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Evaluation of antibacterial photodynamic therapy effects on human dental pulp cell cultures

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Antibacterial photodynamic therapy;
Methylene blue;
Cytotoxicity;
Dental pulp cell;
Red laser

Summary
Background: The antibacterial photodynamic therapy (aPDT) has been used in dentistry against oral microorganisms because of its excellent biocide effect. However, for carious lesions applications, there is little evidence that this therapy is safe for the pulp tissue.
Objective: This study evaluates the effects of an aPDT protocol on human pulp cells *in vitro*.
Methods: Pulp cells isolated from dental pulp were exposed to an aPDT protocol associating methylene blue (MB) at concentrations of 0.0125, 0.025 and 0.050 mg/ml and red laser irradiation using a continuous-wave indium-gallium-aluminum-phosphide (InGaAlP) diode laser ($\lambda = 660$ nm, 40 mW, 2.4 J, 60 J/cm^2 for 1 min). Pre-irradiation time was 5 min for each MB concentration. Cell viability was determined by MTT assay and activity of alkaline phosphatase was assessed by BCIP-NBT assay. Type of aPDT-induced cell death was assessed by flow cytometry. Data was statistically compared (ANOVA followed by Tukey' or Bonferroni's *post hoc* tests).
Results: aPDT was able to kill pulp cells in a dye concentration-dependent manner. The cellular viability was significantly reduced when used MB at 0.025 or 0.050 mg/ml concentrations ($p < 0.0001$). At these concentrations, aPDT-induced cell death occurred mostly by necrosis. Alkaline phosphatase activity was significantly reduced in all experimental groups ($p < 0.001$). Pulp cells showed suitable viability when MB at 0.0125 mg/ml was exposed to laser irradiation.

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Conclusions: aPDT with MB at 0.0125 mg/ml may represent a low-risk therapy for restorative dentistry applications. aPDT protocol using concentrations above 0.025 mg/ml of MB associating red laser irradiation may be harmful for dental pulp cells.

Introduction

The treatment of carious lesions requires the stoppage of the disease progress by the mechanical removal of the infected dentin [1]. The infected dentin is the irreversible altered collagen matrix, highly contaminated with cariogenic microorganisms, that is no more capable for remineralization [2]. The affected dentin is present in the inner part of the carious lesion and despite it is contaminated with toxins and bacterial acids, does not necessarily has to be removed [1]. For the minimal invasive dentistry, the affected dentin has to be preserved. However, it is difficult to determine the limit infected-affected dentin [3] and, consequently, it might lead to an excessive removal of the dental tissue. On the other hand, infected dentin could not be completely removed, which can jeopardize the restorative treatment. For circumvent this problem an alternative therapeutic approach has been proposed, the antibacterial photodynamic therapy (aPDT), which can eradicate, or at least reduce, cariogenic microorganisms *in situ* [4—9].aPDT-induced cell death occurs due to the generation of cytotoxic species when a photosensitizer is activated by a light source at an appropriate wavelength in the presence of oxygen. The excitation of the photosensitizer results in the production of singlet oxygen and free radicals, which damage cell components [6,7,10].

Several studies have shown the effectiveness of aPDT against cariogenic microorganisms in biofilms, cell suspensions and even in dentin tissue, associating different dyes and light sources [4,5,9,11—15]. It was also previously reported that the association of methylene blue and red laser has successful results against *Streptococcus mutans*, a well-know bacterial strain involved in carious lesions initiation [14]. Nevertheless, the analysis of the aPDT effects on dental pulp cells is also of paramount importance considering that the residual dentin layer due to caries disease may lead to photosensitizer penetration, which when activated by light, could be a potential hazardous to the dental pulp tissue. Although the use of a series of photosensitizers in combination with different light sources has been well described on various cell lines over the past 20 years [16—20], there is no single report of application of methylene blue and red laser on dental pulp cells. It is important to establish the safety of this therapy on these cells considering that the dental pulp tissue has limited remodeling potential and is, consequently, sensitive to noxious stimuli. Bearing this in mind, the purpose of this study was to evaluate the effects of aPDT directly applied on human primary pulp cell cultures.

Materials and methods

Cell culture

Ten impacted third molars were extracted for orthodontic purposes from healthy patients (aged 15—21 years). The project was approved by the Universidade Federal de Minas Gerais Ethics Committee (Process Number 229/10). The teeth were collected in carrier medium DMEM (Sigma—Aldrich, St Louis, MO, USA) supplemented with 20% fetal bovine serum (GIBCO/Invitrogen, Grand Island, NY, USA), 1% penicillin, 1% streptomycin and 0.2% fungizone. All teeth were free from cracks and cervical lesions. Under a laminar-flow hood, teeth were cracked open with two forceps and the coronary pulps removed. Minced pulp tissues were digested into 3 mg/ml collagenase type I and 4 mg/ml dispase (GIBCO/Invitrogen, Grand Island, NY, USA) [21]. Dental pulp cells were not previously characterized according to its stemness and multilineage differentiation capabilities. Only cells in the 3rd to 5th passage were used in this study.

Photosensitizer

A stock aqueous solution of 0.050 mg/ml, corresponding to approximately 135 μM of methylene blue (Chimiolux, Aptivalux, Belo Horizonte, MG, Brazil), was used as the highest photosensitizer concentration. Subsequent dilutions of the stock solution were performed in phosphate buffered solution (PBS) to obtain final concentrations of 0.025 and 0.0125 mg/ml. All solutions were sterilized by filtration through a 0.22 μm membrane filter and only those freshly prepared were used for the experiments. The UV—vis absorption spectra of MB in PBS were recorded from 200 to 800 nm using quartz cuvettes on a diode-array spectrophotometer with maximum absorption peak of 665 nm [22]. The maximum absorption peak of MB corresponds to the wavelength emitted by the red laser (660 nm).

Antibacterial photodynamic therapy

Pulp cells were seeded at a density of 1.5×10^5 cells/well in 0.1 ml of growth medium in 96-multiwell plates, with empty wells adjacent to the test well, in order to avoid indirect light exposure [23]. At 48 h post-seeding, cell cultures were exposed to 0.0125; 0.025 or 0.050 mg/ml MB according to the experimental groups described in Table 1. After 5 min of pre-irradiation (PI) time exposure to MB the cells were submitted to laser irradiation using a continuous-wave