTGT 3′
Col1a1	R: 5′ CAGGGAAGCCTCTTTCTCCT 3′
	F: 5′ ACGTCCTGGTGAAGTTGGTC 3′
Dmp-1	Rn01450122_m1

Statistical analysis

Statistical analysis of cell viability and of the number of viable cells was performed by Kruskal-Wallis and Mann-Whitney tests. Data from gene expression assay presented normal distribution and homoscedasticity, being therefore analyzed by the fixed one-way ANOVA and Tukey tests. The level of significance was set on 5 %. Cellular morphology was descriptively analyzed.

Results

The viability of MDPC-23 cells irradiated with an energy dose of 4 J/cm^2 did not differ statistically from that of the control group. However, there was significant reduction in the viability of cells irradiated with a dose of 2 J/cm^2 (Fig. 1a). None of the energy doses increased the number of viable cells, since the counts did not differ from those of the control. However, when the doses were compared with each other, a significantly higher number of viable cells were observed for the 4 J/cm^2 dose (Fig. 1b).

Of the genes investigated, only *Alp* expression was negatively influenced by irradiation with blue LED, irrespective of the energy dose used. Exposure of MDPC-23 cells to blue LED had no effect on the gene expression of *Col-1a1* and *Dmp-1* (Table 2).

The scanning electron microscopy (SEM) analysis (Fig. 2) demonstrated that irradiation under the parameters selected had no negative effects on the morphology of the MDPC-23 cell culture. At the dose of 4 J/cm^2, cytoplasmic and cell membrane projections continued to have an aspect of normality. The 4 J/cm^2-irradiated cells had large cytoplasm emitting multiple slender projections that allowed adhesion to the glass surface, similar to that observed for the control group. Likewise, some cellular mitosis were found both in the control group and the samples exposed to 4 J/cm^2. Considering the dose of 2 J/cm^2, cytoplasmic contraction of the cells adhered to the glass substrate was observed. However, no quantitative alterations were apparent for this group.

