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Evaluation of photodynamic therapy on fibroblast viability and cytokine production

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ABSTRACT

Background: The aim of this study was to evaluate the effects of photodynamic therapy with curcumin (PDT) comparatively to 5% sodium hypochlorite (NaOCl) and saline solution on cell viability and cytokine (IL-1β and IL-6) production by mouse fibroblasts.
Methods: Sixty seconds of pre-irradiation time with curcumin 500 mg/L and Led wavelength (λ) 480 nm, 72 J cm^2, for 300 s was used for PDT. Solutions were diluted in culture medium DMEM (1×10^4 cells) and placed into 24-well cell culture plates with mouse fibroblasts L-929. Culture medium was used as control. After 6, 24 and 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) was used to evaluate the cell viability and the supernatant was collected for cytokine evaluation using enzyme-linked immunosorbent assay (ELISA). The results were statistically analyzed by ANOVA and BonFerroni correction ($p < 0.05$) for MTT and Kruskal–Wallis test and Dunn ($p < 0.05$) for ELISA.
Results: PDT and saline solution presented low cytotoxic effect similar to the control group ($p > 0.05$) while 5% NaOCl was more cytotoxic than PDT ($p < 0.05$) in all periods of time. All materials similarly expressed IL-1β and IL-6 regardless to the experimental period ($p < 0.05$).
Conclusions: PDT with curcumin was not cytotoxic to L929 fibroblasts differently from 5% NaOCl. In all groups occurred similar expression of IL-1β and IL-6.

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1. Introduction

Root canal cleaning and shaping are essential to reduce and/or eliminate the population of micro-organisms (MO) and their toxic products (endotoxins and apical biofilm) present in endodontic infection [1]. The use of irrigating solution that assist cleaning during endodontic treatment is essential to maximize the decontamination by its chemical action [2].

Sodium hypochlorite (NaOCl) is the most employed irrigation solution primarily for its antimicrobial action [2,3]. However, despite the scientific-technical progress, authors show the persistence of MO in root canal system post-treatment [1,2,4]. Therefore, new therapeutic strategies must be investigated to potentiate the combat of endodontic infections.

Recently new methods as photodynamic therapy (PDT) are used on treatments to promote disinfection in periodontitis, dental caries, endodontic diseases, among other dental specialties [5–7]. Photodynamic therapy uses specific wavelength light Laser (light amplification by stimulated emission of radiation) or Led

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(light emitting diode) that activates the photosensitizer (PS) and produces highly reactive specie of oxygen (singlet oxygen) inactivating the target cell [8,9] and assists antimicrobial action without the risk to promote microbial resistance [10]. Several kinds of PS may be associated with Laser or Led: hematoporphyrin derivatives, porphyrin, phenothiazine (toluidine blue and methylene blue), chlorophyll, fluorones (rhodamine B), cyanine phytotherapic agents, phthalocyanines and curcumin [5,11]. Curcumin, a compound from *Curcuma longa* L., the drug has a variety of applications, including treatment of liver diseases, wounds, inflamed joints and microbial effects [6,12,13].

In vitro studies with porphyrin and methylene blue PS [14] and in vivo studies with chlorophyll and methylene blue PS [14–17] demonstrated PDT as a new antimicrobial therapeutic modality aiming to increase the disinfection of root canal system during endodontic treatment. It was also evidenced with toluidine blue PS in PDT against *Enterococcus faecalis* once PS fixes in the cell membrane and reaches the peak of absorption leading to generation of singlet oxygen, which destroys cellular wall and leads to bacterial death [18]. However, Frota et al. [19], did not show total disinfection of root canals PDT against *E. faecalis* with curcumin PS.

Although PDT has been already employed for tumor treatment in oncology [20] its cytotoxicity effect is not completely understood, especially when using curcumin as a photosensitizer. Thus, the aim of this study was to determine the effects of PDT with curcumin comparatively to 5% NaOCl and saline solution on fibroblasts viability and on IL-1β and IL-6 cytokine releasing.

2. Materials and methods

2.1. Fibroblasts culture

L-929 mouse lineage fibroblasts were maintained in culture bottles with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD, USA), streptomycin (50 g/mL), and 1% antibiotic/antimycotic cocktail (300 units/mL penicillin, 300 μg/mL streptomycin, 5 μg/mL amphotericin B and 200 μg/mL of glutamin) (GIBCO BRL, Gaithersburg, MD, USA). The cultures were maintained under standard cell culture conditions (37 °C, 100% humidity, 5% CO_2) [21,22].

