ORIGINAL ARTICLE

The effect of the combination of cetrimide and photodynamic therapy in reducing *Enterococcus faecalis* load from the root canal system

ABSTRACT

Aim: *To evaluate the effect of cetrimide (CT) in combination with photodynamic therapy (PDT) on the reduction of Enterococcus faecalis in the root canal system.*
Methodology: *Forty mesiobuccal canals from extracted human mandibular molars contaminated with the standard strain of Enterococcus faecalis were selected. Instrumentation was performed using the WaveOne Gold Primary file (25.07) and specimens were randomly divided into two groups (n=20): PDT - 0.01% methylene blue photosensitizer was applied for 5 minutes, PDT was performed with 660 nm, 9 J red laser for 90 seconds with fiber optics; CT + PDT – Cetrimide was placed for 60 seconds, photosensitizer was applied, and PDT was performed as described in the PDT group. Samples were collected before instrumentation and after disinfection procedures for each group. The results were subjected to the Kruskal-Wallis statistical test (Student-Newman-Keuls) with a significance level of 5%.*
Results: *There was a microbial reduction before and after PDT and CT +PDT ($p<0.0001$). The use of CT in conjunction with PDT resulted in a significant increase in microbial reduction compared to the use of PDT alone ($p=0.0226$). There was no significant difference between sample groups in microbial counts performed prior to disinfection protocols ($p=0.5448$).*
Conclusion: *The use of CT in conjunction with PDT proved to be highly effective in allowing deeper penetration of the photosensitizer into the dentinal tubules, thus improving the root canal disinfection process.*

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Introduction

The goal of endodontic treatment is to disinfect the root canal system with the reduction of bacteria and microorganisms (1). Techniques include mechanical debridement and shaping of the root canal system with various systems, intracanal irrigation with antimicrobial agents, and intracanal medication (2). However, eliminating microorganisms from the infected canal is a difficult task due to the complex anatomy with its branches, accessory canals, and isthmuses. With the technological advances in microbiological culture and identification, it is already known that in teeth with pulp necrosis and without endodontic intervention, there is a mixed microbial colonization with gram-positive and gram-negative species, with anaerobes predominating (3). The use of auxiliary methods in the disinfection process of the canal system is necessary, especially in the case of persistent microorganisms (4).

Photodynamic therapy (PDT) has been explored as an aid in root canal disinfection (5-7). The technique is based on the use of photosensitizers (PS) that interact with the target cell and are excited in the presence of visible light of appropriate wavelength (1). In the excited state, called triplet, PS can transfer electrons to molecules in the medium or transfer energy to the oxygen molecule. Both reactions produce reactive oxygen species, free radicals, or singlet oxygen, which cause the death of bacteria by damaging the cytoplasmic membrane or DNA.

Several photosensitizers are available for each type of light source which did not induce any damage to the patient and are safe, and the efficiency of PDT depends on the penetration of the photosensitizer on the microbial cell surface (8). The Methylene blue that was the first phenothiazine dye to be synthesized. It is very effective in inactivating gram-positive and gram-negative endodontic bacteria by diode laser irradiation (9). Toluidine Blue is another photosensitizer, that is a thiazine with a π-conjugated structure and has been shown to absorb light in the 596-665 nm wavelength range (10) and has shown to be a highly effective photosensitizer resulted in a significant reduction (P =0.0001) of the initial values of bacteria loads (11). Other photosensitizers solution is the indocyanine Green, that is a water-soluble fluorophore used in clinical research due to its green fluorescence, emitted when excited by near-infrared light, can be detected using dedicated optical systems without affecting the surgical field view (12). Another possible substance to be used as photosensitizer is the cetrimide, that is a cationic surfactant (quaternary ammonium salt) that is in hygroscopic form. It can reduce surface tension (13) and has antimicrobial activity in aqueous solution. According to Bolfoni et al (14), the addition of cetrimide to a 1% NaOCl solution increased the antibacterial activity to a level like 5% NaOCl. Some studies has also shown that cetrimide has good residual activity compared to some antibacterial solutions (15, 16). Wang et al. (17) stated that one of the explanations for the increase in antibacterial activity of cetrimide on dentin could be that surfactants enhance the penetration of the solutions into the dentin tubules by reducing the surface tension of the solutions. There are no published studies in the literature investigating the effect of cetrimide in combination with PDT to reduce the *Enterococcus faecalis* in the root canal system. The aim of this study was to evaluate the effect of combining cetrimide with photodynamic therapy on reducing *Enterococcus faecalis* in the root canal system. The null hypothesis is that there is no difference in microbial reduction in root canals when cetrimide is used PDT.

