Original Article

Comparative *in vitro* study of intracanal *Enterococcus faecalis* reduction using photosensitizers in aPDT

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Aim: To compare *Enterococcus faecalis* reduction after antimicrobial photodynamic therapy (aPDT) used with methylene blue, toluidine blue, tannin, and curcumin as photosensitizers, an adjunct to endodontic chemomechanical preparation (CMP) in root canals of human teeth. **Methods:** A total of 120 single-rooted teeth were divided into 6 groups (*n* = 20): G1- CMP and 2.5% sodium hypochlorite (NaOCl); G2- CMP and saline solution; G3- CMP, 2.5% NaOCl, and aPDT with 0.005% methylene blue; G4- CMP, 2.5% NaOCl, and aPDT with 0.005% toluidine blue; G5- CMP, 2.5% NaOCl, and aPDT with 0.005% tannin; and G6- CMP, 2.5% NaOCl, and aPDT with 0.005% curcumin. A portable semiconductor laser was used (660 nm, 100 mW, 1.8 J, 180s) in groups 1, 2, 3, 4, 5, and a blue LED light-curing (420-480 nm, 1200 mV/cm^2) in G6. For all groups, a 5 min pre-irradiation time was applied. Samples were collected before (initial collection), immediately after (intermediate collection) and 7 days after CMP (final collection) for colony-forming unit (CFU) counting. The Kruskal-Wallis test and analysis of variance (ANOVA) were performed ($p < 0.05$; 95% confidence interval). **Results:** In between-group comparisons, there was no significant difference observed in the number of CFUs at the initial ($p < 0.001$) and final collections ($p < 0.001$) for G2 and G3. In within-group comparisons, the number of CFUs showed a decreasing trend in G4 ($p = 0.007$) and G5 ($p = 0.001$). **Conclusion:** Photosensitizers promoted *E. faecalis* reduction, with better results for tannin and curcumin. Alternative photosensitizers should be the focus of further studies.

Keywords: Photodynamic. Endodontics. Photosensitizing agents. Curcumin. Tannins.

Introduction

The main goal of endodontic treatment is to eliminate or reduce the intraradicular microbial load to a level that allows for the prevention or cure of apical periodontitis[1]. The development and/or persistence of apical periodontitis is closely related to the presence of microorganisms within the root canal, and insufficient root canal disinfection has been suggested as one of the main reasons for treatment failure[2].

In addition to auxiliary chemicals, intracanal medicaments, passive ultrasonic irrigation, magnification, and ultrasound, antimicrobial photodynamic therapy (aPDT) has also been used as an adjunct to conventional endodontic treatment[3-5]. aPDT requires a photosensitizer and a light source emitting at a wavelength close to its absorption peak that will absorb energy from light photons[6]. The photosensitizing agent binds to the microorganism membrane or cell wall or accumulates selectively in them, becoming target to the light irradiated. In the presence of oxygen, photosensitizers generate reactive oxygen species (ROS) – singlet oxygen and free radicals – that, by oxidative reactions, can lead to death of various organisms- fungi, viruses, protozoa and bacteria[7].

Photosensitizers initially act on the cell membrane to increase cell permeability, and then penetrate the cell acting on other intracellular organelles[8]. Methylene blue and toluidine blue, at low concentrations, have been widely used as photosensitizers for not exerting cytotoxic effects on keratinocytes and fibroblasts[9]. Tannins and curcumin are also used in aPDT. Tannins are astringent, hemostatic, polyphenolic compounds that have been used as natural dyes and are major active constituents of plants, which justifies the traditional use of plants as anti-inflammatory and healing agents[10]. They have a strong absorption band at 672 nm and rapid skin clearance. Tannins are non-toxic and have favorable photophysical properties, with high triplet-state yields. Curcumin, a polyphenolic compound extracted from *Curcuma Longa* rhizomes, has been widely used in traditional Chinese medicine and food industry, with anti-inflammatory, antitumor, antifungal, antibacterial, and anticarcinogenic properties[11]. In aPDT, curcumin has high light absorption capacity at wavelengths in the blue spectral region, between 455 nm and 492 nm[12] which is commonly used in dental offices for the light curing of resin-based composites by using a LED or halogen light device.

