Efficiency of NaOCl and laser-assisted photosensitization on the reduction of *Enterococcus faecalis* in vitro

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Objective. To investigate the action of a red laser associated with a photosensitizer on the reduction of *Enterococcus faecalis* in dental root canal in vitro.
Study design. Thirty prepared teeth with single canals were contaminated. The chemical group was irrigated with 0.5% NaOCl and left flooded for 30 minutes. In the laser group, a paste-based photosensitizer was maintained in the root canals for 5 minutes, and then irradiated with a laser at 685 nm using an optical fiber for an E of 1.8 J during 3 minutes. After treatment, the canal content was collected, serially diluted, and cultured to determine the number of colony-forming units.
Results. Photosensitizer alone or laser alone did not have any bactericidal effect. Chemical solution reduced viable bacteria in 93.25%. Laser photosensitization resulted in a reduction of 99.2%, a significantly higher bacterial reduction than NaOCl.
Conclusion. Laser photosensitization was effective for reducing *E. faecalis* in root canals and could be an adjunct to endodontic treatment. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:e93-e98)**

Microorganisms play a crucial role in pulp death and periapical infections.[1] Therefore, the main goal of endodontic treatment is the elimination of bacteria and organic substrate found inside the dental root canal. Contemporary treatment procedures to eliminate the infection include root canal debridement and shaping, irrigation with a disinfectant agent, interappointment dressing with an antimicrobial agent, and sealing.[2] The main causes of treatment failure are the presence of persistent microorganisms and the recontamination of the canal due to an inadequate sealing.[3,4] In case of treatment failure, the use of antibiotics and antiseptics is an alternative approach. The long-term use of chemical antimicrobial agents, however, can be rendered ineffective by resistance developed in the target organisms.[5]

The long-term success rate of conventional endodontic treatment depends on several factors, such as the diverse and complex anatomy of the root canal system and the antimicrobial resistance of the polymorphous microflora, which include anaerobic, facultative anaerobic, and aerobic bacteria.[6-8] Particularly, the probability of teeth with apical periodontitis to completely heal after first treatment or retreatment is 74% to 86%.[7,9]

The root canal microflora presents different susceptibility to antimicrobial solutions and therefore the disinfection process can be complex. Sodium hypochlorite (NaOCl) is a recommended antiseptic for irrigation of the root canal system because of its effective antimicrobial and tissue-disssolving action.[10] Recently, 0.5% NaOCl for 30 minutes has been shown to reduce colony-forming units (CFU) of *Enterococcus faecalis* to zero in vitro.[11,12] *E. faecalis* is associated with persistent endodontic infections. Indeed, *E. faecalis* becomes resistant to common intracanal medication by forming biofilms.[4]

Photodynamic therapy (PDT), or lethal photosensitization, is a new antimicrobial strategy that involves the use of a light source and a photosensitizer. The excited photosensitizer reacts with the substrate, mostly oxygen and water, to produce highly reactive oxygen species, which induce injury and death of microorganisms.[13,14] The selective action of PDT is one of its most important characteristics, because this therapy appears to be lethal to microorganisms in lower concentrations than those required to kill normal cells.[15,16]

Photodynamic therapy has been studied as a promising approach to eradicate oral pathogenic bacteria, and some photosensitizers, such as toluidine blue and methylene blue, have been tested over the last few

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years in association with low-intensity red lasers to promote bactericidal effect in vitro and in vivo.[2,17,18] However, significant reduction of *E. faecalis* has not been achieved by PDT.[19]

The necessary photosensitizer concentration that leads to microorganism death due to photodynamic activity depends upon the dye, the irradiation parameters, and the genus of bacteria. Usually the concentrations of the photosensitizers range from 5 to 200 μmol/L depending on the bacteria susceptibility and the irradiation conditions.[20] In a recent study, Hayek et al.[21] showed that azulene (AZ) is an effective photosensitizer to kill periodontopathogenic bacteria in a ligature-induced peri-implantitis model in dogs.

The purpose of this study was to evaluate *E. faecalis* reduction in root canal following PDT with azulene or 0.5% NaOCl disinfection.