2.2. Group division and photodynamic therapy

It was used 50 μL of each solution diluted in 450 μL of DMEM. The solutions were 20 times more concentrated to get the desired concentration after dilution. A total volume of 500 μL was inserted in each well. The groups were distributed as follow: G1-Culture medium (control); G2-5% NaOCl; G3-Saline solution; G4-PDT.

The PDT was performed with curcumin at 500 mg/L [1,7-*bis*-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (PDTPharma, Cravinhos, SP, Brazil), in the period of 60 s of pre-irradiation as recommended by Paschoal et al., 2013 [6]. Then the PS was activated with blue Led for λ 480 nm, 72 J cm^2, (Physics Institute of São Carlos, University of São Paulo, São Carlos, SP, Brazil) for 300 s [6]. During the pre-irradiation of PS and also during the activation of the LED, the lighting of the laminar flow chamber was turned off and the ambient light in the laboratory was reduced to avoid PS degradation.

2.3. Cytotoxicity testing

L929 fibroblasts were seeded into the 24-well plates (1×10^4 cell/well). The cells were incubated for 24 h in a humidified air atmosphere of 5% CO_2 at 37 °C. The solutions were tested for 6 h, 24 h and 48 h. Tree wells were used for each solutions, and wells with DMEM were used as the control. Viable cells were stained with formazan dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (MTT) (Sigma Chemical Co., St. Louis, MO) that was dissolved in phosphate- buffered saline at 5 mg/mL and filtered in order to sterilize and remove a small amount of insoluble residue. At the experimental times, stock MTT solution (40 μL per 360 μL medium) was added to all wells of an assay, and plates were incubated at 37 °C for 3 h. The medium was then removed by the inversion of the plate and 200 mL of isopropilic alcohol was added to the wells and mixed during 20 minutes in order to dissolve the dark blue crystals. The blue solution was transferred to a 96-well plate, and the absorbance was read in the microplate reader using 570 nm wavelength [23]. The treatments were classified according to the cytotoxic effect of: 0—non-cytotoxic (less inhibition than 25%); 1—slightly cytotoxic (inhibition between 25% and 50%); 2—moderately cytotoxic (inhibition between 50% and 75%) and 3—strongly cytotoxic (greater than 75% inhibition). Assays were performed in triplicate.

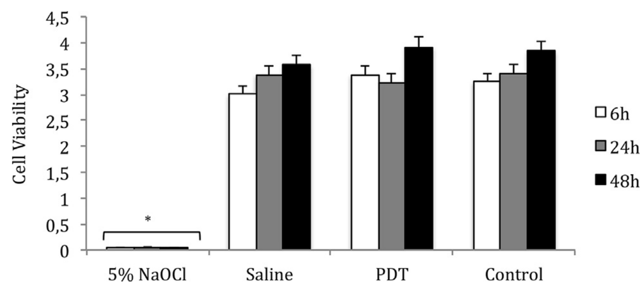

Fig. 1. Viability of fibroblasts in the presence of PDT and irrigating solutions, the periods 6 h, 24 h and 48 h. *($p < 0.05$) indicates significant statistical difference.

2.4. Enzyme-linked immunosorbent assay

For cytokine assay, supernatant samples were used in the experiment originated from cytotoxicity to analyze the production of cytokines (IL-1β and IL-6) by fibroblasts, according to Gomes-Filho et al. [21,22]. After the incubation periods, supernatants were collected and analyzed to detect were measured using Peprotech Murine IL-1β and IL-6 mini ELISA development kits (Manufacturer, Rocky Hill, NJ, USA), according to the manufacturer's instructions. This assay also revealed the presence and levels of IL-1β and IL-6 (pg/ml). Wells with no added GT solution or irrigant, but with cell culture served as a control. The measurements were made in triplicate.

2.5. Statistical analysis

The results were statistically analyzed by ANOVA test with Bonferroni correction ($p < 0.05$) for MTT and Kruskal–Wallis test and Dunn's ($p < 0.05$) for ELISA.

3. Results

MTT assay in the periods of 6 h, 24 h and 48 h is shown in Fig. 1. It was revealed that control, saline solution, and PDT did inhibit less than 25% the cell viability. The same result was not seen in the 5% NaOCl group that was greater than 75% inhibition and was the most cytotoxic ($p < 0.05$).

The mean concentrations of IL-1β for the different groups are shown in Fig. 2a. All groups were able to induce similar IL-1β production observed by ELISA assay ($p < 0.05$).

The expression of IL-6 are shown in Fig. 2b. ELISA assay revealed that the mean concentrations of IL-6 was similar among the groups ($p > 0.05$), although 5% NaOCl had been highly cytotoxic.

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