Reduction of intracanal microorganisms promoted by aPDT ranges from 65 to 99.6% depending on the photosensitizer used, and some studies have been conducted to compare the reducing ability of photosensitizers[12,13]. This way, in this study had compared methylene blue, toluidine blue, tannin, and curcumin for their ability to reduce intracanal *Enterococcus faecalis*.

The purpose of this study was to compare microbial reduction after aPDT (660nm) used with methylene blue, toluidine blue, tannin, and curcumin as photosensitizers as an adjunct to chemomechanical preparation (CMP) in root canals of human teeth contaminated with *E. faecalis*.

Materials and Methods

Study design and setting

This *in vitro* study was conducted after approval by the institutional research ethics committee (approval number 2.332.759). The sample size was calculated using G*Power, version 3.1, based on a pilot data set to obtain a medium to large effect size, with a significance level of 5%, 95% confidence interval (CI), statistical power of 80% (β = 0.20), and a 1:1 allocation ratio. A sample size of 20 specimens per group was required.

Specimen preparation

A total 120 extracted single-rooted mandibular incisors with fully formed apices were selected for this study. All teeth had been donated to the human tooth bank of our institution, and written informed consent was obtained from all patients prior to tooth donation. After periodontal tissue removal and rubber cup and pumice prophylaxis, the crowns were removed with a diamond disc. The remaining 12-mm roots were autoclaved at 120ºC, 1 atm, for 20 min, and the apical third were sealed with Araldite epoxy resin (Brascola, São Paulo, SP, Brazil) to prevent leakage of bacterial cultures after contamination.

Microorganisms and culture medium

The root canals were contaminated with a pure culture of *E. faecalis* (ATCC 29212) in brain heart infusion (BHI) broth (BHI00- Oxoid; Basinkstoke, UK). Isolated colonies were suspended in screw-cap tubes containing 5 mL of BHI broth. The suspension was mechanically shaken and adjusted in a spectrophotometer with absorbance at 800 nm to match a 1.0 McFarland standard (3.0×10^8 bacteria/mL). The specimens were transferred to flasks containing sterile BHI broth and remained incubated at 37°C for 24 h to confirm sterility.

Contamination with E. faecalis

In a laminar flow hood, 5.0 mL of BHI broth were removed and replaced with 4.0 mL of *E. faecalis* suspension. The flasks containing the specimens were sealed and incubated at 37°C in a 10% CO_2 atmosphere for 7 days, with 2.0 mL of contaminated BHI broth being replaced with 2.0 mL of sterile BHI broth every 2 days to avoid medium saturation. Bacterial growth during incubation was determined by turbidity of the culture medium.

Experimental groups

The 120 specimens were divided into 6 groups (*n* = 20) according to the disinfection protocol used during CMP, as shown in Table 1.

	N	DISINFECTION PROTOCOL
G1(Control)	20	CMP + 2.5% Sodium hypochlorite
G2(Control)	20	CMP + Saline solution
G3	20	CMP + Methylene blue + aPDT
G4	20	CMP + Toluidine blue + aPDT
G5	20	CMP + Tannin + aPDT
G6	20	CMP + Curcumin + aPDT

Chemomechanical preparation and photodynamic therapy

The teeth were arranged in a table-top lathe machine and prepared with a size 35.06 WaveOne Gold file (Dentsply). All root canals were prepared and irrigated with 10 mL of 2.5% sodium hypochlorite (NaOCl), followed by irrigation with 10 mL of saline solution, except group 2 that irrigation was realized with saline solution. In groups submitted to aPDT after instrumentation, the canals were irrigated with photosensitizers solution (3 mL) of 0.005% with a 5-min pre-irradiation period. Portable semiconductor laser (Laser DUO®, AlGaAs, InGaAlP, λ880 nm and λ660 nm, MM OPTICS LTDA, São Carlos, SP, Brazil) was used at a wavelength of 660 nm, 100 mW output power, and laser beam area of 3 mm^2, for a total energy of 1.8 J per spot area over an irradiation time of 180s. In final, the canals were irrigated with 10 mL of saline solution to remove the dyes according to the protocol of Gomes et al.[14] In group 6, aPDT irradiation was performed with a blue LED light-curing unit (Gnatus), at a wavelength of 420-480 nm, with an intensity of 1200 mV/cm^2.

