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Nutritional deprivation and LPS exposure as feasible methods for induction of cellular — A methodology to validate for vitro photobiomodulation studies

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ABSTRACT

Previous studies have demonstrated that high biostimulation takes place when cells under stress are subjected to phototherapy by laser or light-emitting-diode (LED) devices. Several studies selected nutritional deprivation by reducing the concentration of fetal bovine serum (FBS) in the culture medium or the exposure of cultured cells to lipopolysaccharide (LPS) as an in vitro cellular stress condition. However, there are no data certifying that these stimuli cause stressful conditions for cultured cells. This investigation assessed the induction of cellular stress by decreasing the concentration of FBS or adding LPS to culture medium. Odontoblast-like cells (MDPC-23) were cultured in complete culture medium (DMEM) containing 10% FBS. After a 12-hour incubation period, the DMEM was replaced by fresh medium containing 10% FBS (control), low concentrations of FBS (0, 0.2, 0.5, 2, or 5%) or LPS from *Escherichia coli* (10 μg/ml). After an additional 12-hour incubation, cell viability, total cell-counting, total protein production, and gene expression of heat shock protein 70 (HSP70) were assessed. Data were statistically analyzed by ANOVA complemented by the Tukey test, with 5% considered significant. Cell viability was negatively affected only for 0% FBS, while reduced viable cell numbers and total protein production were detected for FBS concentrations lower than 2%. Higher HSP70 gene expression was also observed for FBS concentrations lower than 2% and for cells exposed to LPS. The nutritional deprivation model with culture medium lower than 2% of FBS can be safely used to induce cellular stress for in vitro photobiomodulation studies.

1. Introduction

Photobiomodulation using low-level laser therapy (LLLT) or light-emitting diodes (LED) biostimulates different cell types, such as fibroblasts, osteoblasts, epithelial cells, and odontoblast-like cells, enhancing viability, proliferation, and protein synthesis as well as inducing cell differentiation, migration, and gene expression of proteins related to tissue healing [1–7].

The descriptions of these effects are recent, and diverse irradiation protocols have been evaluated in an attempt to standardize this therapy, providing better comparison of results and elucidation of biological issues [8–11].

Previous studies demonstrated that high biostimulation takes place when cells are irradiated under stressful conditions, such as nutritional deprivation [5,8,9,12]. According to the authors, this in vitro stressful cell conditions seem to mimic the stress found in damaged tissues. Another experimental in vitro cell stress stimulus also used for photobiomodulation study is the addition of lipopolysaccharide (LPS) to the culture medium [13]. Nutritional deprivation and LPS exposure were applied by several studies as a cellular stress model [1,13–19]. However, besides decreased cell metabolism was observed, it seems important to validate these models as reliable for inducing cellular stress in vitro.

In this study, the authors used LPS to induce inflammatory mediator synthesis in cultured odontoblast-like cells (MDPC-23). These pulp cells, which were isolated from rodent dental papillae and are phenotypically similar to odontoblasts [20], have been widely used to assess different phototherapy protocols associated or not to nutritional deprivation as stressful condition [8,9,11].

Cellular stress occurs in the presence of harmful stimuli such as temperature deviation, microorganism exposure, local ischemia, or hypoxia, which promote a specific cellular response to reestablish cell and tissue homeostasis [21,22]. In these conditions, several cell functions can be altered, cell viability is decreased, and the expression of heat shock proteins (HSPs) is up-regulated [22]. These proteins are responsible for stimulating some cell functions that promote cell adaptation or even induce cell apoptosis [23]. Therefore, enhanced HSP expression may be related to the induction of cellular stress [22].

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It has been demonstrated that the HSP family consists of approximately 15 different groups of proteins [24]. However, the HSP that has most often been assessed relative to cellular stress is HSP70 [22,25]. Despite the fact that the effects of nutritional stress on cell behavior have already been evaluated in different cell types, the expression of HPS70 by cells under nutritional stress conditions has not been shown. The aim of this study was to validate FBS deprivation and LPS exposure as a feasible method to induce cellular stress by means of evaluation of cell viability, viable cell numbers, total protein production, and gene expression of HSP70 by odontoblast-like MDPC-23.