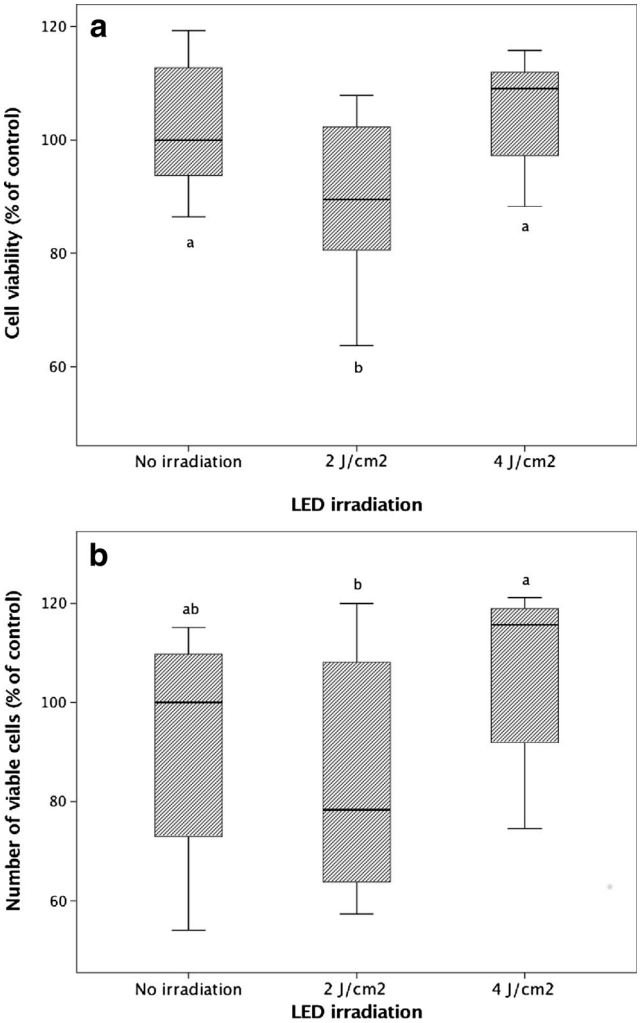


Fig. 1 Cell viability (**a**) and number of viable cells (**b**) of groups after LED irradiation are expressed as a percentage of control. MDPC-23 cells were irradiated with blue LED (455 nm) at energy doses of 2 or 4 J/cm^2. Non-irradiated cells were used as control. Boxes comprise 50 % of data; being the lower limit the 25 % and the upper limit the 75 %. The *horizontal lines* within the boxes consist of the median and the *lines outside bars* above and bellow the boxes indicate maximum and minimum values, respectively, of the dataset within each group ($n = 12$). Groups with the same letter do not differ statistically (Mann-Whitney, $p > 0.05$)

Table 2 Relative gene expression of alkaline phosphatase (*Alp*), collagen type I (*Col-1a1*), and dentin matrix protein (*Dmp1*) in MDPC-23 after blue LED irradiation

Energy doses	Gene expression		
	Alpl	*Col1a1*	*Dmp1*
0 (control)	100.0 ± 13.2^a	100.0 ± 14.5^a	100.0 ± 13.6^a
2 J/cm^2	67.4 ± 8.7^b	84.1 ± 10.2^a	81.4 ± 36.5^a
4 J/cm^2	70.8 ± 8.6^b	83.4 ± 23.5^a	117.4 ± 38.7^a

The housekeeping gene (β-*actin*) normalized the expression of target genes. Analysis of gene expression within groups was made as a percentage of control. Numbers are average ± standard deviation, $n = 6$

^{ab} Within each gene (column), groups identified by the same letter do not differ statistically (Tukey, $p > 0.05$)

