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Original Article

Laser photobiomodulation effect on fibroblasts viability exposed to endodontic medications

Gustavo Danilo Nascimento Lima[1], Luiz Alexandre Chisini[2,3]*, Marcus Cristian Muniz Conde[2], André Luís Faria e Silva[1], Flávio Fernando Demarco[3], Maria Amália Gonzaga Ribeiro[1]

[1] Department of Dentistry, Federal University of Sergipe, São Cristovão, Sergipe, Brazil

[2] Faculty of Dentistry, University of Vale do Taquari, Lajeado, RS, Brazil

[3] University of Pelotas, Pelotas, RS, Brazil

Corresponding author:
Luiz Alexandre Chisini
alexandrechisini@gmail.com

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Aim: The literature has not yet reported investigations about the effect of laser photobiomodulation (LPBM) over the cytotoxicity of drugs for endodontic treatments. Thus, the aim of this study was to evaluate, *in vitro, the* effect of the association between LPBM and intracanal medications on fibroblasts viability in different exposure times. **Methods:** Calcium hydroxide ($Ca(OH)_2$) and iodoform (IO) were used pure or associated to LPBM. Eluates of medications were prepared and placed in contact with the cells in three different periods: 24h, 48h and 72h. Laser irradiation (emitting radiation λ 660nm, power density of 10mW, energy density of 3 J/cm²) has been performed in two sessions within a six hour interval, for 12s per well. After each experimental time, the colorimetric assay (MTT) has been performed. Statistical analysis was applied for Mann-Whitney test with 5% α error admitted test. **Results:** At 24h, the use of LPBM did not increase cell viability while after 72h cell proliferation was stimulated in the group without medications. LPBM application did not increase cell viability in $Ca(OH)_2$ group and IO at any tested time. $Ca(OH)_2$ cytotoxicity at 24h was higher than iodoform, while at 72h not difference was observed. Therefore, after 72 hours was no statistical difference between the IO and $Ca(OH)_2$ groups. **Conclusion:** LPBM was able to increase cell viability in 72h in the group without medication, although no improvement was observed in the other groups. Thus, LPBM was not able to reduce the cytotoxic effects of the materials on fibroblasts *in vitro*.

Keywords: Endodontics. Low-level light therapy. Fibroblasts.

Introduction

Decontamination of the root canal system is the critical step to achieve success in endodontics, being performed through effective chemical-mechanical preparation[1-3]. With the introduction of rotatory systems, the working time has decreased and, consequently, the contact of disinfectant agents with the microorganisms presents in the root canal[4]. Thus, several irrigation solutions in different concentrations has been applied to achieve quick and effective disinfection[2]. However, some microorganisms can remain in the root canal system[1,2]. In this way, decontaminant pastes, as calcium hydroxide – $Ca(OH)_2$ – and iodoform – IO – are essential to combat the microorganisms resisting of root canal system[5].

These medications should be biocompatible, not carcinogenic, and/or genotoxic to periradicular tissue since they remain in contact with the periodontium[6]. Products of materials degradation in contact with existing cells in periradicular tissue can result in chemical irritation and inflammation, with a huge variety of chemical inflammatory mediators, which in high levels can cause tissue destruction and delay on healing process[7-9]. Therefore, these medications should present the ability to induce repair in the injured area without interfering with osteogenesis and cementogenesis[9-11]. Searching strategies to minimize tissue damage, the association between intracanal medications and laser photobiomodulation (LPBM) has been proposed[12,13]. LPBM is the term applied to describe the wide range of laser applications with low-energy densities and based on photochemical mechanisms where the energy is transferred to the intracellular mitochondrial chromophores and respiratory chain components[12,14]. Thus, LPBM is an electromagnetic radiation source with characteristics (monochromaticity, coherence, and one-pointedness) that differentiate it from other light fonts showing several clinical applications with anti-inflammatory, analgesic, and trophic-regenerative effects[12,13]. LPBM can induce cell's metabolic changes through a series of cascading reactions through photochemical and photoelectric with primary and secondary effects on exposed tissue to laser irradiation[12].

Its ability to stimulate the proliferation of various cell types such as fibroblasts, epithelial cells, lymphocytes, and odontoblasts, participating directly in the cell and tissue repair is directly linked to parameters used, including wavelength[15-18]. To the best of our knowledge, there is no report evaluating the effect of LPBM on fibroblasts viability exposed to different endodontic medications. Thus, this study aimed to evaluate *in vitro* the effect of the association between different exposure times to LPBM and intracanal medications on the viability of fibroblasts.