2. Materials and Methods

2.1. Cell Culture

Immortalized odontoblast-like MDPC-23 cells [9,11,18] were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco), 2 mmol/l glutamine (Gibco), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in an atmosphere of 5% CO_2 until sufficient cells were obtained for the study.

2.2. Induction of Cellular Stress

Cells were seeded (4×10^4 cell/cm^2) with plain DMEM complemented by 10% FBS in wells of 24-well plates (TPP, Techno Plastic Products, Trasadingen, Switzerland) for 12 h, after which experimental cellular stress was induced by replacement of the complete DMEM with fresh DMEM containing different concentrations of FBS or *Escherichia coli* (LPS, 10 μg/ml), giving rise to the following groups: G1 — DMEM + 10% FBS (control); G2 — DMEM + 5% FBS; G3 — DMEM + 2% FBS; G4 — DMEM + 0.5% FBS; G5 — DMEM + 0.2% FBS; G6 — DMEM + 0% FBS; and G7 — DMEM + LPS. Cells were incubated in contact with the DMEM containing different concentrations of FBS or LPS for 12 h.

2.3. Cell Viability — MTT Assay

Cell viability was assessed by the methyltetrazolium (MTT) assay [9], which determines the activity of the SDH enzyme, which is a measure of cellular (mitochondrial) activity and can be considered the metabolic rate of cells. For this purpose, a 900-μl volume of serum-free DMEM was added to each sample, followed by the addition of MTT solution (5 mg/ml in sterile PBS) (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated at 37 °C for 4 h. Thereafter, the culture medium with the MTT solution was aspirated and replaced by 600 μl of acidified isopropanol solution (0.04 N HCl) to solubilize the violet formazan crystals resulted from the cleavage of the MTT salt by the SDH enzyme, resulting in a homogenous solution. After agitation to improve the homogeneity of the solutions, three 100-μl aliquots of each well were transferred to a 96-well plate (TPP). Cell viability was determined by absorbance measurement of the violet solution in a spectrophotometer (Synergy H1, BioTek, Winooski, VT, USA) at 570 nm.

2.4. Total Cell Number — Trypan Blue Assay

Total cell number was determined by the trypan blue assay [10]. After treatments, cells were detached from the cell culture plates with 0.25% trypsin (Gibco) for 10 min at 37 °C. Then, 10-μl aliquots of the cell suspension aliquots were mixed with 10 μl of 0.04% trypan blue solution (Sigma-Aldrich) and incubated at room temperature for 2 min. For cell-counting, 10 μl of the total solution was added to a cell-counting slide (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and inserted into an Automated Cell Counter (TC-10, Bio-Rad). Non-viable cells were determined by the intake of trypan blue dye, which characterizes the presence of membrane damage [25].

2.4.1. Total Protein Production

Total protein production was analyzed by the Lowry method [9]. Briefly, cells were treated with 0.1% sodium lauryl sulphate (Sigma-Aldrich) for 40 min at room temperature to promote cell lysis. Then, 1 ml of Lowry reagent (Sigma-Aldrich) was added to each sample, followed by incubation for 20 min. After that period, 0.5 ml of Folin–Ciocalteu's phenol reagent solution (Sigma-Aldrich) was added. 30 min later, three 100-μl aliquots of each sample were transferred to a 96-well plate, and the protein concentration was assessed by measurement of the solution's absorbance in a spectrophotometer (Synergy H1) at 655 nm, according to a standard curve containing established concentrations of bovine albumin (Sigma-Aldrich).

2.5. HSP70 Gene Expression

After treatment periods, gene expression of HSP70 was assessed by real-time polymerase chain reaction (PCR), as described below [10].