MATERIAL AND METHODS

A preliminary experiment in test tubes was performed to determine the effect of the paste-based photosensitizer over the microorganism. A commercial solution of azulene 25% (w/v) (Byofhitus Pharmacy, São Paulo, Brazil) was used. The paste was composed by urea peroxide 10%, detergent 15% (Tween 80) and vehicle 75% (carbowax). Four hundred microliters of azulene was used per gram of paste, resulting in a 0.01% (w/w) final concentration.

Enterococcus faecalis ATCC 1494 was the studied microorganism maintained by subculturing on brain-heart infusion (BHI; Difco, Detroit, MI). The organism was aerobically grown in BHI broth at 37°C for 24 h.

Test tubes with 0.5 mL BHI were contaminated with 0.5 mL of *E. faecalis* in suspension and incubated at 37°C for 24 h, giving a final cell concentration of 4×10^7 CFU/mL. Five surveys were performed using 3 test tubes in each group: Experiment 1 (L−AZ−): 1 mL bacterial suspension was incubated with 1 mL saline without the use of laser or azulene; experiment 2 (L+AZ−): 1 mL bacterial suspension and 1 mL saline were exposed to red laser without photosensitizer for 3 min; experiment 3 (L−AZ+): 1 mL bacterial suspension was incubated in the dark with 1 mL 0.01% AZ paste for 5 min (preirradiation time (PIT)) (400 μL 25% AZ was used per gram of paste); experiment 4 (L+AZ+): 1 mL bacterial suspension was exposed to red laser in the presence of 1 mL 0.01% AZ paste after 5 min PIT; experiment 5 (NaOCl): 1 mL bacterial suspension was incubated with 1 mL 0.5% NaOCl for 8 min, corresponding to PDT total time.

The irradiations were performed from the bottom to the top for 180 s, using aGaAlAs diode laser at $\lambda = 685$ nm, P = 50 mW, and E = 9 J (Kroman, São Paulo, Brazil).

The suspensions were serially diluted in saline solution, and 200-μL aliquots were spread over plate surfaces with BHI agar. Plates were incubated for 24 h at 37°C, and the CFU/mL was counted.

In the second experiment, thirty freshly extracted human single-rooted teeth (upper central incisors and upper canines), with straight canals confirmed by radiographic examination, extracted for periodontal reasons, were collected and stored in sterile saline until employed in the experiment. The crowns were removed using a diamond disc, and the roots were shortened to a length of 13 mm. The canals were enlarged to an apical size of #35 using Kerr files (Maillefer Instruments, Ballaigues, Switzerland) and cleaned with 10 mL 0.5% NaOCl between each file. The root surfaces were sealed with 2 layers of nail polish to avoid external contamination. The apical foramen was subsequently closed with composite material (Filtek Z 250; 3M, Campinas, Brazil). The root canals were irrigated with 17% EDTA for 2 min followed by irrigation with saline solution. Prior to inoculation, the specimens were sterilized by autoclaving for 15 min at 121°C.[2,6]

Afterward, the root canals were filled with 10 μL bacterial suspension containing approximately 1×10^9 bacteria/mL in stationary growth phase and incubated for 24 h under standard aerobic conditions at 37°C. After 24 h, the teeth were randomly separated into 3 groups of 10 teeth each, and initial samples were collected from all teeth using sterile paper points maintained inside the canals for 1 min to confirm the contamination and to obtain the initial number of viable microorganisms. In the NaOCl group, the canals were irrigated with 0.5% NaOCl using a 1 mL plastic syringe with a 27 gauge needle and left filled for 30 min[11]; in the PDT group, the canals were filled with AZ paste for 5 min and thereafter, the irradiation was performed with the diode laser. The light was distributed by means of an optical fiber ($\phi = 365$ μm) inside the root canal. The fiber was placed in the apical portion of the root canal (approximately 1 mm from the apices) and helicoidal movements, from apical to cervical, were manually performed to improve the diffusion of the red light inside the canal lumen.[22] The laser power output, with the optical fiber, was 10 mW, and the delivered energy was 1.8 J. The exposure time was unchanged and remained 180 s. In the control group, the canals were filled with BHI broth and incubated for 24 h. After each treatment, the root canals were washed with sterilized saline solution, eliminating the photosensitizer paste and the NaOCl solution from the canal lumen prior to the harvest of the microorganisms.

