Journal of Physical Chemistry & Biophysics

de Souza et al., J Phys Chem Biophys 2013, 3:4
DOI: 10.4172/2161-0398.1000124

Research Article Open Access

Effect of Photodynamic Therapy with Different Formulations of Methylene Blue in Teeth Contaminated by *Enterococcus faecalis*

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Abstract

The aim of this study was to compare the disinfection of dentine using photodynamic therapy with methylene blue in different formulations. Thirty bovine teeth roots were autoclaved and incubated with a suspension of *Enterococcus faecalis*. The specimen were randomly divided into three groups: G1, the roots were filled with 10 mM methylene blue dissolved in water; G2, the roots were filled with 10 mM methylene blue dissolved in a mixture of glycerol: ethanol: water; G3, roots filled with 100 mM methylene blue dissolved in water. The groups were irradiated with a 660 nm diode laser with an output power of 100 mW for 4 min, energy density of 850 J/cm^2 and after this procedure, the sensitizer was removed and microbial samples were collected from within the root canals. The samples were plated on mEnterococcus to count the colony-forming units (CFU/mL). The means were: Group 1=513×10^3, Group 2=1431×10^3 and Group 3=2.96×10^3. The statistical analysis detected higher disinfection achieved by G3 when compared with groups G1 and G2, and no significant difference between the groups G1 and G2 (P>0.05). The increase of the concentration of methylene blue dye achieved higher disinfection in photodynamic therapy.

Keywords: *Enterococcus faecalis;* Methylene blue

Introduction

The presence of microorganisms in the root canal system is reported to be the main cause for failure of endodontic treatment due to its metabolic products as well as the resulting formation of a focus of infection. Moreover, the existence of resistant microorganisms inside the root canal system – especially in cases of re-treatment whenever the first treatment was not successful - makes it so that even when endodontic therapy is well performed, using strict measures for controlling infection, it is not effective in ensuring successful treatment.

Thus, new resources against intra and extra-canal (apical biofilm) infection are being pursued through tests with irrigation solutions, intra-canal medications, laser radiation and others. As a resource to be tested photodynamic therapy has the advantage of not inducing microbial resistance, which is currently very promising for use in areas where there are resistant microorganisms that may lead to unfavorable prognosis [1-6].

Enterococcus faecalis is a resistant microorganism that is frequently detected in cases of secondary infections in Endodontics [7]. However, its occurrence has also been reported in primary infections with techniques such as the PCR, in teeth as well as in periapical lesions [3,4,8] Furthermore, this microorganism is able to co-aggregate with *Fusobacterium nucleatum*, which the authors believe plays an important role in periapical lesions [8].

Several studies have shown that chemical-surgical preparation is able to achieve high levels of disinfection, but is unable to completely reduce the microbiota of the root canal system [9-12].

Photodynamic therapy (PDT) is a reaction between photosensitizers and light, producing a cytotoxic effect, usually via oxidative reactions. PDT, which is widely used in the disinfection of blood products, is efficient in the inactivation of viruses, resistant bacteria and yeast [13-17].

In Dentistry, photodynamic therapy has proven its effectiveness in reducing infection in peri-implantitis, both in *in vitro* and *in vivo* studies [18-20]. In endodontic therapy, PDT reduced the root canal infection *in vitro* significantly [21-24]. In *in vivo* studies, there were also an increase in the level of disinfection achieved using photodynamic therapy as an aid to endodontic treatment [6,25]

George and Kishen [26] tested methylene blue as a sensitizer, dissolved in water and in a mixture of glycerol, ethanol and water (MIX) in an *E. faecalis* suspension. The authors found that the sensitizer dissolved in the MIX solution was more effective in reducing the concentration of the microorganism. This is thought to be due to a higher interaction between the methylene blue and the cell acquired by this MIX.

The aim of this study was to test the effectiveness of methylene blue at two different concentrations diluted in water and also dissolved in a glycerol, ethanol and water solution in the disinfection of extracted bovine teeth infected with *E. faecalis*.

Materials and Methods

The crowns were cut off of twenty extracted bovine teeth and their root canals were emptied. They were then immersed in 1% sodium hypochlorite and agitated in an ultrasonic basin in order to remove pulp residue.

Following these procedures, the apical third of these teeth was enlarged by a #60 K-file (Dentsply-Maillefer, Ballaigues, Switzerland) in order to standardize the specimen. The roots were then externally

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Received August 01, 2013; **Accepted** August 26, 2013; **Published** August 28, 2013

Citation: de Souza EB, Lorenzetti Simionato MR, Lage-Marques JL, Antoniazzi JH (2013) Effect of Photodynamic Therapy with Different Formulations of Methylene Blue in Teeth Contaminated by *Enterococcus faecalis*. J Phys Chem Biophys 3: 124. doi:10.4172/2161-0398.1000124

waterproofed using cyanoacrylate (Super Bonder® - Loctite Henkel, Itapevi, SP, Brazil) and dried at room temperature for 24 hours. Next, the roots were placed in 1.5 mL polypropylene tubes and the set was sterilized in an autoclave at 134°C for 15 minutes (Figure 1).