Microbiologic collection

Samples were collected at 3 time points: before CMP (initial collection), immediately after CMP (intermediate collection), and 7 days after CMP (final collection). All procedures were performed in a laminar flow hood.

Initial collection

Before CMP, the specimens were irrigated with 10 mL of saline solution, dried with 15-mm diameter sterile paper points for 1 min (Figure 1), and stored in Eppendorf tubes (Figure 2).

Figure 1. Microbiologic collection with a sterile paper point.

Figure 2. Eppendorf tube with the paper point after collection

Intermediate collection

CMP was performed after the initial collection, and the canals were dried with sterile paper points and stored individually in Eppendorf tubes, following the same protocol used for the initial collection. The specimens were filled with BHI broth and sealed with a gutta-percha stick to prevent contamination. The specimens were placed in individual Eppendorf tubes containing 60 μL of sterile BHI broth to maintain hydration, so that the BHI broth did not reach the middle third of the canal to prevent infiltration into the gutta-percha sealing. The tubes were incubated at 37°C in a 10% CO_2 atmosphere for 7 days.

Final collection

After 7 days, the root canals were again irrigated with 10 mL of saline solution, dried with 35-mm diameter sterile paper points, and placed in individual Eppendorf tubes containing 1 mL of sterile BHI broth. After collection, the Eppendorf tubes were vortexed (Biomixer-QL-901) for 60 s to homogenize the solution.

Dilution and plating

After mechanical agitation, the collected samples were diluted 1:10 and 1:100 in sterile BHI broth. A 50-μL aliquot of the 10^{-2} dilution was seeded on plates containing BHI agar, in triplicate, and the plates were incubated at 37°C in a 10% CO_2 atmosphere. After 48 h, colonies on plates were counted to determine the colony-forming units (CFUs). The number of CFUs was multiplied by 2000 to account for dilutions during sample preparation (100 times) and 50-μL plating (20 times less than 1 mL), thus yielding the number of CFUs per mL of sample (CFU/mL). The average of the 3 plates of each collection was taken as the final count (Figure 3).
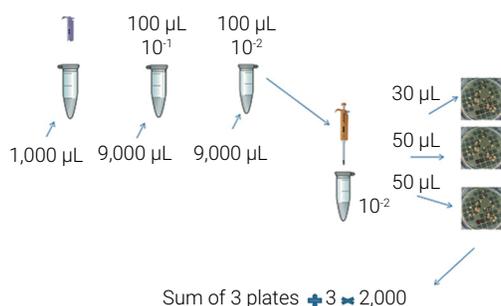
Figure 3. Schematic representation of microbiologic collection in the initial, intermediate, and final stages

Statistical analysis

Data were analyzed using BioStat 4.0. The Shapiro-Wilk test was used to assess the normality of data distribution. The Kruskal-Wallis test and analysis of variance (ANOVA) were used for nonparametric data, at a significance level of $p < 0.05$ and 95% CI.

Results

Table 2 shows the results of the comparative analysis of bacterial load (CFU/mL) in each group at the 3 assessment time points. In between-group comparisons, a significant difference was observed in the number of CFUs at the initial ($p < 0.001$) and final collections ($p < 0.001$). In within-group comparisons, the number of CFUs showed a decreasing trend in groups 4 ($p = 0.007$) and 5 ($p = 0.001$). When comparing initial and final values, percentage reductions in relation to mean CFUs were 68.2% for group 4 and 69.9% for group 5.

