Photodynamic therapy in endodontic root canal treatment significantly increases bacterial clearance, preventing apical periodontitis

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Objective: To analyze the antimicrobial activity of photodynamic therapy as an adjunct to conventional endodontic treatment, particularly against *Enterococcus faecalis*. **Method and materials:** A total of 42 single-rooted teeth obtained from 33 patients with apical periodontitis were included. Sampling was developed in three stages: (1) immediately after accessing the root canal, (2) after chemical and mechanical instrumentation, and finally, (3) after photodynamic therapy application. The bacterial load of each sample was quantified by seeding on blood agar plates and selective M-*Enterococcus* agar. All growing colonies were identified using MALDI-TOF (Bruker; matrix-assisted laser desorption/ionization time-of-flight), and the entire bacterial microbiota composition was determined in the first sample by PCR-DGGE (polymerase chain reaction denaturing gradient gel electrophoresis), using 16 rDNA primers and selective nucleotide sequencing. **Results:** The endodontic therapy obtained a mean reduction in the cultivable bacterial load of 1.12 log, whereas the photodynamic therapy combination significantly increased the bacterial clearance ($P < .0001$). Viable cells of *E faecalis* were detected in 16.6% of root canals, with a mean value of 93 CFU per tooth, which was reduced to 67 and 9 CFU/tooth after conventional endodontic and photodynamic therapy treatments, respectively. Molecular *E faecalis* detection demonstrated that this species was present in 23.2% of baseline samples. DGGE analysis demonstrated the existence of a more complex microbiota than those observed using classical cultures. **Conclusion:** Photodynamic therapy as an adjunct to root canal treatment produces a significant reduction in *E faecalis* bacterial load, and it should be considered in the prevention of apical periodontitis. *(Quintessence Int 2019;50: 782–789; doi: 10.3290/j.qi.a43249)*

Key words: apical periodontitis, endodontics, *Enterococcus*, oral microbiota, photodynamic therapy

Bacterial infection plays an important role in establishing plural inflammation, which may lead to subsequent pulp necrosis and the formation of periapical lesions.[1] Complete eradication, or at least a significant reduction, of the bacterial load during root canal treatment is an important factor determining the final prognosis of endodontic therapy. In fact, negative microbiologic cultures from the root canal system have been correlated with an endodontic success rate close to 94%, whereas in positive cultures the success rate drops to 68%.[2] In the USA, more than 20 million root canal treatments are performed annually.[3] Development of apical periodontitis has been reported in 44.9% of studied cases in Austria.[4] In most cases, the etiology of endodontic failure is related to persistent or secondary endodontic infections.[5] Antibacterial irrigation solutions such as sodium hypochlorite (NaOCl) can penetrate up to 130 µm into dentinal tubules, while some bacterial species are able to penetrate more than 250 µm deep and adhere to collagen present in human serum, leaving bacteria harboring in deeper layers,

accessory canals, anastomoses, and fins.[6] Secondary infections are often linked to facultative anaerobic Gram-positive microorganisms, particularly *Enterococcus faecalis*, which has been shown to be highly resistant to conventional antimicrobial agents and able to invade dentinal tubules, causing reinfection of the root canal system.[7,8]

Recently, novel approaches to enhancing root canal disinfection have been proposed, with photodynamic therapy (PDT) being one of the most promising.[9-13] PDT has been described as a broad spectrum therapy effective against a wide range of microorganisms, including Gram-positive bacteria.[14,15] The therapy combines a light source with phenothiazinium antimicrobial photosensitizers, which bind selectively to bacterial cells without damaging host cells.[16,17] The emitted light is absorbed by the photosensitizer, which enters an excited state and reacts with oxygen to generate cytotoxic particles with bactericide activity.[18,19]

The aim of this work was to analyze the antimicrobial activity of PDT as an adjunct to conventional endodontic treatment, as well as its specific effect against *E faecalis*, with a null hypothesis (H0) stating that there would be no difference between the levels of disinfection achieved with conventional endodontic procedure and those achieved using PDT as an adjunct to conventional endodontic therapy.