Materials and Methods

Cell culture: Fibroblast cells 3T3/NIH (previously cryopreserved[19]) were cultivated in DMEM (Dulbecco's Modified Eagle Medium- Cultilab, Campinas, SP, Brasil) supplemented with 10% fetal bovine serum (FBS) (Sigma Chemical Co. St. Louis, MO, USA) and 1% of penicillin (105UI/mL) (GibcoBRL, Gibco-Invitrogen, Grand Island, NY). The cells were maintained in an incubator (37°C, 5% CO_2, and 95% humidity)[20]. The culture medium was changed every two days. After reaching subconfluence (80%),

the medium has been removed and the cells were washed with phosphate-buffered saline (PBS). Tripsine/EDTA (Gibco BRL) was added for five minutes to create a cell suspension and inactivated with DMEM/FBS. Cells were counted in the Neubauer chamber and $2x10^4$ cells DMEM/FBS solution was applied in each well, in a 96 well plate. Cells were stored in an incubator for 24h to allow cell adhesion.

Manufacture specimens: For the made the specimens of calcium hydroxide group (HC) was used 1g PA calcium hydroxide (Biodynamic Chemicals & Pharmaceuticals LTD, PR, Brazil) + 1.1ml of distilled water. For Iodoform Group (IO) was used 1.5g of iodoform (K-dent, Quimidrol SC, Brazil) + 600 µL of distilled water. The materials from both groups were spatulate and placed in an array with the following formats ISO guidelines for cytotoxicity assays (ISO 10993-5: 2009). They were sterilized with ultraviolet (UV) radiation for 1 hour. Eluate was produced by dipping the samples in a solution of 1 ml of DMEM + 10% FBS and storing them in an oven at 37°C for 24 hours. After 24 hours of cell adhesion, the adhered cells on the plate were washed with PBS and 200 uL of the eluate was added to each well. Each eluate filled 4 plate wells (n=8 for the group).

After one and six hours were conducted at the experimental plates irradiation with the laser medium inserted directly into the cell monolayer and the different groups. Laser application was performed with wave-length in the red spectrum 660nm – InGaAlP- Twin-laser (MMOptics®, Equipamentos Ltda., São Carlos, São Paulo, Brasil) and $3J/cm^2$ for 12 seconds[18] (Table 1). Controls did not receive laser irradiation (n=8).

Table 1. Irradiation parameters for the groups $Ca(OH)_2$, IO and Control.

Irradiation Parameters	
Emission Mode (CW)	Continuous
Length (nm)	660
Active medium	InGaAlP
Optical power of the laser (output)(mW)	10
Optical power of the laser (input) (mW)	40
Field of beam spot (mm)	4
Area (cm^2 - $A=\pi.r^2$)(cm^2)	0,04
Power density (PD)(W/cm^2)	0,25
Energy density (ED)(J/cm^2)	3
Time per point (s)	12
Total energy (J)	0,24
Energy per session (J)	0,12 J/well
Divergence of the beam perpendicular to the junction	17º
Point angle	50º

Cell viability assay: After irradiation, the plates returned to the humid atmosphere for cytotoxicity testing 24h, 48h, and 72h. Cell viability was determined by colorimetric method MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium] (MTT

5 mg / ml DMEM). The MTT was maintained in contact with cells for 4 hours to allow the cells to metabolize and consequently reduce the MTT salt by dehydrogenases present in mitochondria of fibroblasts, thus forming formazan crystals[21]. After this period, the medium was sucked, and formazan crystals resuspended in 200µL of DMSO (dimethyl sulfoxide). The DMSO was placed in contact with the cells for 15 minutes then the plate was placed for 5 minutes on a shaker (150 rpm). Spectrophotometry universal reader ELISA, at a wavelength of 540 nm, assessed the results which were considered the values of absorbance as an indicator of cell viability. Assays were realized in triplicate.

Statistical analysis: Data were analyzed for the Mann-Whitney Rank Sum Test with 5% α error admitted. Sigmastat 3.5® software was used in the analysis.

Results

After 72hs LPBM group demonstrated the best results for cell viability maintenance, however, it has been observed exclusively in the group without medication ($p < 0,05$). In groups containing medications, LPBM was not able to increase cell viability (Table 2). In contrast, LPBM combined with IO demonstrated a decrease of absorbance in 24h. $Ca(OH)_2$ presented high initial toxicity which remained at all periods (24, 48 and 72h) while IO showed more cell viability in 24h compared to $Ca(OH)_2$. In a longer period (72h), IO showed toxicity similar to $Ca(OH)_2$.

Table 2. Median absorbance (nm) (25% / 75%) for the groups submitted to LBPM according to medications and time (n = 8) (Mann-Whitney test, $p \leq 0.05$).