Table 2. Comparison of bacterial load at 3 assessment time points

Time point	Group						
	G1	G2	G3	G4	G5	G6	p-value[1]
Initial Collection							
Mean	116,36[A,Ba]	8,37[Aa]	225,12[Ba]	269,66[Ba]	272,30[Ba]	267,39[Ba]	**< 0,001***
SD	207,46	9,46	215,97	283,75	259,81	286,61	
Intermediate Collection							
Mean	31,73[Aa]	85,00[Aa]	74,26[Ab]	192,92[Aa,b]	54,67[Ab]	292,07[Aa]	< 0,078
SD	35,23	200,93	66,68	209,94	88,20	408,84	
Final Collection							
Mean	74,34[A,Ba]	1856719,65[Bb]	272,40[Ca,b]	85,77[A,Bb]	81,83[A,Bb]	12,23[Aa]	**< 0,001***
SD	159,94	8303252,97	280,49	91,62	164,47	18,38	
***p-valor*[2]**	0,630	**< 0,001***	**0,007***	**0,007***	**0,001***	0,089	
% inhibition (initial-final)	**36,1%**	-	-	**68,2%**	**69,9%**	**95,4%**	

SD = standard deviation; values with different superscript letters are significantly different ($p < 0.05$); [1] uppercase letters compare values in the same row (between-group comparisons); [2] lowercase letters compare values in the same column (within-group comparisons).

The control group 2 (with saline) and group 3 (with methylene blue) showed no reduction in CFUs. All other groups showed bacterial reduction between the initial and final collections, but group 6 (with curcumin) had a marked standard deviation, and the result was not statistically significant.

Discussion

E. faecalis is a valuable microbiologic marker for *in vitro* studies because of its ability to colonize the root canal in a biofilm-like style[2]. In addition, *E. faecalis* does not require strict culture conditions: it grows in culture media supplemented with blood or serum[15], justifying the use of BHI agar as a culture medium, as previously done by Silva Garcez et al.[16] (2006). The incubation period was 7 days, according to Dametto et al.[17] (2005).

The procedures for microbiologic collection vary between studies. Bonsor et al.[18] (2006) used an endodontic file to remove dentin debris from the root canal and this instrument together with the swarf sample (dentin debris) was analyzed, while Soukos et al.[19] (2006) and Fimple et al.[9] (2008) analyzed the solution leaking from the apical foramen. In the present study, microbiologic samples were collected from within the root canal by using sterile paper points, according to previous studies[14,16,20,21].

In all groups, the auxiliary chemical used was 2.5% NaOCl, because its bactericidal effect and ability to dissolve necrotic and living tissues[22,23]. In control group 1 (CMP with 2.5% NaOCl), there was only a 36.1% reduction in bacterial growth, which was not statistically significant (p = 0.63). Higher concentration of NaOCl would improve its properties[24-26] but increase the risk of accidents[27] because substances are less biocompatible with peri radicular tissues[24,28]. Increasing the volume of the solution to compensate for the effects of concentration[22,29-32] would improve its bactericidal properties.

The present study compared the efficacy in reducing *E. faecalis* of 4 dyes used as photosensitizers in aPDT: methylene blue, toluidine blue, tannin, and curcumin. Methylene blue is the most commonly used dye in endodontics[9,19], followed by toluidine blue[14,18,20,21]. They are known to be more effective against gram-positive than gram-negative species[33]. Methylene blue is used at various concentrations, ranging from 1 μg/mL to 25 μg/mL, as is toluidine blue, ranging from 12.5 μg/mL to 100 μg/mL. In accordance with the study by Gomes et al.[14] (2008) the 4 photosensitizers tested in this study were used at a concentration of 5 μg/mL (0.005%). An unexpected statistically significant (p<0.001) bacterial growth was observed between the initial and final collections using methylene blue. However, when a higher concentration was used there was a reduction of 80%[9] and 97%[19]. Besides to the concentration, better results could also be achieved using more energy density of light (J/cm²)[9] and combining it with passive ultrasonic irrigation[34,35]. In contrast to methylene blue, toluidine blue at a concentration of 0.005% promoted a statistically significant reduction of 68.2%, in accordance with other studies[12,18,36].