Methods and materials

Study design

Forty-two single-rooted posterior teeth (20 maxillary and 22 mandibular premolars) were successively treated in 33 patients with apical periodontitis at the Dental Centre of Innovation and Advanced Specialties at Alfonso X El Sabio University (Madrid, Spain) between February 2015 and January 2016. The inclusion criteria were patients 21 to 35 years of age and in good health, who presented with signs and symptoms of apical periodontitis and required root canal treatment on teeth with closed apices. Patients who were not within the selected age range, who did not present with systemic pathologies, who had a different diagnosis or treatment plan, and patients with open apices, multi-rooted tooth, a previously treated tooth, or an affected tooth other than a premolar were excluded. All procedures followed the ethical guidelines established by the Declaration of Helsinki and the CONSORT Statement, and all were approved by the Alfonso X El Sabio University Ethics Committee (Process No. 01/2015). All patients gave their informed consent to take part in the study.

Clinical procedure

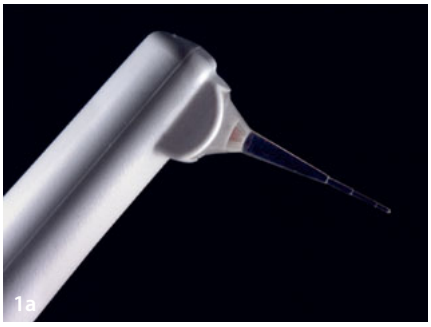
The teeth involved in this study were nonsensitive to both thermal (Endo-Ice, Coltène/Whaledent) and electrical pulp tests (Parkell). Periapical radiographs were taken to confirm the diagnosis of apical periodontitis. Endodontic treatment was performed using infiltrative anesthesia with lidocaine 2% and 1:100,000 epinephrine (Artinibsa, Inibsa). Rubber dams (Hygenic Dental Dam, Coltène Whaledent) were disinfected with a povidone-iodine solution (Betadine, Meda). The crown and rubber dam were disinfected with 5 mL of 30% H_2O_2 (Cinfa) for 30 seconds and 5 mL of 2.5% NaOCl (Clorox) for another 30 seconds after caries removal. NaOCl deactivation was achieved by irrigating with 5 mL of 5% sodium thiosulfate for 30 seconds. Subsequently, the pulp chamber was opened to enable access to the root canal system.

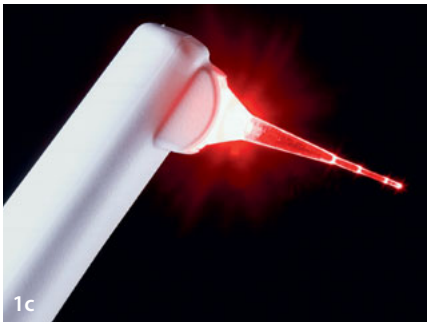
Canal instrumentation was performed with a 10/.02 K-file (Dentsply Maillefer), and 1 mL of sterile saline solution (Braun) was used to irrigate the canal and detach any bacteria adhered to dentin. The first sample ("baseline") was collected using three sterile paper points (Dentsply Maillefer) inserted into the root canal system for 1 minute.

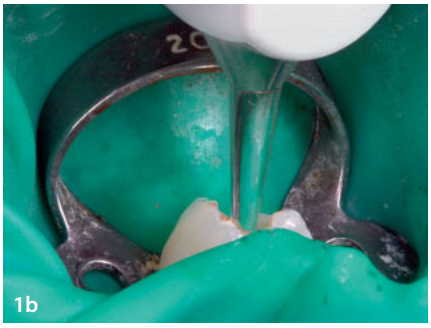
The working length of the root canal was determined using an electronic apex locator (Raypex 6, VDW) and verified with a working length radiograph using a 20/.02 K-file. Each canal was prepared using an R25 rotary file (Reciproc, VDW) and irrigated with 5 mL of 5.25% NaOCl, 5 mL of 17% ethylenediaminetetraacetic acid (EDTA; SmearClear, SybronEndo), and 5 mL of sterile saline solution (Braun) using an endodontic needle (Miraject Endo Luer, Hager & Werken) with a diameter of 0.3 mm inserted 1 mm into the working length. To collect the second sample ("root canal treatment"), the root canal system was dried with three sterile paper points inserted into the root canal.