Time	Laser	Median absorbance (25% 75%)		
		Medications		
		$Ca(OH)_2$	IO	Control
24h	No	#Aa 0.32 (0.26 / 0.35)	#Ba 1.49 (1.36 / 1.66)	#Ba1.17 (1.10 / 1.67)
	Yes	#Aa 0.29 (0.22 / 0.35)	*Aac 0.90 (0.18 / 1.1)	#Ba 1.67 (1.23 / 1.96)
48h	No	#Aa 0.29 (0.27 / 0.33)	#Bb 0.70 (0.55 / 0.85)	#Ca 1.08 (1.01 / 1.35)
	Yes	#Aa 0.29 (0.27 0.32)	#Ba 0.66 (0.41 / 0.89)	#Ba 1.11 (0.54 / 1.70)
72h	No	#Aa 0.28 (0.26 / 0.28)	#Ac 0.27 (0.25 / 0.29)	#Bb 0.39 (0.33 / 0.85)
	Yes	#Aa 0.27 (0.26 / 0.32)	#Ac 0.29 (0.26 / 0.31)	*Ba 1.55 (1.31 / 1.90)

Symbols (#, *) represents statistical differences of laser application at the same time
Uppercase letters represent differences in a row (different medications)
Lowercase letters represent differences over time within the same laser application or not

Discussion

LPBM has been attracting more interest in dentistry, with increasing applicability in this field. Several approaches have incorporated LPBM as an adjuvant to reduce the inflammatory process, aiming a modulation effect, as well as a restorative stimulus for bone, muscular and neural lesions[12,13,15,17,22,23]. The LPBM was used in this study, with the perspective of minimizing possible cytotoxic effects of endodontic drugs in contact with cells *in vitro*. The irradiation parameters used in this study were cho-

sen because the radiation in the visible wavelength is absorbed by the mitochondrial photoreceptor, resulting in photochemical effects, triggering a cascade of metabolic events resulting in a response to biomodulation[16,18,24]. Previous studies[18,25] also reported that the same energy density was able to show positive results in stimulation of fibroblasts' metabolism increasing the number of viable cells and their proliferation. In this way, we chose a wavelength of 660nm because recent studies have reported that shorter wavelengths, ranging from 600 to 700 nm, could be considered best to treat superficial tissue[18,26]. On the other hand, wider wavelengths, ranging from 780 to 950nm, should be chosen to deeper tissues[27,28]. Moreover, several studies[18,29] propose that variations from 3 to 10 J/cm^2 could produce the desired stimulation of metabolic activity.

Fibroblasts were used in this study due to the fact presented as the major constituent of connective tissue, being the most predominant cell type in the periodontal ligament and the largest producer of collagen, elastin, glycosaminoglycans and glycoproteins[30,31]. Intracanal medications when in contact with the periapical tissues cause an inflammatory response and the LPBM could provide an enhanced cellular response in the affected area. Although the LPBM proved better maintenance of the fibroblasts viability after 72h, it has not been observed in the presence of $Ca(OH)_2$ or IO. The basic biological mechanisms behind the effects of LPBM occurs through absorption of red light by chromophores mitochondrial cytochrome C oxidase, which is contained in the respiratory chain of mitochondria. Then, a cascade of events occurs, which leads to biostimulation of various processes, leading to increased enzymatic activity, mitochondrial respiration, transport of electrons, and production of adenosine triphosphate. The LPBM, in turn, alters the redox cell that induces the activation of many intracellular signaling pathways and alters the affinity of transcription factors related to cell proliferation, survival, tissue repair, and regeneration[32]. Therefore, when light is applied to cells, mitochondria are the initial sites of absorption. The photon absorption leads to nitric oxide dissociation, e reactive oxygen species production, and increased ATP synthesis[28]. Thus, photons at the red and infrared wavelengths can interact with specific photoreceptors located within the cell presenting beneficial results *in vivo* models[33].

$Ca(OH)_2$ and IO containing groups presented lower cell viability than the control, indicating the drugs' cytotoxicity effect. $Ca(OH)_2$ showed to be more cytotoxic than IO at 24 and 48 hours when compared to the group without medications. Besides, when it has been associated with the LPBM, there was none positive changes in the results. This fact may be related to pH increases promoted by $Ca(OH)_2$[24,34,35] which promotes enzymatic denaturation and destruction of the cell membrane, causing the cell's death[30] neutralizing the possible positive effect of LPBM. After 72h, there was no difference between $Ca(OH)_2$ and IO. In this way, a recent systematic review evaluating clinical studies showed that LPBM has a positive effect by avoiding dental pain after endodontic treatment since it reduces tissue inflammation[22]. Delay in the onset of pain or reduction in the pain severity and duration were also related to LPBM in endodontic treatment with different laser parameters, despite the studies have not investigated the combination with intracanal medications as the outcome[22,36].

Similar results were found by Sarigol et al.[37] (2010) in which an IO-base substance showed increasing cytotoxicity in fibroblasts over time. In contrast, it was noted in 24h greater aggressiveness of iodoform when compared to calcium hydroxide[38]. The laser therapy's modulator mechanisms are not yet fully understood. It is assumed that some effects from IO association with LPBM may be related to increased production of hydrogen peroxide by modulating the redox activity of mitochondria and/or the redox state of the cell can stimulate cell cycle and protein synthesis at lower concentrations, but which are very cytotoxic at higher concentrations[39].

In conclusion, LPBM was able to increase cell viability in 72h in the group without medication, whereas no improvement was observed in the other groups. Thus, LPBM was not able to reduce the cytotoxic effects of the materials on fibroblasts *in vitro*.

Compliance with Ethical Standards

Conflict of Interest: No conflict of interest.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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