The liquid content of the root canals, after treatment, was absorbed with 3 standardized #30 sterile paper

Table I. Means of the viable bacteria after treatment of *E. faecalis* in test tubes

Antimicrobial agent	*CFU/mL ($\times 10^6$)*	*Standard deviation (CFU/mL $\times$ 10^6)*	*Reduction in the number of viable cells (%)*
L−AZ−	40	1	0
L+AZ−	40	1.5	0
L−AZ+	40	1.62	0
L+AZP+	0.46	0.13	99.89
NaOCl	1.18	0.16	97.06

L−AZ−, suspensions not exposed to laser light or azulene paste; L+AZ−, suspensions exposed to laser light only; L−AZ+, suspensions exposed to azulene paste only; L+AZ+, suspensions exposed to laser light and azulene paste; NaOCl, suspensions exposed to 0.5% sodium hypochlorite.

points (Tanari, Manaus, Brazil). The paper points were rubbed and maintained inside the canals for 1 min each.

Immediately after the harvest, the paper points were transfered to 1 mL sterile saline. The solutions were agitated for 30 s, and serial dilutions were prepared. Two hundred microliters of each dilution was inoculated onto BHI agar plates and incubated for 24 h. The CFU recovered from each treated root canal were calculated.

After counting the CFU, the mean values and the percentage variation between the number of viable bacteria before and after treatment, were computed. In the test tube experiment, the effects of saline solution alone, laser irradiation alone, photosensitizer for 5 min without irradiation, PDT, and NaOCl were compared using the mean values. In the extracted tooth model experiment the effects of PDT and NaOCl were compared with a control group without any treatment. The intragroup values were calculated as percentage variation from the initial and final mean values. Statistical analysis of the experimental data was performed using the one-way analysis of variance, followed by the Tukey method. Significance was accepted at $P < .05$.

RESULTS

The effects of paste-based AZ and 0.5% NaOCl on the reduction of *E. faecalis* in test tubes are presented in Table I. Neither irradiation of the organism for 3 min in the absence of AZ paste nor incubation with AZ paste for 5 min (PIT) in the absence of laser irradiation had a significant effect on the viability of *E. faecalis.* The average cell recovery in experiments 1, 2, and 3 was 4×10^7 cells/mL. The treatments with paste-based azulene plus light (PDT) and with NaOCl achieved significant reductions of cell viability (99.89% and 97.06%, respectively). Statistically significant differences were observed between PDT and 0.5% NaOCl ($P < .0001$).

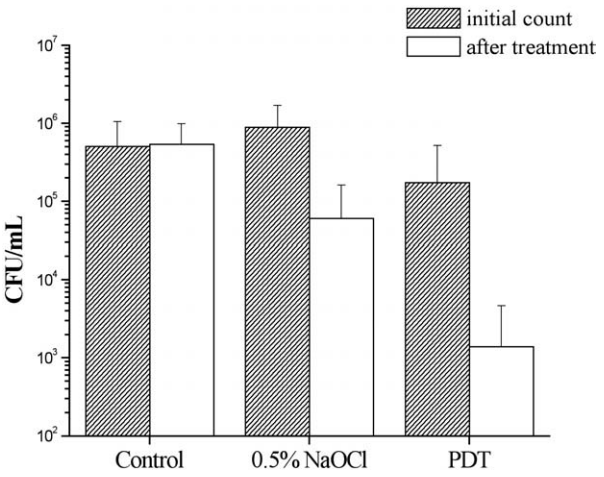

Fig. 1. Mean and standard deviation of viable bacteria (CFU) after treatment of *E. faecalis* inside root canals before and after treatment in the 3 experimental groups. The control group did not present any bacterial reduction. The antimicrobial effect was significantly higher in the PDT (99.2%) compared to the NaOCl group (93.25%).