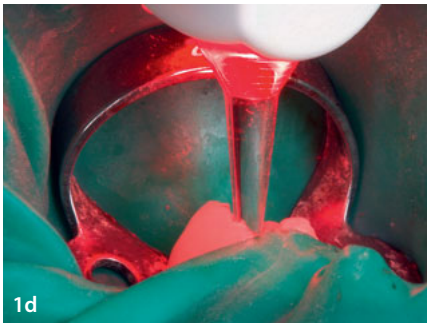
Subsequently, the root canal system was treated with PDT. A photosensitizer (FotoSan 630, CMS Dental) was applied inside the root canal (0.5 mL) for 2 minutes. The canal was then irradiated using a 50/.03 endoscopic threaded imaging port (EndoTIP, CMS Dental) (Fig 1) and fiber-coupled infrared light-emitting diode (LED) (FotoSan 630). The LED emitted light with a wavelength of 630 ± 20 nm and intensity of 2,000 mW/cm^2. The fiber tip was placed at 1 mm of the working length and light applied for two cycles of 30 seconds each. A brand new fiber tip was used for each patient. The canal was then irrigated with 5 mL of sterile saline solution to flush the photosensitizer out of the root canal, and finally the third sample ("PDT") was collected.

Each root canal system was sealed using a warm gutta-percha system (Calamus, Dentsply Maillefer) and an epoxy-amine









Figs 1a to 1d Photodynamic therapy in a mandibular right second premolar. Detail of the EndoTIP before *(a and b)* and after *(c and d)* infrared LED emission.

resin-based sealer (AH Plus, Dentsply DeTrey). Finally, cavity access was restored with a flowable composite resin (Filtek Supreme XTE, 3M) and follow-up appointments were scheduled at 3, 6, and 12 months to assess the treatment outcome.

Microbiologic processes

After sample collection, the sterile paper points were transferred to a sterile Eppendorf tube containing 1 mL of Nutrient Broth medium (Difco) and immediately frozen at −20°C. All samples were processed together at the end of the study. After being slowly defrosted at 4°C for 24 hours and vortexed for 5 minutes, 100 µL from each tube were seeded onto 5% sheep blood agar plates (bioMérieux) and M-*Enterococcus* agar plates (Difco). After 24 to 48 hours of incubation at 37°C, colonies were counted and identified using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF; Bruker).

Total DNA of the baseline samples was obtained using the QIAamp kit (Qiagen), and bacterial microbiota were detected with the universal polymerase chain reaction (PCR) method using 16 rDNA primers for the V3 to V4 region (968GC-F: 5´-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC-3′ and Uni1401R: 5´-CGGTGTGTACAAGACCC-3′). The amplicons obtained were separated using denaturing gel gradient electrophoresis (DGGE) in vertical electrophoresis polyacrylamide gels (8%) at 60°C, with a urea-formamide denaturing gel gradient (30% to 50%) subjected to 130 V for 330 minutes. Gels were visualized after staining with ethidium bromide, and specific bands were extracted from the gel, re-amplified by PCR, and sequenced to identify the relevant microorganism. Finally, the presence of *E faecalis* was also tested by PCR in the baseline samples using the specific ddl primers E1, 5′-ATCAAGTACAGTTAGTCT-3′ and E2, 5′-ACGATTCAAAGCTAACTG-3′.

Statistical analysis

Statistical analysis of all variables was carried out using SPSS 22.00 (IBM) and GraphPad Prism 7.0 (GraphPad Software). Descriptive statistics were expressed as means and standard deviation (SD) for quantitative variables and as absolute numbers and percentages for qualitative variables. Comparative analysis was performed by comparing the mean colony-forming unit (CFU) count for each group before and after intervention, using the Mann-Whitney U test because variables did not have a normal distribution. The statistical significance was set at $P < .05$.

