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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′*