2.5.1. RNA Isolation and cDNA Synthesis

Briefly, after 12 h of incubation, total RNA was isolated by the Trizol method (Invitrogen, Carlsbad, CA, USA) [27]. After treatment, Trizol (Invitrogen) solution was added for 5 min, and samples were stored at −80 °C until RNA isolation. For this, samples were defrosted, treated with 0.2 ml of chloroform (Sigma-Aldrich), and incubated at room temperature for 2 min, followed by centrifugation (12,000 rpm, 15 min, 4 °C; microcentrifuge Eppendorf 5415R, Hamburg, Germany). After that, a three-phase solution was obtained. The first phase (aqueous) containing total RNA was collected and transferred to another tube. Isopropanol (0.5 ml; Sigma-Aldrich) was added, and samples were centrifuged (12,000 rpm, 10 min, 4 °C). The supernatant fraction was discarded, and 75% ethanol (Sigma-Aldrich) was added, followed by centrifugation (7500 rpm, 5 min, 4 °C). After the supernatant was discarded, samples were dried by inversion for 45 min.

The precipitated fraction was suspended in ultrapure water and incubated at 55 °C for 10 min. Samples were then subjected to evaluation of RNA concentration in a biophotometer (Eppendorf, RS 323C) at 1:49 dilutions.

cDNA was synthesized using the High Capacity cDNA Reverse Transcriptions Kit (Applied Biosystems, Foster City, CA, USA), as described by the protocol. The reagent mix was prepared for all samples (10 × RT buffer, 10 × RT random primers, 25 × dNTP mix and reverse transcriptase) and transferred to 200-μl tubes (10 μl per sample). After concentration measurement, one aliquot of 0.5 μg of RNA from each sample was added to each corresponding tube, and total volume was adjusted to 20 μl using ultrapure water.

Samples were subjected to cycling (25 °C, 10 min; 37 °C, 120 min; 85 °C, 5 s; and 4 °C thereafter) and were stored at −20 °C until the quantitative PCR protocol (qPCR).

2.5.2. qPCR

After cDNA synthesis, HSP70 expression was evaluated by real-time PCR. Reactions were prepared with Syber Green PCR Master Mix (Applied Biosystems), in addition to primer sets for HSP70 and βActin (endogenous control) (Table 1).

Fluorescence amplification was detected by Step One Plus (Applied Biosystems) and analyzed by Step One Software 2.1 (Applied

Table 1
Primer sequences for the evaluation of HSP70 gene expression by MDPC-23 cells subjected to FBS deprivation or LPS treatment.

Target gene	Primer sequences
HSP70	*Forward – 5′ CGACCTGAACAAGAGCATCA 3′* *Reverse – 5′ CCAAGGTCACCTCGATCTGT 3′*
βActin	*Forward – 5′ AGC CAT GTA CGT AGC CAT CC 3′* *Reverse – 5′ CT CTC AGC TGT GGT GGT GAA 3′*

Biosystems). All reactions were performed in the same conditions. Results of amplification cycles of HSP70 were normalized according to endogenous control expression (βActin).

2.6. Statistical Analysis

The resulting data on cell viability (MTT), cell-counting (TB), total protein production, and HSP gene expression were evaluated by ANOVA complemented by Tukey's test, at 5% significance level.

3. Results

Cell viability was affected only by total FBS deprivation, i.e., no addition of FBS to the culture medium (Fig. 1). The number cells, determined by trypan blue assay, showed a significant decrease in the amount of pulp cells in those groups in which <2% FBS were added to the culture medium (Fig. 2). LPS added to the culture medium did not change the number of viable cells. Similar results were observed for total protein production, since cells maintained in culture medium containing low concentrations of FBS exhibited a significant decrease in total protein production (Fig. 3). LPS did not affect the production of total protein by the cultured pulp cells.

Gene expression of HSP70 after selected stimuli demonstrated that LPS promoted the highest HSP70 expression. For FBS concentrations, 0, 0.1, 0.5, and 2% also increased such expression when compared with that in the control group (G1), in which 10% FBS was used (Fig. 4).

4. Discussion

Several in vitro studies that assessed the responses of cells subjected to photobiomodulation therapy have induced cellular stress before irradiation. It seems that this previous procedure causes the cells to be more sensitive to photobiomodulation [1,9,11,28].