Figure 1 shows the data obtained from the infected root canal experiment. The control group showed confluent growth at the 24 h time point. The highest reduction for *E. faecalis* was achieved with PDT (99.2%), whereas the use of 0.5% NaOCl for 30 min reached a reduction of 93.25%. In the PDT group, 3 samples were rendered bacteria free, and none in the 0.5% NaOCl group was without some growth. There was a statistically significant difference between PDT and NaOCl groups ($P = .0171$).

DISCUSSION

Enterococcus faecalis has been associated with persistent endodontic infections presenting low sensitivity to conventional treatment.[11] *Enterococcus faecalis* is a gram-positive facultative anaerobic microorganism resistant to many antimicrobial agents[8,23,24] and has been identified in infected root canals,[25] acute apical abscesses,[26] and root-filled canals with persistent infections.[25] The resistance of enterococci to endodontic treatment has long been recognized; in fact, even calcium hydroxide had been considered ineffective against *E. faecalis.*[27,28] Besides the genus of the bacteria, the growth phase seems to be an important determinant of bacterial resistance to antimicrobial approaches. Almost all antimicrobial agents are more effective in killing rapidly growing cells.[29] Likewise, the bacterial resistance to PDT seems to be greater in slow-growth cells, although there is not a consensus in the literature about the effect of growth phase on the susceptibility of bacteria to PDT.[30] In this work, the cells used were in

a slow growth rate to increase the antimicrobial challenge, and, according to the results, PDT is an effective approach even in such a condition.

In a first attempt to demonstrate the effect of PDT with azulene paste as photosensitizer, a pilot study was carried out in test tubes comparing the bactericidal effect of PDT and 0.5% NaOCl against *E. faecalis*. The results showed that AZ paste is an effective photosensitizer to kill *E. faecalis.* Paste-based AZ plus light (99.89% of reduction) was more efficient than 0.5% NaOCl (97.06% of reduction) in the test tube experiment.

Photosensitizers are molecules that have the special property, upon absorption of the light, of using the energy to carry out chemical reactions in cells. The photosensitizers generate cytotoxic oxygen species when irradiated with light of an appropriate wavelength. Upon the absorption of a photon, the photosensitizer molecule will be excited to a higher energy level. The molecule will then lose its energy, and during this process an electron transfer reaction may occur or the energy may be transfered to the environment. The energy transfer processes may lead to oxidative reactions, which are toxic to cells, mostly if they occur in the vicinity of the microbe or in the intracellular space.[31]

In this study, a significant reduction of *E. faecalis* was obtained after PDT, using AZ as photosensitizer. Azulene is a fused-ring, planar, polycyclic aromatic hydrocarbon (PAH); chemically, it corresponds to cyclopentacycloheptene. Azulene is an essential oil derived from the German plant *Matricaria chamomile*; it is used in face and body creams, sunburn remedies, burn ointments, and bath salts.[32] Azulene and its derivatives have been found to possess antiallergic, antiinflammatory, and antiulcer properties.[33] According to FDA regulations, azulene can be used in low concentrations (~1%) in cosmetic products. The toxic dose for oral intake (LD_{50}) of azulene in rats is 4,000 mg/kg.[34] In this study, the final concentration of azulene in the paste is approximately 0.01% w/w. It should be emphasized that as a cosmetic used in skin creams, azulene is constantly exposed to sunlight, thus it is exposed to all wavelengths for a prolonged period of time. The concentration that was used in this study is 100 times smaller than the concentration considered safe by the FDA regulations.

Some reports indicate that the phototoxicity of a PAH is predominately caused by photodynamic mechanisms requiring oxygen.[35,36] It is known that oxygen radicals, i.e., hydroxyl, superoxide, or singlet oxygen, may damage cell membranes via lipid peroxidation and may damage DNA.[13] As used in this work, the 25% AZ solution in a paste-based delivery resulted in a final concentration of 0.01% (w/w). In this concentration, there is no evidence of direct toxicity. Moreover, Hayek et al.[21] used the same concentration in an animal model, and no direct side effect was reported by the authors.