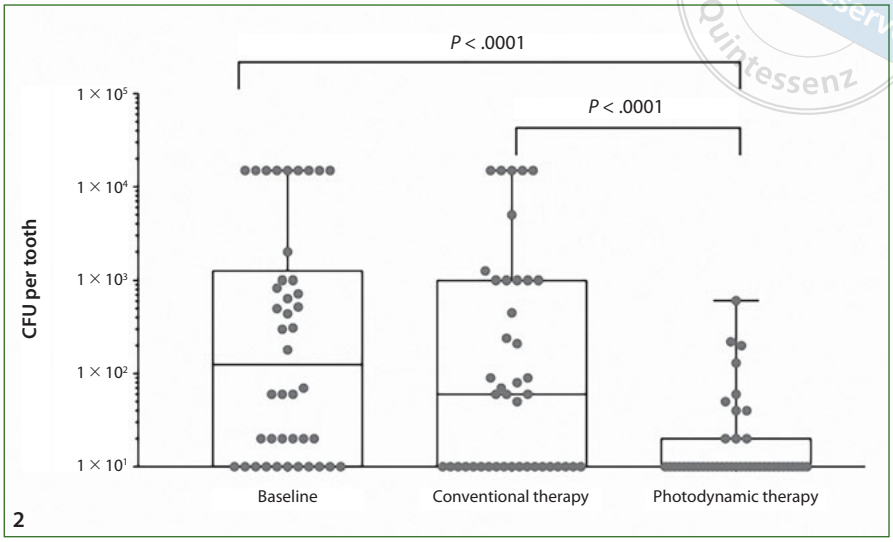


Fig 2 Mean ± standard deviation of the colony-forming unit (CFU) per tooth in the three different samples of the study. The individual values are also plotted using gray dots.

Results

Significant reductions were observed in bacterial load, including detected microorganisms, from the baseline sample (median value ± SD; 113.5 ± 130 CFU/tooth) to the second sample (26.52 ± 72 CFU/tooth), with further reductions observed in the third sample, which was taken after PDT (4.2 ± 13 CFU/tooth) (Fig 2).

In five teeth, cultivable microorganisms were not detected in any of the three microbiologic samples. Additionally, the baseline sample was sterile in four other teeth. Similarly, cultivable bacteria were not detected in 12 and 21 teeth of the root canal treatment and PDT samples, respectively (Table 1). The teeth with no bacterial cultures were excluded from the sample. A mean bacterial reduction of 71.39% was observed in the root canal treatment sample when compared with the baseline sample, with a mean bacterial reduction of 96.86% from the baseline sample to the PDT sample. A bacterial reduction of 25.47% was observed in the PDT sample when compared with the root canal treatment sample (Table 1).

Identification of viable endodontic microbiota revealed up to 32 bacterial species, with *Staphylococcus epidermidis* being the most common (54.7%), followed by *Kocuria* (21.4%), *Micrococcus* (14.2%), *E faecalis* (11.9%), *Microbacterium* (11.9%), *Bacillus* (7.1%), *Rothia* (4.7%), and *Brevibacillus* (4.7%). The minority species were *Actinomyces*, *Clostridium*, *Dietzia*, *Massilia*, and *Pseudomonas*, detected in 2.3% of the samples. Curiously, 68.7% of the cultivable species corresponded to obligate aerobes and 18.7% to facultative anaerobes, with one facultative aerobe species and one obligate aerobe species (6.2% each).

E faecalis colonies were observed in 16.6% of the first sample (baseline) with a mean value of 93 CFU/tooth. Following conventional endodontic treatment, *E faecalis* counts were reduced by up to 26 CFU (28% of the bacterial load), whereas PDT achieved a significant additional reduction of 84 CFU, representing a 90.3% decrease in the bacterial count ($P < .0001$). Other endodontic pathogens such as *Stenotrophomonas maltophilia* (2.3% of teeth, with a mean value of 19 CFU/tooth) were also identified inside the root canals, although these were completely eradicated after combined endodontic treatment and PDT.

The molecular detection of *E faecalis* by specific PCR showed that this species was present in 23.2% of baseline samples. Presence of *E faecalis* was also confirmed by DGGE analysis. This technique also definitively demonstrated the existence of a more complex microbiota than that observed in classical cultures (Fig 3a). Each band of the DGGE pattern represents independent bacterial species, observing a median value of 4 to 5 bands per tooth, ranging from 1 to 13 bands per tooth. Contrary to the results of the microbiologic culture, all baseline samples yielded positive amplicons, demonstrating universal bacterial colonization of the root canal. Only the widest bands could be identified in the DGGE experiments (Fig 3b).