FBS nutritional deprivation and LPS exposure are two standard protocols widely cited as feasible methods for the induction of cellular stress before the application of experimental in vitro photobiomodulation therapy [8,13,18]. The first protocol is commonly selected for cell proliferation, migration, and differentiation studies [11,28], while the second is often used for inflammatory modulation studies [13]. According to previous reports, the application of 5% FBS previously to cell irradiation causes a decrease in cell metabolism, creating an optimal condition to assess the phototherapy effects on cultured cells. However, besides the feasibility of these methods to reduce cell metabolic activity, there is a gap on the confirmation of these conditions as stress-inducing protocols, mainly for photobiomodulation investigations. Despite the decreased cell metabolism or viability, the induction of cellular stress may not be based on a single protocol application or a single cell function assessment.

In the present study, we assessed the cell viability, total cell-counting, total protein production, and gene expression of a heat shock protein (HSP70) to confirm the induction of cellular stress by nutritional deprivation (FBS reduction) or LPS exposure for MDPC-23 cells. Previous studies have demonstrated that, in stressful conditions, several cell functions can be affected in response to different endogenous and exogenous factors, like growth factors and glucose concentration [21,22]. Based on these findings, the results of the present study confirmed that nutritional deprivation can decrease cell viability and the numbers of cells as well as total protein synthesis. These results confirm the decreased cell metabolism demonstrated by previous studies that applied the nutritional deprivation model [1,9,11,28]. Conversely, cells exposed to LPS showed no significant functional inhibition.

The reduced number of cells for 0, 0.1, 0.5, and 2% FBS concentrations, detected by cell counting, may be related to inhibition cell proliferation, since the low FBS concentrations are also associated with decreased disposable growth factors for the cultured cells. Therefore, the authors suggest that cells presented a FBS-dependent proliferation rate. In addition, the decreased growth factors concentration in the culture medium may lead to the activation of different cellular pathways, such as NFκb, increasing ATP formation and mitochondrial activity. Taken together, these data seem to represent an attempting of the MDPC-23 cells to maintain their homeostasis during a stressful condition.

HSP70 has been proven to be up-regulated during conditions of cellular stress [29,30]. Previous studies have demonstrated that the HSP70 protein plays an important role in cell metabolism, showing both a housekeeping function (building components of crossing and signal transduction pathways) and a quality control function (reviewing the structure of proteins and repairing misfiled conformers) [28]. Moreover, HSP70 can promote cell survival by interfering with different

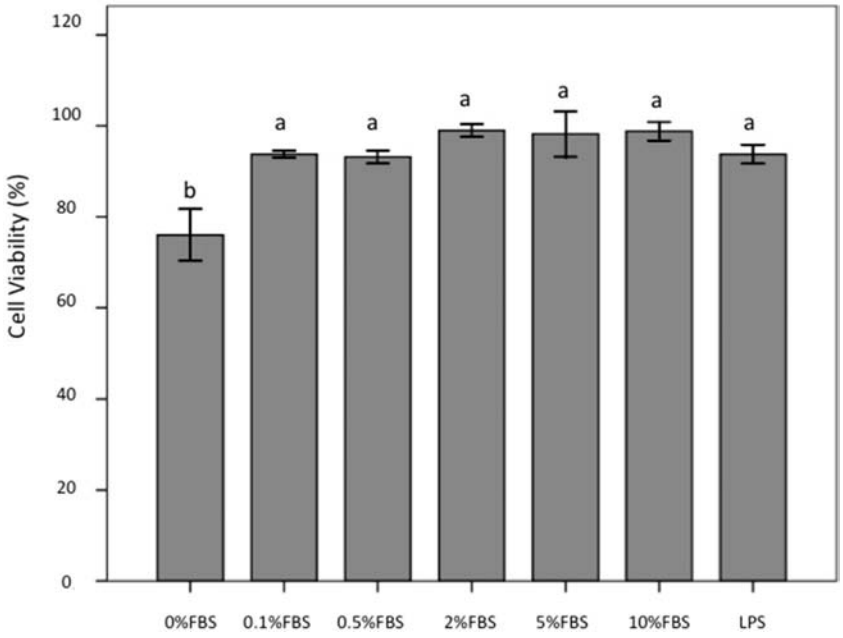

Fig. 1. Cell viability of odontoblast-like cells after nutritional deprivation by decreasing FBS concentration and LPS treatment. Different letters indicate statistically significant difference among groups (Tukey, $p < 0.05$).