Materials and Methods

Forty recently extracted human mandibular first and second molars were collected after approval by the ethics committee of the local dental research center (CAAE: 510555921.5.0000.5374). The inclusion criteria was fully formed roots and foramen, multi-rooted teeth with distinct mesio-vestibular and mesio-lingual canals, mesial canals with moderate curvature between 10° and 20° (18) and root canals with an initial anatomical diameter compatible with

a K#10 file. The exclusion criteria were teeth with previous endodontic treatment, teeth with internal/external apical resorption, teeth with radicular carious lesion, teeth with root cracks visible under the operating microscope and calcified root canals. The tooth integrity was assessed under magnification of 16X (DFV, Valença, Brazil).
Teeth were extracted and preserved in 0.1% thymol solution (Farmarim, Colatina, Brazil). The root surfaces were scraped with a No. 14 periodontal curette (Hu-Friedy, USA) to remove any remaining periodontal ligaments. Prophylaxis was performed with a Robinson brush (Microdont, São Paulo, Brazil), a pumice stone (Asfer, São Caetano do Sul, Brazil), and water. After this step was completed, the teeth were rinsed and stored in distilled water until the time of the study.

Standardization of the samples
All specimens were radiographed in the ortho-radial direction to determine the degree of mesio-vestibular root curvature. Dental crowns were cut at the cementoenamel junction using a double-sided diamond disk (KG Sorensen Ind. e Comércio Ltda. São Paulo, Brazil) to standardize root length to 15 mm and root canals with an initial anatomical diameter compatible with a K#10 file. The distal root was cut and discarded. The cervical-apical dimension of the mesial root was measured with a digital caliper (MTX, Salto de Pirapora, Brazil) and the measurement was transferred to a millimeter ruler (Angelus, Lindóia, Brazil). The apical foramen was sealed with epoxy resin (Araldite, São Paulo, Brazil) and the outer surface of the roots, except for the root canal opening, was sealed with two coats of cosmetic nail polish (Impala, Guarulhos, Brazil).

Preparation of the Enterococcus faecalis suspension
The roots were distributed in 24-well cell culture plates. Standard Enterococcus faecalis strain ACTT 19433 (LAB CENTER Campinas, Brazil) was reactivated at brain-heart infusion (BHI) (Difco- Detroit, USA) and incubated in an incubator with 5% CO2 at 37 °C for 24 hours. The 24-hour culture was grown in a Petri dish containing BHI agar and incubated for 24 hours in an incubator with 5% CO2 at 37 °C. After microbial growth, the culture suspension was prepared in a test tube containing 10 mL of sterile saline (0.9% NaCl) at a concentration compatible with standard 10 of the Mc Farland scale (19) Then, in a sterile test tube, 5 mL of the prepared suspension was mixed with 5 mL of BHI broth to obtain a suspension of the final concentration.

Contamination of teeth with Enterococcus faecalis
To facilitate contamination of specimens, teeth were first instrumented to working length (WL) with manual No. 15, 20, and 25 K files (Dentisply Maillefer) and rinsed with sterile saline. The teeth were sterilized in an autoclave at 121 °C for 15 minutes (20). Twenty microliters of the suspension at the final concentration were introduced into the root canal using a BD 10 mL syringe (Plastipak, Curitiba, Brazil) with a BD 20x0.55 24G injection needle (Injex Indústria Cirúrgica LTDA, Guarulhos, Brazil), and a sterile cotton swab soaked with the Enterococcus faecalis suspension was inserted into each root canal entrance. Absorbent cotton soaked with sterile distilled water was placed in 4 wells of each cell culture plate to ensure room humidity. The lid of the plate was closed and sealed with tape and the set was incubated in an incubator at 37 °C and 5% CO2 for 21 days. Every two days, 20 µL of BHI broth was added to the root canal using a BD 10 mL syringe with a BD 24G injection needle, and cotton moistened in distilled water was replaced in the wells of the plates (21).

