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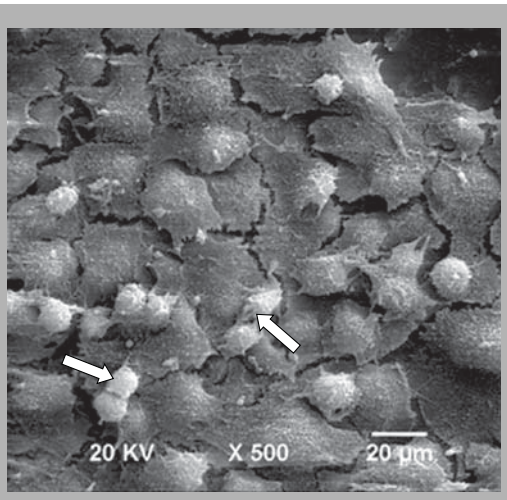
Abstract: In spite of knowing that cells under stress are biostimulated by low level laser (LLL) irradiation, the ideal condition of stress to different cell lines has not yet been established. Consequently, the aim of the present *in vitro* study was to evaluate the effects of a defined parameter of LLL irradiation applied on stressed odontoblast-like pulp cells (MDPC-23). The cells were seeded (12500 cells/cm^2) in wells of 24-well plates using complete culture medium (DMEM) and incubated for 24 hours. Then, the DMEM was replaced by a new medium with low concentrations (nutritional stress condition) of fetal bovine serum (FBS) giving rise to the following experimental groups: G1: 2% FBS; G2: 5% FBS; and G3: 10% FBS. The cells were irradiated three times with LLL in specific parameters (808±3 nm, 100 mW, 1.5 J/cm^2) every 24 hours. No irradiation was carried out in groups G4 (2% FBS-Control), G5 (5% FBS-Control), and G6 (10% FBS-Control). For all groups, the cell metabolism (MTT assay) and morphology (SEM) was evaluated. The experimental groups showed enhanced cell metabolism and normal cell morphology regardless of FBS concentration. A slight increase in the cell metabolism was observed only in group G2. It was concluded that cell nutritional stress caused by reducing the concentration of FBS to 5% is the most suitable method to assess the biostimulation of LLL irradiated MDPC-23 cells.



MDPC-23 cells adhered to the glass substrate exhibited a spindle-shaped morphology and some mitosis were observed (arrows); SEM ×500

Nutritional stress enhances cell viability of odontoblast-like cells subjected to low level laser irradiation

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1. Introduction

Low level laser (LLL) has been extensively investigated in the search for ideal conditions of cell biomodulation [1–5]. The LLL interacts with different types of tissues and LLL therapy (LLLT) is known to cause varied reactions and modulate several biological processes. It has been demonstrated that the photo-stimulation mediated by the LLLT, in addition to increase ATP synthesis [6], produces an analgesic effect [7–9] and stimulates cell division [10], accelerating tissue healing [11–13]. Some specific laser types also have the capacity to stimulate the synthesis and deposition of collagen matrix by fibroblasts [14–16], and the increase of mitochondrial activity and cell metabolism [8] and proliferation [12].

It has been demonstrated that different cell types under stress conditions are more sensitive to the biomodulatory

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effect of laser irradiation [17–20]. The cell stress mechanisms may range from alterations in the oxidative agents and the temperature of the culture medium to nutritional deficit (decrease of fetal bovine serum (FBS) concentration in the culture medium), the latter being more effectively used to evaluate the effects laser irradiation on cell metabolism [13,17,21–23].

A.N. Pereira, et al. in 2002 [24] reported an increase in the proliferation of NIH 3T3 cells placed under stress condition, that is seeded in a culture medium supplemented with 2.5% FBS. On the other hand, L. Almeida-Lopes et al. in 2001 [17] reported that fibroblasts seeded in culture medium without FBS supplementation did not show growth, while the addition of 5% FBS to the medium resulted in a lower cell proliferation rate than that obtained with the ideal FBS supplementation (10%), but high enough to induce a stress condition *in vitro*.

To date, no study has established a safe nutritional deficit to induce cell stress without causing cell damage [25]. The purpose of this study was to evaluate the effects of LLLT with a dose of 1.5 J/cm^2 on odontoblast-like cell (MDPC-23) cultures subjected to different nutritional deficit conditions, using a near infrared InGaAsP diode laser prototype specifically designed to provide a uniform irradiation of the wells with pre-established laser parameters.

2. Material and methods

2.1. MDPC-23 cell culture

Immortalized cells of the MDPC-23 cell line were defrost and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), with 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM/L glutamine (Gibco, Grand Island, NY, USA) in an humidified incubator with 5% CO_2 and 95% air at 37 ° C (Isotemp; Fisher Scientific, Pittsburgh, PA, USA). The MDPC-23 cells were subcultured every 3 days until an adequate number of cells were obtained for the study.