Table 1 Individual readings of CFU values of each tooth, as well as reduction values (R) between the samples (S)

				Bacterial reduction (%)		
				R1	R2	R3
Tooth	S1 (CFU)	S2 (CFU)	S3 (CFU)	S1–S2	S2–S3	S1–S3
1	150	81	0	54	100	100
2	100	10	0	90	100	100
3	24	8	0	67	100	100
4	35	10	2	71	80	94
5	40	0	0	100	0	100
6	2	0	0	100	0	100
7	5	0	0	100	0	100
8	300	106	63	65	41	79
9	50	22	0	56	100	100
10	52	0	0	100	0	100
11	100	10	1	90	90	99
12	100	10	6	90	40	94
13	0	0	0	0	0	0
14	100	0	0	100	0	100
15	0	0	0	0	0	0
16	2	0	0	100	0	100
17	79	1	0	99	100	100
18	200	100	0	50	100	100
19	44	0	0	100	0	100
20	100	80	21	20	74	79
21	137	120	40	15	67	71
22	1,000	280	6	72	98	99
23	100	12	3	88	75	100
24	125	32	20	74	38	100
25	20	0	0	100	0	100
26	400	10	1	98	10	100
27	98	30	2	69	93	98
28	1,000	145	4	86	97	100
29	80	10	1	88	10	99
30	10	0	0	100	0	100
31	0	0	0	0	0	0
32	17	10	4	41	60	77
33	31	0	0	100	0	100
34	35	10	1	71	10	97
35	6	2	0	67	100	100
36	12	4	0	67	100	100
37	72	0	0	100	0	100
38	26	1	0	96	100	100
39	2	0	0	100	0	100
40	102	10	1	90	100	99
41	0	0	0	0	0	0
42	0	0	0	0	0	0