Confirmation of contamination
Confirmation of the viability and purity of the microorganisms in the root canals was performed weekly by random sampling on two teeth using a sterile paper cone #25 (Endopoints, Rio de Janeiro, Brazil). The cones were left in the canal for 1 minute, seeded in BHI broth, and incubated for 24 hours in an incubator at 37 °C with 5% CO_2 (22). After growth, smear and gram stain were performed for morphological and staining confirmation of the microorganisms.

Microbiological collection before root canal instrumentation

After 21 days of contamination, samples were collected before root canal instrumentation by inserting a sterile cone of N°. 25 absorbent paper into each sample. The cone was held in the root canal for 1 minute and then transferred to a polypropylene flask (Eppendorf, Hamburg, Germany) containing 1 mL of NaCl 0.9% shaken for 30 seconds in a tubular shaker (Vortex AD 56, Phoenix, Araraquara, Brazil).

Serial dilutions of this suspension were prepared to a concentration of 10^5. Aliquots of 0.1 mL of the suspension and each dilution were seeded onto Petri plates containing BHI agar. The cultured plates were incubated in a 5% CO_2 incubator at 37 °C for 24 hours. Colony-forming units (CFUs) per plate were then counted, and the number of CFUs/mL was calculated.

Biomechanical preparation of the root canals

Instrumentation and irrigation were performed by the same operator, a specialist experienced in the use of the system used in the study. "Before canal preparation, each specimen was fixed used a table bench vise and isolated with a rubber dam to simulate clinical conditions." During instrumentation, a sterile gauze (Cremer, Blumenau, Brazil) soaked in sterile saline (Eurofarma, Itupeva, Brazil) was used to clean the active part of the files and remove the adherent dentin debris. A K#25 file (Dentsply Maillefer, Ballaigues, Switzerland) was inserted into the canal until its tip was visible through the surgical microscope (DFV, Valença, Brazil) at 8x magnification in the apical foramen. From this measurement, 1 mm was subtracted to determine the WL.

All roots were prepared using the same instrument protocol and standardization of canal diameter. The mesiobuccal canals were instrumented using the reciprocating technique (23) with the WaveOne Gold 25.07 file (Dentisply, Maillefer) driven by the endodontic motor (Saevo), alternating with the K#10 file used to maintain foraminal patency. A 5 mL of sterile saline was used at each instrument change or at each 1/3 of the prepared root, so that the total volume per canal was 15 mL. Irrigation was performed using a 5 mL disposable syringe (Injex, São Paulo, Brazil) and an Endo-Eze 27 G irrigation tip (Ultradent, Indaiatuba, Brazil), with in-and-out movements to WL. Simultaneously with the irrigation, the irrigation solution was aspirated from the root canal using a metal cannula positioned at the entrance of the canal and connected to a vacuum pump. All canals were dried with a paper cone.

Classification of the treatment groups

Samples were calculated based on the results of the pilot procedure performed with 10 results from the microbial counts of the sample groups using the ANOVA (one-way) test (G Power 3.1.9.4, Franz Faul, College of Kiel, Germany) with α=0.05 and β=0.80, effect size f=0.9. The minimum number of samples calculated for each group was 20. Roots were randomly distributed (www.random.org.br) to the following groups (n=20) (Figure 1).

PDT: 0.01% methylene blue photosensitizer was placed using endo-eze tips with a lateral exit as an endodontic irrigation needle for 5 minutes as a pre-irradiation period. PDT was performed with a low-intensity laser (Lasersmile Hand) using fiber optics (Lasersmile Hand) with 9 J of energy, a power of 100 mW, and a wavelength of 660 nm por 90 seconds. The spot size was 0.0028 cm^2.