2.2. LLLT on the MDPC-23 cells

The MDPC-23 cells at 12500 cells/cm^2 concentration were seeded in 12 wells of sterile acrylic 24-well plates using plain DMEM supplemented with 10% FBS (total of 24 wells in each group). The plates were maintained in the humidified incubator (Isotemp; Fisher Scientific, Pittsburgh, PA, USA) with 5% CO_2 and 95% air at 37°C for 24 hours. Thereafter, the culture medium was aspirated and fresh DMEM supplemented with either 2 or 5% FBS (for induction of stress by nutritional deficit) or 10% FBS (ideal condition) was applied to the cells for 24 hours. After this period and immediately before laser irradiation, the culture medium was renewed, maintaining the FBS concentrations (2, 5, or 10%), giving rise to the experimental and control groups shown in Table 1.

Laser dose	**% FBS**			**Irradiation time**
	2%	5%	10%	
1.5 J/cm^2	G1	G2	G3	1 min 20 s
no irradiation	G4 (Control)	G5 (Control)	G6 (Control)	0 (no LLLT)

N = 12 specimens per group. FBS: fetal bovine serum. LLLT: low level laser therapy.

Table 1 Experimental and control groups formed according to the laser dose and cell stress condition (FBS concentrations)

The LLL device used in this study was a near infrared indium gallium arsenide phosphide (InGaAsP) diode laser prototype (LASERTable; 808±3 nm wavelength, 100 mW maximum power output) specifically designed to provide a uniform irradiation of the wells in which the cells were seeded.

The radiation originated from the LASERTable was delivered on the base of each 24-well plate at the pre-established laser dose. Although this diode laser has an output power of 100 mW, the laser light reached the MDPC-23 cells on the bottom of each well with a final power of 70 mW. The cells were irradiated every 24 hours totalizing 3 applications during 3 consecutive days [21,22,26–28]. After the last irradiation cycle, the 24-well plates were maintained in the humidified incubator (Isotemp; Fisher Scientific, Pittsburgh, PA, USA) with 5% CO_2 and 95% air at 37°C for an additional period of 3 hours [8]. Next, the cells were evaluated for the production of the succinic dehydrogenase (SDH) enzyme. The cells in the control groups received the same treatment as that of the experimental groups. The 24-well plates containing the cells in the control groups were maintained in the LASERTable for the same time used for irradiation of the corresponding experimental groups, though without activating the laser source. One control group was established for each experimental condition because the different periods that the cells remained out of the incubator for laser irradiation should be simulated in the control groups.

2.3. Analysis of cell viability (MTT assay)

Twelve of each experimental and control group were used for analysis of cell viability. Cell metabolic activity was evaluated by SDH activity, which is a measure of the mitochondrial respiration of the cells. For such purpose, the methyltetrazolium (MTT) assay was used [29].

Each well with the MDPC-23 cells received 900 μL of DMEM associated with 100 μL of MTT solution (5 mg/mL sterile PBS). The cells were incubated at 37 ° C

for 4 h. Thereafter, the culture medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) with the MTT solution were aspirated and replaced by 700 μL of acidified isopropanol solution (0.04 N HCl) in each well to dissolve the violet formazan crystals resulting from the cleavage of the MTT salt ring by the SDH enzyme present in the mitochondria of viable cells. After agitation and confirmation of the homogeneity of the solutions, three 100 μL aliquots of each well were transferred to a 96-well plate (Costar Corp., Cambridge, MA, USA). Cell viability was evaluated by spectrophotometry as being proportional to the absorbance measured at 570 nm wavelength with an ELISA plate reader (Multiskan, Ascent 354, Labsystems CE, Lês Ulis, France). The values of absorbance obtained from the three aliquots were averaged to provide a single value. Then, the inhibitory effect of the different groups on cell mitochondrial activity was calculated and expressed as medians.

2.4. Analysis of cell morphology by scanning electron microscopy (SEM)

Two specimens were used for analysis of cell morphology by SEM. However, for these two specimens per group, sterile 12-mm-diameter cover glasses (Fisher Scientific) were placed on the bottom of the wells immediately before seeding the MDPC-23 cells. After the experimental conditions, as reported above, the culture medium was removed and the viable cells that remained adhered to the glass substrate were fixed in 1 mL of buffered 2.5% glutaraldehyde for 24 hours and post-fixed with 1% osmium tetroxide for 1 hour. The cells adhered to the glass substrate were then dehydrated in a series of increasing ethanol concentrations (30, 50, 70, 95, and 100%) and immersed in 1,1,1,3,3,3-hexamethyldisilazane (HMDS; Acros Organics, Springfield, NJ, USA) for 90 min, as described elsewhere (C.A. de Souza Costa et al., 2008) and stored in a desiccator for 24 h. The cover glasses were then mounted on metallic stubs, sputter-coated with gold and the morphology of the surface-adhered L929 and MDPC-23 cells was examined with a scanning electron microscope (JEOL-JMS-T33A Scanning Microscope, Tokyo, Japan).