All *specimens* were checked for a lack of contamination by inoculating with sterile TSB, before the experiment.

Preparation of inoculation and contamination of the specimen

A suspension of 50 µL of the *E. faecalis* ATCC 29212 was incubated in 5 mL of Tryptic Soy Broth (TSB - Difco- Becton Drive, NJ, USA) at 37°C for 24 hours. The concentration of the inoculation was adjusted using the optic density at 600nm, corresponding to 3×10^8 CFU/mL.

Next, the roots of the *specimen* were filled with the inoculation and were incubated over 2 weeks at 37°C with fresh culture media complemented every day. All the procedures were done in a flow chamber.

Experimental groups

All Groups: The canals of the teeth were filled with 0.5 mL of photosensitizer, which was left for 2 minutes (prior to irradiation). Irradiation was performed using a diode laser (Thera Lase® - DMC Equipamentos Ltda, Sao Carlos, SP, Brazil), 660 nm wavelength, 100 mW power for 4 minutes and energy density of 850 J/cm² (Figure 2). After the canals were irradiated, they were irrigated and aspirated with 10 mL of sterile saline solution to eliminate the photosensitizer.

Group 1: photosensitizer was methylene blue at a 10 mM concentration diluted in distilled water.

Group 2: photosensitizer was methylene blue at a 10 mM concentration diluted in a glycerol: ethanol: water (30:20:50) solution.

Group 3: photosensitizer was methylene blue at a 100 mM concentration diluted in distilled water.

Afterwards, the canal was filled with peptonated water solution and the walls were filed with a #60 K-file; the content was later aspirated with micropipettes and those contents were transferred to 1mL of peptonated water solution and were vortexed (Genie 2™- Fisher Scientific, Bohemia, NY, USA), to get the samples homogenized. Serial dilutions of 10^{-1} to 10^{-4} were done.

Twenty-five µL was plated on the surface of mEnterococcus Agar® (Difco - Becton Drive, NJ, USA) in triplicate, a selective media for *E. faecalis*–– in order to count the colonies of *E. faecalis*. The cultures were incubated at 37°C for 48 h.

Results

The means obtained were: Group 1=513×10^3, Group 2=1431×10^3 and Group 3=2.96×10^3 CFU/mL (Table 1). Statistical analysis was performed (Kruskall-Wallis and Student-Newman-Keuls tests) and it was detected higher disinfection achieved by G3 when compared with groups G1 and G2, and no significant difference between the groups G1 and G2 ($P>0.05$) (Table 1).

Discussion

Photodynamic therapy has long been used in Medicine, especially in Oncology therapy. In Dentistry, and more specifically in Endodontics, it is used because of the antimicrobial properties. Photodynamic therapy is safe and efficient as an aid to endodontic treatment in terms of an increase in intracanal disinfection. Some studies have shown

Figure 1: Picture of roots in polypropylene tubes for sterilization in an autoclave.

Figure 2: To illustrate how the irradiation was performed, showing the light spreading all over the surface dimensionally.

Experimental groups	CFU/mL means	Standard deviation	Minimum - maximum CFU/mL
Group 1	513×10^3	1025786.8	$640 – 32\times10^5$
Group 2	1431×10^3	2308552.2	$160 – 72\times10^5$
Group 3	2.96×10^3	4187.7	$300 – 13.6\times10^3$

Table 1: CFU/mL means in the experimental groups.

the efficacy of this therapy against *E. faecalis* [17,22-26]. These studies present different options of sensitizers and different concentrations as well as different light wavelengths and application protocols, the most widely used sensitizer, however, is methylene blue, a phenothiazinic dye that is well known in Dentistry. In this study it was tested two different solutions (G1 and G2) for the same concentration (10 mM) of the dye, aiming at comparing the influence of solution in the antimicrobial effect of the PDT. These results did not show statistical difference between them which is contrary to the findings of George and Kishen [26] using methylene blue dissolved in a solution with glycerol:ethanol:water, perhaps because the protocol performed in this study was different of them. The parameters chosen were set by clinical conditions and the time of irradiation was similar to Garcez et al. [25] in their studies because the lack of consensus among authors towards optimal time-set and their good results in their clinical studies. When comparing the concentrations of the sensitizer the higher one (Group 3) was able to reduce a larger number of microorganisms, which implies that the concentration of the sensitizer is a factor that has influence in the antimicrobial effect of PDT and these findings are in accordance

with other studies [18-20]. In the present study an increase of ten times in the concentration decreased the microbial load more than a hundred times. These results show a promising effect of the PDT in root canal disinfection. Besides the concentration of the dye or the type of solution that is made, variables such as prior irradiation time and irradiation time also can interfere in the efficacy of photodynamic therapy and there is not an established protocol followed by all researchers. Further studies must be carried out in order to get the most effective protocol of PDT towards endodontic infection. Moreover it is known that the endodontic microbiota can vary highly among the patients and even in different teeth of the same individual, thus it is crucial to develop more *in vivo* studies to achieve the benefits of the photodynamic therapy.

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