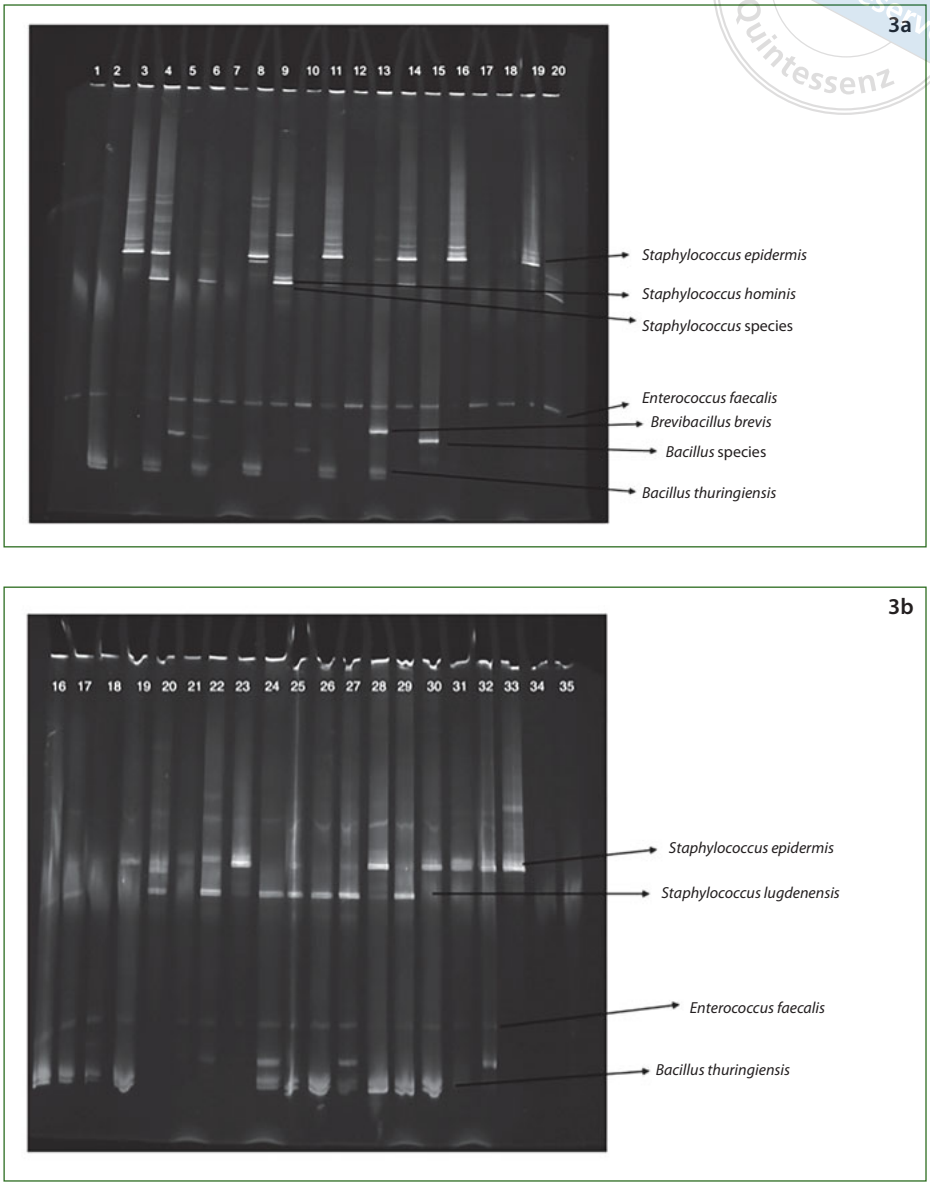
Discussion

The results obtained in the present study rejected the null hypothesis (H0) that states that there would be no difference between the levels of disinfection achieved with conventional endodontic procedure versus PDT as an adjunct to conventional endodontic therapy. Conventional endodontic treatment of infected root canals includes mechanical instrumentation, clearance by means of various irrigation solutions, and the administration of antimicrobial medication in the canal. These techniques lead to a considerable reduction in bacterial load, although complete sterilization is virtually impossible.[20,21] However, complementing conventional endodontic treatment with PDT appears to be very effective in reducing the complete microbiota, and particularly *E faecalis*.[22] Some ex vivo studies using PDT in combination with conventional endodontic therapy have reported mean reduction values higher than those reported in in vivo studies.[21,23]

Root canal systems affected by apical periodontitis usually contain complex, variable pathologic microbiota that also tend to form biofilms.[18] Great care must be taken during the process of sample collection inside the root canal system and subsequent culturing in order to ensure microbial growth and avoid ambient contamination.[24] Nevertheless, this remains the most effective short-term means of evaluating in vivo disinfection of root canals.[25]

In the present study, the root canal microbiota has been characterized using not only classical cultures but also molecular detection. In general, the results showed considerable concordance between both techniques, and although the molecular detection was observed to be more sensitive, the detection of nonviable bacterial cells might also be taken into account. Despite the efficacy of culture-based methods in the surveillance of microbial diversity profiles in primary and secondary endodontic infections, it is possible that some microorganisms present in the root canal remain unidentified, especially when the microbial count is low.[25] The past decade has seen many advances in microbial molecular diagnostics, with PCR technology and its derivatives being the most frequently used. PCR and other molecular methods would appear to offer a means of better identification and thus greater understanding of the causative agents involved in endodontic infections.[26]

A significant number of studies have identified *E faecalis* as the most prevalent species after root canal treatment,[5,27] a finding that is corroborated by the results of the present study. The resistance of enterococci to endodontic treatment has long been recognized, and even calcium hydroxide has been deemed



Figs 3a and 3b Denaturing gel gradient electrophoresis (DGGE) of the baseline sample (from 1 to 35) after universal bacterial amplification by PCR using 16 sDNA primers. Identified bands are marked.

ineffective against *E faecalis*.[28] Nevertheless, a median bacterial reduction of 10^5 CFU/mL has been observed after irrigation with NaOCl or chlorhexidine (CHX) and calcium hydroxide placement for 7 days on extracted single-rooted teeth previously inoculated with *E faecalis*. This in vitro study determined that the use of irrigant solutions was responsible for the increase in the antibacterial efficacy of calcium hydroxide.[29] Bacterial resistance to PDT appears to be higher in slow-growing cells, including *E faecalis*, although there is no consensus in the literature with regard to the effect of bacterial growth rate on susceptibility to PDT.[30] Some authors have used cells with a slow growth rate, posing a challenge to antimicrobial treatment, but PDT was found to be an effective approach in spite of this slow growth rate.[21] Some reports indicate that PDT phototoxicity is mainly caused by photodynamic mechanisms that require oxygen.[31,32] It has been established that oxygen radicals such as hydroxyl, superoxide, or singlet oxygen can damage cell membranes through lipid peroxidation and may also damage DNA.[33]

Previously published results of bacteria eradication by PDT[18,21] do not report eradication rates as high as those in the present study. This may be due to the optical fiber inserted in the channel to access the entire cavity, which likely provided

more uniform illumination, reaching the more recondite areas of the canal.[34]