Discussion

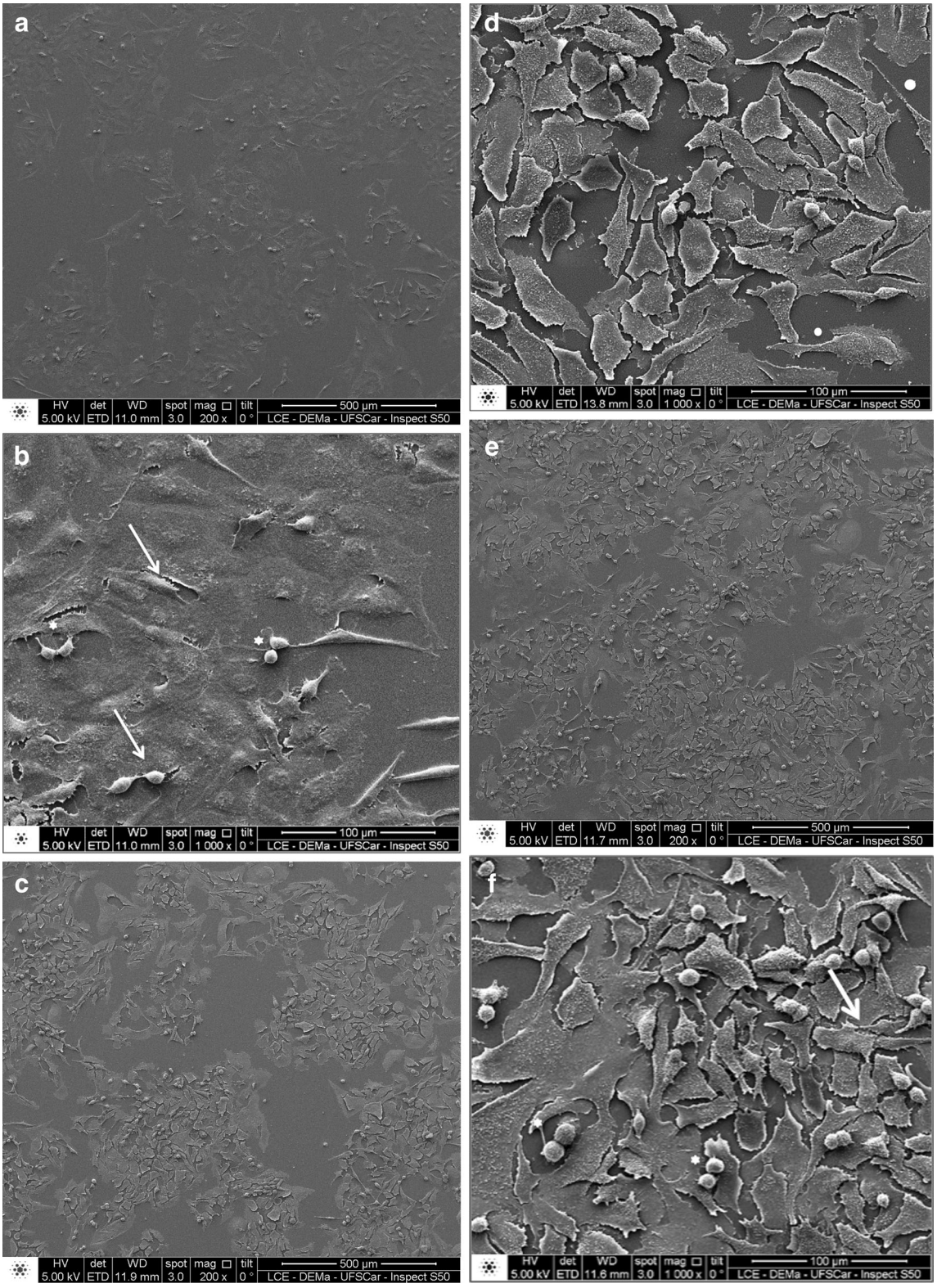
Light sources with various wavelengths have been used with the purpose of biostimulating cell lineages and/or promoting tissue repair [6–8, 10, 12, 14]. However, there are few studies about blue wavelength, which is widely used in dentistry as an instrument for light activating polymers [2, 3]. In the present study, we investigated the effect of LED in the blue wavelength (455 nm) on the metabolism of MDPC-23 cells, which present phenotypic characteristics of odontoblasts. These cells are essential to the pulp tissue repair process and are generally the first to suffer the effects of aggressions imposed on this tissue [24], by pathological processes such as dental caries or due to the treatment of these pathologies.

The physical parameters of phototherapy have an important effect on cell and tissue response [8, 9]. The energy doses used in the present study were 2 and 4 J/cm^2 and have been extensively cited in the literature as recommended energy dose for low level light therapy [9, 12, 19, 25, 26]. Those energy doses differ from that commonly used for light activation of dental materials (16 J/cm^2) [13].

The low level light therapy is primordially performed to biostimulate cells and tissues and must be within the limit of tolerance so that it does not cause cell death [8]. This is, perhaps, the greatest challenge in light therapy: to define the best set of physical parameters for each cell lineage and tissue [9], in order to allow reproducibility of the technique [16].

The viability of MDPC-23 cells was not negatively affected by blue LED at an energy dose of 4 J/cm^2. However, exposure to 2 J/cm^2 had a deleterious effect, promoting reduction in the cell respiratory metabolism. It may be suggested

Fig. 2 SEM photomicrographs representative of MDPC-23 cell morphology. Non-irradiated cells (control group) are shown on panels **a** and **b** indicate. **a** General vision of adhered cells on glass cover slip **b** MDPC-23 cell exhibited typical morphological, spindle-shape, as well wide cytoplasm with cytoplasmic processes (*arrow*) originating from their membrane that seemed to keep them attached to the glass substrate. It was observed also total adhesion to glass and some mitosis (*asterisk*). Representative images of cells after irradiation with blue LED (455 nm) delivering the energy dose of 2 J/cm^2 are presented on panels **c** and **d**. Overall view of adhered cells on the glass cover slip (**c**). It was seen that MDPC-23 cells exhibited typical spindle-shape, as well as multiple thin cytoplasmatic processes (*arrow*). Some cells had a contracted cytoplasm (*pointer*). Representative images of cells irradiated with blue LED (455 nm) delivering 4 J/cm^2 are shown on panels **e** and **f**. General view of adhered cells on glass cover slip (**e**) and **f** MDPC-23 cell exhibiting typical spindle-shape morphology and normal cytoplasm with several cytoplasmatic processes (*arrow*). It was also observed total adhesion to the glass slip and some mitosis (*asterisk*) as seen in the control group