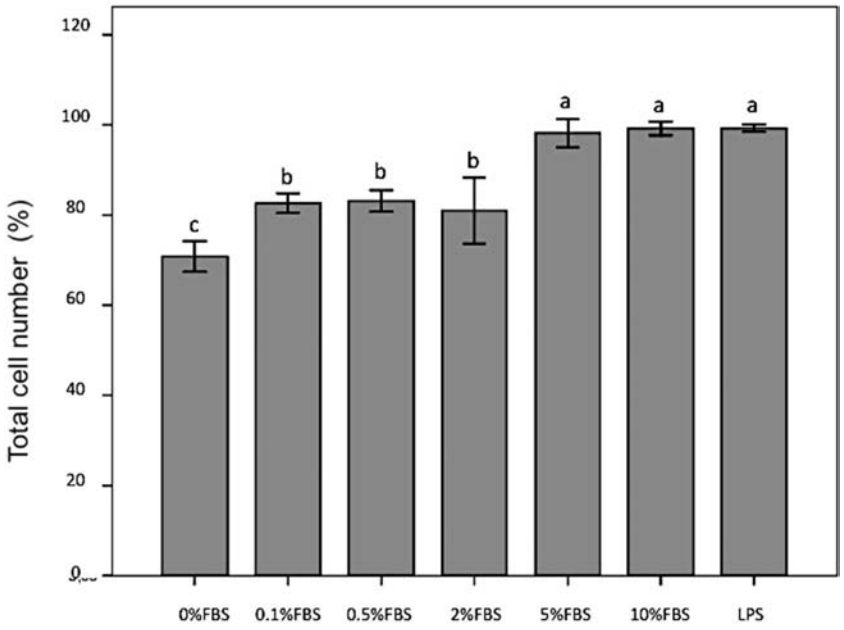

Fig. 2. Total cell-counting of seeded cells after induction of cellular stress by FBS deprivation and LPS treatment. Different letters indicate statistically significant difference among groups (Tukey, $p < 0.05$).

apoptosis components [30]. Therefore, results of the present study also showed that nutritional deprivation, actually termed nutritional stress, by decreasing FBS concentrations in the culture medium, can be used as a cellular stress induction method for odontoblast-like cells. However, only FBS at concentrations lower than 2% produced a significant increase in HSP70 expression, indicating that 5% of FBS can still be considered as non-stressful, at least for MDPC-23 pulp cells. Therefore, the confirmation of cellular stress induction relies on the application of various methods to assess cell functions using different protocols, the careful evaluation of all data obtained, as well as the combination of affected cell functions for different cell lines.

Richter et al., [22] demonstrated that once gene expression of HSP70 is stimulated, several cell functions can be modulated, such as protein synthesis, cell migration, proliferation, and apoptosis. In this way, biostimulation of cells under stress by photobiomodulation therapy may be related to HSP70 activation. Conversely, one may suggest that lack of photobiostimulation of cells in homeostasis (maintained in DMEM + 10% FBS conditions) or even incubated in culture medium containing only 5% FBS occurred because of the low sensitivity of those non-stressed cells to the experimental photobiomodulation therapy [9]. Only scarce data are available concerning the pathway by which mammalian cells are biostimulated by light irradiation [7,31, 32]. However, the results of the present in vitro study, demonstrating that specific stressful cell conditions can be safely induced in cultured pulp cells, especially for MDC-23 cells, may drive further studies concerning phototherapy, allowing researchers to better understand