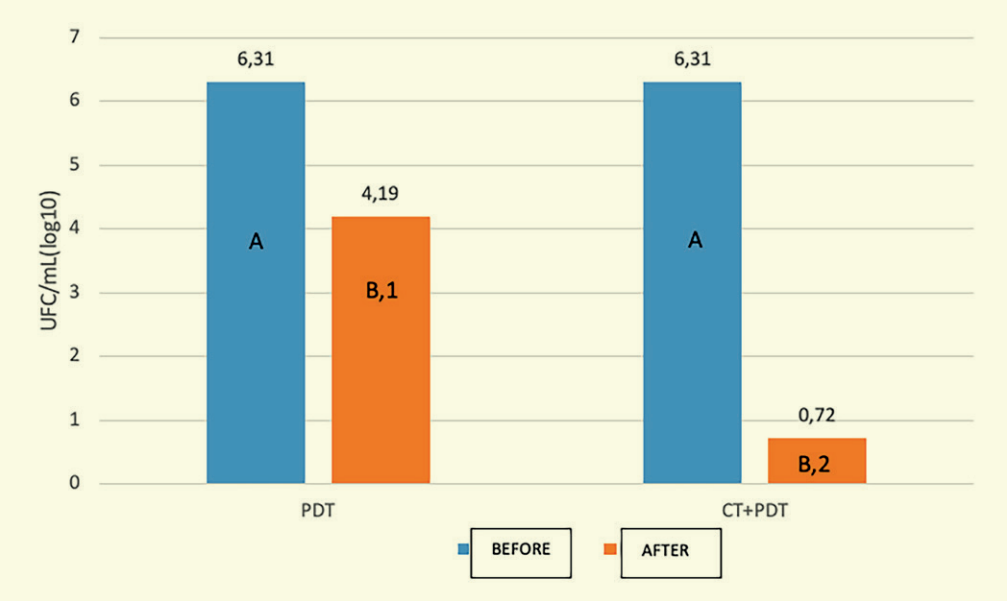
CT+PDT: Before PDT, 2% cetrimide was applied with an irrigation syringe and an endo-eze irrigation tip for 60 seconds. The excess cetrimide was removed with a sterile paper absorbent tip. PDT was then performed as described in the previous group (24).

Sample collection

Samples were collected before instrumentation and after application of PDT and cetrimide+PDT. Samples were collected by inserting a sterile cone of N°. 25 absorbent paper into the root canals. The cone was left in the canal for 1 minute and then transferred to a polypropylene vial containing 1 mL of 0.9% NaCl, which was homogenized on the tube shaker for 30 seconds.

Serial dilutions of this suspension were prepared to a concentration of 10^5. Aliquots

Figure 1
Arithmetic means and Kruskal-Wallis (Student-Newman-Keuls) statistical test for the number of colony-forming units/mL (log10) in the sample groups.



PDT: Photodynamic Therapy; CT+PDT: Cetrimide+Photodynamic Therapy, capital letters and different numbers in horizontal direction: statistically significant differences.

of 0.1 mL of the suspension and each of the dilutions were cultured on Petri dishes containing BHI agar. The colonized plates were incubated in a 5% CO_2 atmosphere at 37 °C for 24 hours. The number of CFU per plate was then counted and the number of CFU/mL was calculated.

Statistical analysis
The results were analyzed using the BioEstat 5.3 program and subjected to the Shapiro-Wilk normality test. The sample showed non-normal behavior. The results were subjected to the Kruskal-Wallis statistical test (Student-Newman-Keuls) with a significance level of 5%.

Results

There was a microbial reduction after PDT and CT+PDT (p<0.0001). The use of CT associated with PDT resulted in a significant increase in microbial reduction compared to performing PDT alone (p=0.0226). There was no significant difference between sample groups in microbial counts performed prior to disinfection protocols (p=0.5448) (Table 1).

Discussion

This *ex vivo* study evaluated the effect of CT associated with PDT to reduce *Enterococcus faecalis* from the root canal system.

Table 1

Medians (MD), interquartile deviations (ID), arithmetic means (MA), standard deviations (SD) and Kruskal-Wallis (Student-Newman-Keuls) statistical test of the colony forming units/mL (log10) counts of the sample groups.

	PDT		CT+PDT		(p-KW)
	before	after	Before	after	
MD(DI)	6.68(0.57)^{A}	4.40(1.18)^{B,1}	6.33(0.71)^{A}	0.00(0.28)^{B,2}	0.0000
MA(DP)	6.31(1.56)	4.19(1.22)	6.31(0.49)	0.72(1.52)	

Legend: PDT: Photodynamic Therapy; CT+PDT: Cetrimide+Photodynamic Therapy, capital letters and numbers different in horizontal direction: statistically significant differences.

The use of CT with PDT resulted in a significant increase in microbial reduction compared with performing PDT alone, so the null hypothesis was rejected. Effective disinfection of the canal is paramount to the success of endodontic treatment (25). *Enterococcus faecalis* is one of the most common microorganisms in necrotic root canal infections (26) and is frequently used in *in vitro* models to evaluate the efficacy of antimicrobial agents (27).