Several studies support the clinical use of PDT in both permanent and primary dentition.[35] It has been found that this could be a promising adjunct therapy requiring only a single session, with favorable results in comparison with conventional treatment after 90 days, with PDT achieving an absence of inflammatory cells, moderate fibrogenesis, and neoangiogenesis. Another radiographic study compared the periapical healing of teeth treated with and without PDT.[36] Both therapies promoted an increase in periapical healing over time, but the PDT showed better results after a 6-month follow-up when compared with conventional endodontic treatment alone. However, another study evaluated the response of apical and periapical tissues in induced periapical lesions, comparing a one-session treatment with and without PDT to a two-session treatment combined with a calcium hydroxide-based intracanal dressing. Three months after treatment therapy, the two-session treatment was shown to result in significantly smaller periapical lesions, characterized by progressive repair, when compared with the one-session therapy with PDT.[37]

The teaching objective derived from this study is that applying PDT as an adjunct to endodontic therapy results in a further reduction of endodontic bacterial counts, especially those of *E faecalis*.

To date, PDT has proven to be an efficient adjunct therapy to endodontic treatment, and it offers promising results as an adjunct therapy for the treatment of endodontic infections. Nevertheless, further research is needed to determine the potential of PDT in treating endodontic pathologies.

Conclusion

PDT as an adjunct to root canal treatment significantly reduces bacterial load, including that of *E faecalis*. This technique can help to reduce the endodontic bacterial load, decreasing the risk of endodontic failure caused by the presence of persistent endodontic pathogens.

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Declaration

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References

1. Siqueira JF Jr, Rôças IN. Polymerase chain reaction–based analysis of microorganisms associated with failed endodontic treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2014;97:85–94.
2. Sjögren T, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J 1997;30:297–306.
3. Brown LJ, Nash KD, Johns BA, Warren M. The economics of endodontics. ADA Health Policy Resources Center Dental Health Policy Analysis Series. Chicago: ADA Health Policy Institute, 2003.
4. Kielbassa AM, Frank W, Madaus T. Radiologic assessment of quality of root canal fillings and periapical status in an Austrian subpopulation: an observational study. PLoS One 2017;12:e0176724.
5. Siqueira JF Jr. Aetiology of root canal treatment failure: why well-treated teeth can fail. Int Endod J 2001;34:1–10.
6. Kirsch J, Basche S, Neunzehn J, et al. Is it really penetration? Locomotion of devitalized *Enterococcus faecalis* cells within dentinal tubules of bovine teeth. Arch Oral Biol 2017;83:289–296.
7. Love RM. *Enterococcus faecalis*: a mechanism for its role in endodontic failure. Int Endod J 2001;34:399–405.
8. Siqueira JF Jr. Endodontic infections: concepts, paradigms, and perspectives. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;94:281–293.
9. Armand A, Khani M, Asnaashari M, AliAhmadi A, Shokri B. Comparison study of root canal disinfection by cold plasma jet and photodynamic therapy. Photodiagnosis Photodyn Ther 2019;26:327–333.
10. Plotino G, Grande NM, Mercade M. Photodynamic therapy in endodontics. Int Endod J 2019;52:760–774.
11. Pourhajibagher M, Bahador A. An in vivo evaluation of microbial diversity before and after the photo-activated disinfection in primary endodontic infections: Traditional phenotypic and molecular approaches. Photodiagnosis Photodyn Ther 2018;22:19–25.
12. De Miranda RG, Colombo APV. Clinical and microbiological effectiveness of photodynamic therapy on primary endodontic infections: a 6-month randomized clinical trial. Clin Oral Investig 2018;22:1751–1761.
13. Rabello DGD, Corazza BJM, Ferreira LL, Santamaria MP, Gomes APM, Martinho FC. Does supplemental photodynamic therapy optimize the disinfection of bacteria and endotoxins in one-visit and two-visit root canal therapy? A randomized clinical trial. Photodiagnosis Photodyn Ther 2017;19:205–211.
14. Takasaki AA, Aoki A, Mizutani K, et al. Application of antimicrobial photodynamic therapy in periodontal and peri-implant diseases. Periodontol 2000 2009;51:109–140.