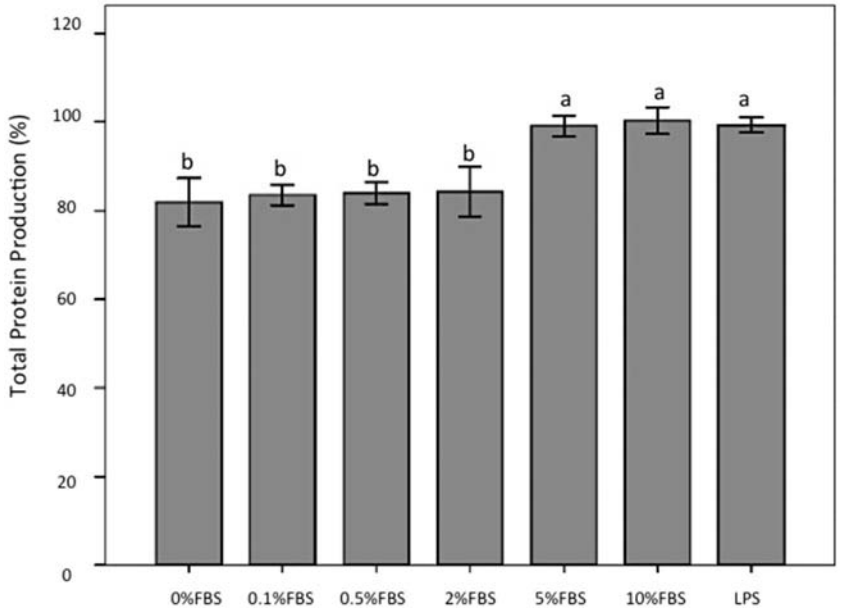

Fig. 3. Total protein production by MDPC-23 cells subjected to nutritional deprivation or LPS treatment. Different letters indicate statistically significant difference among groups (Tukey, $p < 0.05$).

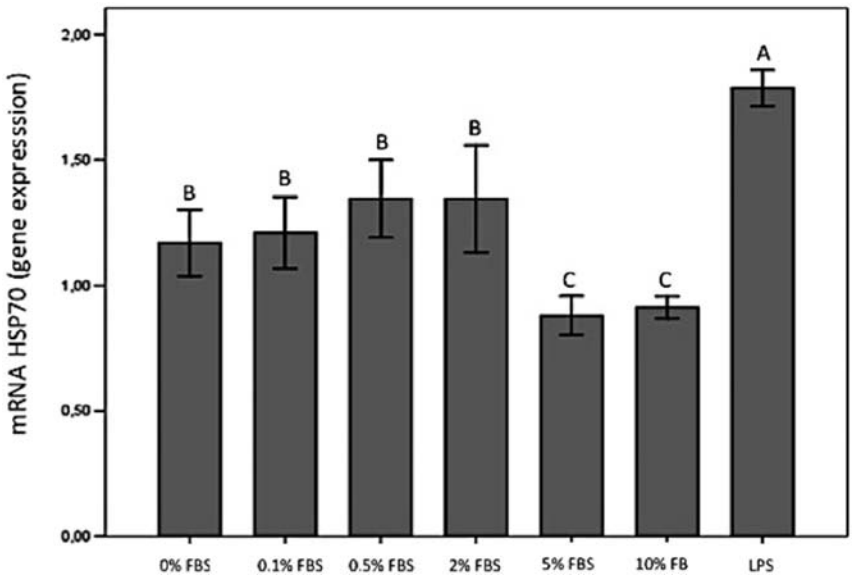

Fig. 4. HSP70 gene expression by MDPC-23 cells subjected to nutritional deprivation or LPS treatment. Different letters indicate statistically significant difference among groups (Tukey, $p < 0.05$).

the mechanisms of light/cell interactions. On the other hand, further studies are also needed to determine specific protocols for inducing stress in different cell lines.

5. Conclusion

According to the methodology and limitations of the present study, it can be concluded that concentrations of FBS lower than 2% or LPS exposure may be used to promote cellular stress for in vitro photobiomodulation studies using MDPC-23 cells.

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