Bacterial biofilms are complex three-dimensional structures formed by a matrix of extracellular polymeric matrix in which bacteria are embedded. For this purpose, several chemical agents are often used that can act on the biofilm, including surfactants. CT has a cytotoxic, bactericidal effect (28), does not irritate host tissues, and has the ability to reduce the surface tension of fluids, which facilitates its penetration into hard-to-reach areas such as the dentinal tubules (29). CT 2% eradicated *S. mutans* in most samples and also increased the rate of biofilm removal (24). In combination with chlorhexidine, it was effective against *Enterococcus faecalis* (30) Another study showed that CT 0.2% had a longer lasting substantivity compared to chlorhexidine 0.2% and almost as long as that of chlorhexidine 2% in a dentin model (31). This could be related to the cationic nature of CT, which is able to interact with dentin. These studies demonstrated the antimicrobial ability of cetrimide and are consistent with the results of the present work, in which the use of CT in conjunction with PDT reduced the load of *Enterococcus faecalis*.

Cetrimide is a cationic surfactant with bactericidal activity and the capacity to decrease the biofilm's mechanical stability (32). Solutions of 2% chlorhexidine and 0.2% cetrimide, when applied for 1 min alone or in final irrigation protocols, can completely inhibit the 24-h *Enterococcus faecalis* biofilm formation in dentin (33), while the combination of 2% chlorhexidine+0.2% cetrimide after the use of chelating agents (34) has been proposed as an effective alternative for final irrigation in root canals because of its antimicrobial action over time.

Although 0.2% cetrimide showed a high residual activity (median: 27 days), a result closer to the one obtained with 2% chlorhexidine had been anticipated, given its greater ability to kill *Enterococcus faecalis* (32) and comparable substantivity determined in a volumetric-dentin unit (35). The present study involved the use of roots where the low surface tension of cetrimide facilitated its diffusion in the main root canal, but the penetration of the solutions into dentinal tubules may be compromised (36). However, it has been shown that the addition of cetrimide in the disinfecting solutions increased their antibacterial effects against *Enterococcus faecalis* in the dentinal tubules (37). In this sense, the results obtained in this study suggest that the antibacterial residual effect of cetrimide would depend on its concentration and the length of its application time (32) or its association with antiseptic agents (35, 36).

PDT is an antimicrobial technique that consists in the application of light to activate a photosensitive agent in the presence of oxygen, generating reactive oxygen species (such as singleto-oxygen) *in situ* and leading to the lysis of bacteria (38). When used in conjunction with chemical-mechanical preparation, PDT has shown a high success rate in primary or secondary infections (39). In this study, an energy of 9 J was used, although PDT cycles above 12 J significantly increased bacterial clearance in another study (40). Alves-Silva et al. (7) demonstrated the efficacy of 0.005% methylene blue followed by red laser irradiation in reducing the total number of bacteria in primary apical periodontitis. Da Silva et al. (41) demonstrated the efficacy of PDT and 0.1% methylene blue in reducing the burden of *Enterococcus faecalis*, *C. albicans*, and bacteria domain. The results are consistent with those of the present study, as PDT was able to significantly reduce the burden of *Enterococcus faecalis*. It is worth noting that penetration of the photosensitizer into the root canal system presents difficulties. The structure of the dentinal tubules, with 1-2 µm lumen and 2-3 mm length, poses challenges to all disinfection methods. PDT is no exception, as light

propagation, and penetration of the photosensitizer into the dentinal tubules is limited (42) The combination of CT with PDT was able to promote deeper penetration of the photosensitizer (methylene blue 0.01%) into the tubules, allowing better disinfection of the root canal. Based on the results of this *ex vivo* study, PDT in combination with CT was 2% more efficient than PDT alone in reducing *Enterococcus faecalis* counts.

The combined protocol using cetrimide and PDT could have some advantages, such as enhancing the antimicrobial efficacy. Cetrimide can disrupt microbial cell membranes, making them more susceptible to the PDT. The cetrimide may help in better penetration of the photosensitizer into microbial cells, increasing the overall effectiveness of PDT (43).

As an *ex vivo* study, it has limitations: the study utilized *an ex vivo* model using extracted human teeth, which may not fully represent the complex biological environment of an *in vivo* root canal system. To validate the findings, further research should be conducted using in vivo models to assess the effectiveness of CT in combination with PDT in real clinical scenarios. It could be concluded that the use of CT in conjunction with PDT resulted in increased microbial reduction and may provide an alternative for disinfection of the root canal system.

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