The paste used as a base in the present experimen, has urea peroxide, which reacts with water and forms hydrogen peroxide (H_2O_2).[37] It is possible that the paste had facilitated the photodynamic mechanism through an increased production of reactive oxygen species, because Kashima-Tanaka et al.[38] reported that when H_2O_2 is exposed to red He-Ne laser there is generation of free radicals.

When H_2O_2 is exposed to laser radiation, the amounts of free radicals formed vary according to the exposure time and H_2O_2 concentration. The hydroxyl radical (OH) is generated from H_2O_2 by the Fenton reaction or via the Haber-Weiss reaction. A bactericidal effect could occur when OH is generated from a low concentration of H_2O_2.[38] Thus, the reaction of the urea peroxide on the paste with the water content on the AZ solution may result in H_2O_2 and urea. Usually 33% of the urea peroxide is converted into hydrogen peroxide.[39] The illumination of the H_2O_2 inside the root canal could promote an increased bactericidal effect due to a higher amount of active oxygen species formed during illumination.

In root canals, PDT using paste-based azulene showed 99.2% *E. faecalis* reduction, whereas 0.5% NaOCl achieved 93.25%. Interestingly, these findings differ from Silbert et al.,[19] who found 40% killing of *E. faecalis* using methylene blue and a 670 nm diode laser in infected root canals. Seal et al.[2] achieved a 99.9% reduction of the *Streptococcus intermedius* biofilm using a He-Ne laser and toluidine blue as a photosensitizer on the disinfection of root canals. These contradictory reports could be explained by the conditions involved on the success of this therapy. For instance, different bacterial species present different sensitivity to PDT; consequently, reactive oxygen species are not equally toxic to all microorganisms.[20] The light wavelength, photosensitizer absorbance, light energy, light intensity, and exposure time may also play a part in the results, because to achieve the best result the photosensitizer has to be efficiently excited by the light source.[13] In a pilot study, the optical absorbance of AZ, methylene blue, and toluidine blue were obtained (data not shown), and AZ was chosen because it was the most resonant with the wavelength of the laser system employed in this study ($\lambda = 685$ nm). The introduction of an optic fiber into the canal to access the whole cavity may be another important factor, because it provided a more uniform illumination, and this method of light delivery is expected to be able to reach cosseted oral sites.[18]

In this study, PDT was more efficient than 0.5% NaOCl to reduce bacteria in root canals. In contrast, Seal et al.,[2] using 3% NaOCl for 10 min, showed that the chemical agent was more efficient than PDT. According to Radcliffe et al.,[11] a period of 30 min with 0.5% NaOCl achieved a zero viable count of *E. faecalis*; the increased efficiency of higher concentrations of NaOCl appeared to be related with the exposure time, because using a regression analysis, the authors showed a significant interaction between time and concentration; thus, higher concentrations of NaOCl would achieve the same result in a period shorter than 30 min. Moreover, Siqueira et al.[40] reported that different concentrations of NaOCl solution showed large inhibition zones against *E. faecalis*. It is well known that higher concentrations of NaOCl could be considered to irrigate root canals owing to its antimicrobial capabilities.[41] Nevertheless, the aim of this study was not to propose the use of PDT to replace NaOCl; PDT has been proposed as an adjunct of the conventional treatment, perhaps allowing the use of smaller concentrations of NaOCl, thus diminishing its irritating effects. Tanomaru Filho et al.[42] reported that even at low concentration, NaOCl solution causes tissue reactions leading to an intense inflammatory response on the connective tissue.

Our results suggest that the use of PDT as an adjuvant to the conventional endodontic treatment may lead to a reduction of pathogens in a short period of time. A tendency to practice the single-visit endodontic treatment, especially in cases without apical periodontitis, has been reported.[43] Therefore, an effective and fast method of killing bacteria inside dental root canal is desirable.

By now, PDT has proved to be an efficient alternative to antibiotics and chemical agents against microbial infections, mostly in vitro. It is a noncumulative local treatment, which may be an appropriate approach for the treatment of infections mainly in the oral cavity. Further studies are required to determine the exact scope of PDT in oral infections. Overall, the PDT of a single species grown in a tooth model was interestingly effective compared to the chemical agent.

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