2.5. Statistical analysis

The SDH enzyme activity data were analyzed statistically by two-way analysis of variance complemented by Tukey tests for pairwise comparisons. A significance level of 5% was set for all analyses. The means obtained in the control groups (non-irradiated cells) were used as indicative of 100% cellular metabolism in order to calculate the percentage of metabolism after irradiation.

Irradiation	**% FBS**		
	2%	5%	10%
1.5 J/cm^2	(G1) 0.211 (0.021)^{*,a,b}	(G2) 0.184 (0.035)^{b,c}	(G3) 0.194 (0.047)^{a,b,c}
no irradiation	(G4) 0.238 (0.040)^{a}	(G5) 0.166 ((0.046)^{c}	(G6) 0.206 (0.020)^{a,b,c}

* The values represent mean (standard deviation), n=12.

^{a} Same lowercase letter indicate no statistically significant difference (Tukey, $p > 0.05$).

Table 2 SDH enzyme activity (optical density) detected by the MTT assay according to the irradiation and FBS concentration (%FBS)

3. Results

3.1. SDH enzyme activity (MTT assay)

The SDH enzyme activity data (MTT assay) for the irradiated (1.5 J/cm^2) and non-irradiated cells according to the percentage of FBS added to the culture medium are presented in Table 2.

For the non-irradiated cells the supplementation of the culture medium with 5% FBS (G5) resulted in statistically significant lower activity of SDH enzyme when compared with the activity seen when only 2% FBS (G4) was used. However, 5% and 10% FBS did not differ statistically as well as 2% and 10%. Comparing the concentrations of FBS for the irradiated group, the values did not differ statistically when the culture medium was supplemented with 2% (G1), 5% (G2), or 10% (G3).

For all the concentrations of FBS, the irradiated group did not differ statistically from the respective control group ($p > 0.05$). However, in groups G1, G2, and G3, the percentage of cell metabolism recorded for the irradiated cells considering the control groups as 100% metabolism was 81.7%, 118.2%, and 97.2%, respectively. Therefore, a slight increase in the activity of SDH was recorded only when the culture medium was supplemented with 5% FBS (G2).

3.2. SEM analysis

In all the control groups (G4, G5, and G6), a large number of MDPC-23 cells that remained attached to the glass substrate was close to the confluence. Those cells with spindle-shaped morphology presented several thin cytoplasmatic processes originating from their large membrane. The cytoplasmatic processes seemed to maintain the cells adhered to the glass discs (Fig. 1a and Fig. 1b). For all the experimental groups (G1, G2, and G3), in which the MDPC-23 cells were submitted to LLLT irradiation, no

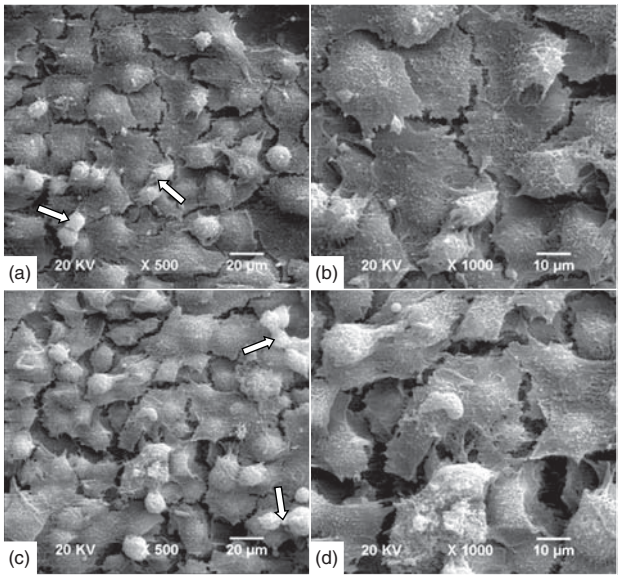

Figure 1 (online color at www.lphys.org) Panel of SEM micrographs representative of cell morphology in each group. (a) – (representative of all control groups): MDPC-23 cells adhered to the glass substrate exhibited a spindle-shaped morphology and some mitosis were observed (arrows); SEM ×500. (b) – high magnification of Fig. 3a. Note that the odontoblast-like cells exhibit several thin cytoplasmatic prolongations originating from their membrane; SEM ×1000. (c) – (representative of all irradiated groups): MDPC-23 cells with similar morphology covering the entire glass substrate are seen. As observed in control the groups, mitosis frequently occurred (arrows); SEM ×500. (d) – high magnification of Fig. 3c. The cells that remained attached to the glass substrate exhibit large cytoplasmic membrane, from which and a number of thin and short prolongations are originated. SEM ×1000

significant morphological alterations were observed in either of the FBS concentrations, and the results were very similar to those observed in the control groups (Fig. 1c and Fig. 1d).