that these energy dose values were not capable of stimulating the flavoproteins and cell chromophores, sensitized by the wavelength of blue light, which belong to site I of the electron transport chain present in the cell respiratory cycle [10, 27].

The phototherapy may exert cell stimulatory effects by accelerating the electron transport in the respiratory chain and consequently increasing ATP production [9, 28]. However, such effect was not seen in the present study, since none of

the irradiated groups had an increase in cell proliferation in comparison to the non-irradiated group. The effects of phototherapy have been recently considered biphasic [9], producing a nonlinear response [18]. The biphasic effect could explain the results seen in the present study since the 2 J/cm^2 dose reduced cellular viability, while 4 J/cm^2 dose did not differ significantly from the control group. Other studies have also demonstrated similar results by employing other physical parameters of light exposure [29–31]. Together, these studies ratify the concept that the effects of phototherapy are not linear.

Light in the blue wavelength may also be capable of increasing the production of reactive oxygen species (ROS) in a non-modulated manner [17]. This increase promotes damage to the cell cycle and effects on the integrity of DNA [32, 33]. This oxidative stress may lead to a reduction in the potential of the mitochondrial membrane and alter the distribution of this organelle in the cytoplasm [34].

Protein expression, such as Collagen and Dentin Matrix Protein, suffered no influence from exposure to blue LED light. However, both energy doses had deleterious effects on the gene expression of *Alp*. These proteins are related to dental extracellular matrix synthesis and its mineralization [35]. When using blue light LASER (405 nm) at a power density of 100 mW/cm^2, Kushibiki et al. [15] [15] observed an increase in collagen expression in prechondrogenic cells. Obviously, the differences between the studies do not allow direct comparison of the results; however, they reinforce the concept that positive results for light therapy depend on the cell stimulation threshold, defined by power density parameters, cell phenotype, and embryonic origin [10].

Positive effects were obtained with the irradiation of keratinocytes and fibroblasts with blue LED light [10, 36, 37]. Exposure to LED and Plasma Arc, using power density parameters of 140 and 466 mW/cm^2, resulted in an increase in human fibroblast cell viability, 72 h after exposure [37]. The period of evaluation after light irradiation is another aspect that must be considered. In previous investigations [12, 14] using the same cell line, the after-irradiation period of analysis used in the present study (72 h) was proven able to detect significant metabolic changes in odontoblast-like cells culture.

Pulp cells could be directly, in cases of pulp exposure or pulpotomies, or indirectly photostimulated. A dentin barrier between the cells and the light source was not used in the present study. However, the anisotropic property and the heterogeneity of dentin are known factors that change the direction of light propagation [38, 39]. Therefore, future investigations should also consider the effect of dentin on the light propagation that would better simulate a clinical situation where odontoblast cells would be irradiated throughout a dentin barrier in deep and very deep cavities.

The lack of standardization of studies makes it extremely difficult to make comparisons between the results [40], and at times, makes the clinical use of the therapy unfeasible. Once it has been demonstrated that each cell type present an optimal energy dose for exposure, known as the biphasic effect [41], other studies may be conducted, with variation of the physical parameters so that pulp cells receive the ideal stimulus for potentiating their action of pulp tissue defense and repair.

Conclusions

Considering the physical parameters investigated, blue LED (455 nm) had no biostimulatory effects on MDPC-23 odontoblast-like cells. When irradiated with 2 J/cm^2, the metabolism of these cells was negatively influenced.

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Compliance with ethical standards

Conflict of interest We declare no conflicts of interest.

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