15. Dougherty TJ. An update on photodynamic therapy applications. J Clin Laser Med Surg 2002;20:3–7.

16. Xu Y, Young MJ, Battaglino RA, et al. Endodontic antimicrobial photodynamic therapy: safety assessment in mammalian cell cultures. J Endod 2009;35:1567–1572.

17. Kübler AC, Stenzel W, Rühling M, Meul B, Fischer JH. Experimental evaluation of possible side effects of intra-operative photodynamic therapy on rabbit blood vessels and nerves. Lasers Surg Med 2003;33:247–255.

18. Garcez AS, Nuñez SC, Hamblin MR, Ribeiro MS. Antimicrobial effects of photodynamic therapy on patients with necrotic pulps and periapical lesion. J Endod 2008;34:138–142.

19. Jori G, Fabris C, Soncin M, et al. Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications. Lasers Surg Med 2006;38:468–481.

20. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol 1995;33:24–27.

21. Garcez AS, Nuñez SC, Lage-Marques JL, Jorge AOC, Ribeiro MS. Efficiency of NaOCl and laser-assisted photosensitization on the reduction of *Enterococcus faecalis* in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:e93–e98.

22. Maisch T, Wagner J, Papastamou V, et al. Combination of 10% EDTA, Photosan, and a blue light hand-held photopolymerizer to inactivate leading oral bacteria in dentistry in vitro. J Appl Microbiol 2009;107:1569–1578.

23. Fimple JL, Fontana CR, Foschi F, et al. Photodynamic treatment of endodontic polymicrobial infection in vitro. J Endod 2008;34:728–734.

24. Pinheiro ET, Gomes BP, Drucker DB, Zaia AA, Ferraz CC, Souza-Filho FJ. Antimicrobial susceptibility of *Enterococcus faecalis* isolated from canals of root filled teeth with periapical lesions. Int Endod J 2004;37: 756–763.

25. Wade WG. Non-culturable bacteria in complex commensal populations. Adv Appl Microbiol 2004;54:93–106.

26. Anderson AC, Hellwig E, Vespermann R, et al. Comprehensive analysis of secondary dental root canal infections: a combination of culture and culture independent approaches reveals new insights. PLoS One 2012;7:1–12.

27. Lewis MAO, MacFarlane TW, McGowan DA. Quantitative bacteriology of acute dento-alveolar abscesses. J Med Microbiol 1986;21:101–104.

28. Haapasalo M, Orstavick D. In vitro infection and disinfection of dentinal tubules. J Dent Res 1987;66:1375–1379.

29. Noetzel J, Nonhoff J, Bitter K, Wagner J, Neumann K, Kielbassa AM. Efficacy of calcium hydroxide, Er:YAG laser or gaseous ozone against *Enterococcus faecalis* in root canals. Am J Dent 2009 ;22:14–18.

30. Gad F, Zahra T, Hasan T, Hamblin MR. Effects of growth phase and extracellular slime on photodynamic inactivation of gram-positive pathogenic bacteria. Antimicrob Agents Chemother 2004;48:2173–2178.

31. Arfsten DP, Schaeffer DJ, Mulveny DC. The effects of near ultraviolet radiation on the toxic effects of polycyclic aromatic hydrocarbons in animals and plants: a review. Ecotoxicol Environ Saf 1996;33:1–24.

32. Yu HT. Environmental carcinogenic polycyclic aromatic hydrocarbons: photochemistry and phototoxicity. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2002;20:149–183.

33. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 1998;42:13–28.

34. Komerik N, Nakanishi H, MacRobert AJ, Henderson B, Speight P, Wilson M. In vivo killing of *Porphyromonas gingivalis* by toluidine blue–mediated photosensitization in an animal model. Antimicrob Agents Chemother 2003;47:932–940.

35. Okamoto CB, Motta LJ, Prates RA, et al. Antimicrobial photodynamic therapy as a co-adjuvant in endodontic treatment of deciduous teeth: case series. Photochem Photobiol 2018;94:760–764.

36. Miranda RG, Colombo APV. Clinical and microbiological effectiveness of photodynamic therapy on primary endodontic infections: a 6-month randomized clinical trial. Clin Oral Investig 2017;22:1751–1761.

37. Borsatto MC, Correa-Afonso AM, Lucisano MP, et al. One-session root canal treatment with antimicrobial photodynamic therapy (aPDT): an in vivo study. Int Endod J 2016;49:511–518.

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