Extracted from guava leaves (*Psidium guajava* L. Myrtaceae), 'espinheira-santa' leaves (*Maytenus* sp., Celastraceae), and Brazilian peppertree leaves (*Schinus terebinthifolius* Raddi), tannins are astringent, hemostatic substances[37] commonly used in the leather tanning industry and paint industry. The hydrolyzable tannins have aroused the interest of researchers to be tested against microorganisms that infect the root canal sys-

tem because they are non-toxic and have favorable photophysical properties with high triplet-state yields[9]. The use of tannin in Group 5 promoted a statistically significant bacterial reduction of 69.9% (p = 0.001), better than groups 3 and 4 (methylene blue and toluidine blue, respectively) and group 1 (NaOCl alone). According to Verza et al.[38] (2012) hydrolyzable tannins have a stable binding to central glycose residues and consequently increased solubility and binding of its molecules to low-density lipoproteins, facilitating entry into the target cell.

Given the 69.9% bacterial load reduction and results superior to those of methylene blue and toluidine blue, the present authors hypothesize that tannin may be indicated in cases of persistent *E. faecalis* infection. Tannins are easily extracted from plants such as guava tree, '*espinheira-santa*' (*Maytenus* sp., Celastraceae), and Brazilian peppertree at a low cost. So, they have become a highly promising dye for use in aPDT. Curcumin is a pigment extracted from Curcuma Longa, a plant used as a spice in cuisine that also has anti-inflammatory, antitumor, antifungal, antibacterial, and anticarcinogenic properties, in addition to antiviral effects against hepatitis B and H1N1 viruses. It has an antimicrobial effect against gram-negative and gram-positive bacteria when used in aPDT and can be purchased at a compounding pharmacy. Curcumin has the advantage of being activated by a blue spectral range between 455 nm and 492 nm, which allows for the use of LED or halogen light devices[12]. In this study, the curcumin group (Group 6) showed a marked standard deviation and the 95,4% bacteria reduction was not statistically significant. Da Frota et al.[12] (2015) evaluated the efficacy of aPDT using curcumin in the elimination of Enterococcus faecalis from root canals, varying the time of LED irradiation (5 or 10 min) after 5 min of pre-irradiation time. They observed that 5min reduced more bacterial viability than 10 min LED irradiation. Mahdi et al.[39] (2015) investigated the effect of aPDT using a combination of curcumin (60 μM), H2O2 (0.3 mM), and erythrosine (22 μM) in planktonic cultures of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* and observed a 100% bacterial reduction after 5 min of blue light activation (450 nm).

In sum, the control group 2 (CMP with saline) and group 3 (CMP + aPDT with methylene blue) showed no reduction in CFUs. All other groups showed bacterial reduction between the initial and final collections, but group 6 (CMP + aPDT with curcumin) had a marked standard deviation and a not statistically significant reduction. Group 5 (CMP + aPDT with tannin) showed the best results, introducing tannin as an alternative dye with less toxicity and good antibacterial activity.

Despite the fact of all advances in rotary and reciprocating instruments, microbiology should always be the focus of endodontic treatment. It is well- known these instruments become instrumentation of radicular canal faster, reducing clinical time. In addition, studies have shown effectiveness of these instruments systems on microbial reduction[40]. However, the present authors have a concern if clinicians and specialists are, in fact, irrigating successfully in such reduced clinical time. aPDT emerges in this scenario improving and ensuring microbial load reducton inside radicular system canal. This study presents tannin and curcumin as alternatives to methylene blue and toluidine blue, showing promising results. However, randomized controlled trials are warranted to further test them in aPDT given their scientific relevance. Alternative photosensitizers and other concentrations in aPDT should be the focus of further *in vitro* and *in vivo* studies.

In conclusion, aPDT using a wavelength of 660 nm, 100 mW as light source during 180s in association with toluidine blue or tannin at a 0.005% concentration used as an adjunct to CMP with 2.5% NaOCl reduced *E. faecalis* significantly. Methylene blue at a 0.005% concentration and curcumin did not promote microbial reduction.

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