4. Discussion

The light source has been applied in several health fields, as in the photodynamic therapy (PDT) used as therapeutic modality for both various cancers as cisplatin-resistant ovarian [30], using some photosensitzer, as protoporphyrin IX efflux for ALA-PDT [31] and 2-devinyl-2-(1-methoxyl-ethyl) chlorin f (CPD4) [32], oxygen and irradiation. However, the direct or indirect mechanisms of action of the LLLT on cells remain unclear [8,22]. It is speculated that the laser light is first absorbed by the tissue. In a second moment, this energy is transferred to intracellular components, promoting photoelectrical effects that regulate the cell system by means of a process known as biomodulation. When applied at adequate doses, lasers with wavelength in the near infrared spectral region, such as the InGaAsP diode laser used in the present study, can reach the mitochondrial membrane of the cells and be absorbed by the chromophores, thus stimulating the cell functions [7]. Among these functions, the most frequently investigated with respect to biomodulation are proliferation cell, tissue healing associated with the release of proteins and cytokines, and the synthesis of collagen matrix [28,33–35].

Laser research studies have used nutritional deficit as a means to cause stress to different cell cultures. DMEM supplementation with 2% and 5% FBS to induce cell stress is based on the findings of previous studies that demonstrated that cell cultures responded more effectively to an irradiation stimulus when subjected to nutritional deficit conditions, since the mechanisms of cell response were sensitive to the absorption of irradiation [17,21–23]. In line with this concept, the present study evaluated different low FBS concentrations in odontoblast-like cell cultures, but respecting the threshold not to cause a harmful cell stress condition [17]. L. Almeida-Lopes et al. in 2001 [17] have demonstrated that FBS-free culture medium applied on laser-irradiated fibroblasts did not produce a satisfactory bioestimulatory response when compared to the 5% FBS-enriched medium. In the same way, C.F. Oliveira et al. in 2008 [25] did not observe biostimulation after irradiation of odontoblast-like cells seeded in culture medium without FBS. However, the lack of scientific data demonstrating the influence of the different FBS concentrations present in the culture medium on cell metabolism makes it difficult to compare the results to those of studies on laser irradiation of cell cultures.

The present study evaluated the influence of 2, 5, and 10% FBS supplementation of DMEM on odontoblast-like cells irradiated with specific LLL parameters. Regarding the metabolic activity, the MDPC-23 cells responded more favorably to the 5% FBS concentration when exposed to the laser light at 1.5 J/cm^2, though without statistically significant difference from the other groups. This result is in accordance with the findings of L. Almeida-Lopes et al. in 2001 [17]. A.N. Pereira et al. in 2002 [24] also observed an increase in the proliferation of NIH 3T3 cells subjected to stress conditions (supplementation of the culture medium with 2.5% FBS). On the other hand, in the present study, the irradiation of MDPC-23 cells seeded in a culture medium supplemented with 2% FBS reduced significantly the metabolism of this cell type. Therefore, it may be suggested that FBS concentration to produce a non-deleterious stress stimulus that has a bioestimulatory effect on the cells is specifically related to each cell type.

The increase of cell metabolism and proliferation are not the only desirable effects of LLLT. Regarding specifically the MDPC-23 cells used in this study, the biomodulation would also stimulate the synthesis of typical dentin matrix proteins, such as collagen, fibronectin and alkaline phosphatase, providing local tissue healing without causing morphological cell alterations [33]. The results suggest

that the decrease of FBS concentration did not cause damage to the cells under any of the tested conditions since the SEM analysis did not show significant morphological alterations and the irradiated groups maintained the similar characteristics as those of the control groups. The laser prototype (LASERTable) used in the present study standardizes the irradiation protocol and thus did not interfere in this result.

Finally, the results of the present study are relevant for understanding the mechanisms of interaction of the laser light with odontoblast-like cells. An *in vitro* investigation is the first step for understanding the cell/light interaction. Therefore, defining adequate irradiation parameters and culture medium are key factors since this is basic knowledge to establish standardized research protocols for the development of future LLLT studies in the dental research related to the healing of the dentin-pulp complex.

5. Conclusion

Under the tested conditions, the cells irradiated with a laser dose of 3 J/cm^2 and experimentally subjected induced nutritional deficit (culture medium supplemented with 5% FBS) presented an increase in cell metabolism, and this condition was the most favorable for biomodulation with LLLT.

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