Ex Vivo Evaluation of the Efficacy of Photodynamic Therapy in Eliminating *Enterococcus faecalis* from Dentinal Tubules by Confocal Laser Scanning Microscopy

Roberta de Castro Leitão Godoy, DDS, MSc,[*] Carlos Eduardo da Silveira Bueno, PhD,[*] Alexandre Sigrist De Martin, DDS, MSc, PhD,[*] Rina Andrea Pelegrine, DDS, MSc, PhD,[*] Carlos Eduardo Fontana, DDS, MSc, PhD,[†] Marco Antônio Hungaro Duarte, DDS, MSc, PhD,[‡] Rodrigo Ricci Vivan, DDS, MSc, PhD,[‡] Wayne Martins Nascimento, DDS, MSc,[*] Ana Grasiela da Silva Limoeiro, DDS, MSc, PhD,[*] and Daniel Guimarães Pedro Rocha, DDS, MSc, PhD[§]

ABSTRACT

Introduction: The aim of the present study was to investigate *ex vivo* by confocal laser scanning microscopy the antibacterial effect of photodynamic therapy (PDT) on dentinal tubules in the apical 5 mm of human mandibular premolars contaminated with *Enterococcus faecalis*. **Methods:** Thirty-four teeth were standardized to 20 mm and foraminal anatomic diameters using a #20 K-file (Dentsply Maillefer). Samples were contaminated for 21 days and divided into the following 3 experimental groups ($n = 10$): the PDT group (instrumented canals and PDT), the passive ultrasonic irrigation (PUI) group (instrumented canals and PUI), and the PUI-PDT group (instrumented canals, PUI, and PDT), along with a control group ($n = 4$) (noninstrumented canals). The canals in the experimental groups were instrumented with ProTaper Next (Dentsply Maillefer) up to X3 and rinsed with EDTA and sodium hypochlorite. The photosensitizer used was 0.01% methylene blue with a preirradiation time of 5 minutes and a diode laser with 4 J energy and a 660-nm wavelength. Cross sections were made 5 mm from the apex of all samples, which were analyzed using confocal laser scanning microscopy. The results were analyzed using the Shapiro-Wilk and Kruskal-Wallis (Dunn) tests. **Results:** There was a lower percentage of live bacteria in the PUI-PDT group, with a statistical difference compared with the control and PDT groups ($P < .05$). There was no statistical difference in the percentage of live bacteria between PUI-PDT and PUI ($P > .05$). **Conclusions:** It was concluded that the PUI-PDT association was most effective in disinfecting root canals compared with the control group and PDT. *(J Endod 2023;49:889–893.)*

KEY WORDS

Laser scanning confocal microscopy; low-power laser therapy; photodynamic therapy

SIGNIFICANCE

In clinical practice, the combination of PUI and PDT may provide better disinfection of the root canal by killing more bacteria in the dentinal tubules.

Antimicrobial adjuvant chemicals promote the dissolution of organic tissue and the removal of the smear layer and are essential for successful endodontic treatment. The dentinal tubules adjacent to the root canal walls harbor microorganisms, and this is a potential cause of persistent endodontic infections[1]. *Enterococcus faecalis* is 1 of the predominant bacteria in persistent endodontic infections. They can survive in the root canal system under unfavorable conditions, such as alkalinity and malnutrition[2]. They may also invade dentinal tubules and be resistant to various antibiotics, making complete removal by mechanical instruments and intracanal medications difficult[3]. Endodontic treatment has become faster, resulting in a reduction of the irrigation time during chemical-mechanical preparation. Even with the use of safer techniques, some areas of the canal wall may remain untouched and covered by biofilm[4]. Therefore, the use of passive ultrasonic irrigation (PUI) becomes relevant as an additional approach to root canal disinfection[5].

From the *Faculdade São Leopoldo Mandic, Department of Endodontics, Instituto de Pesquisas São Leopoldo Mandic, Campinas; †Pontifical Catholic University of Campinas, Programa de pós-Graduação em Ciências da Saúde, Center of Life Sciences, Campinas; ‡Faculdade de Odontologia de Bauru, University of São Paulo, Bauru, São Paulo; and §Pontifical Catholic University of Campinas, Department of Endodontics, Center of Life Sciences, Campinas, São Paulo, Brazil

Address requests for reprints to Dr Ana Grasiela da Silva Limoeiro, Faculdade São Leopoldo Mandic, R. Dr. José Rocha Junqueira, 13, Campinas-SP, Brazil, 13045-755.
E-mail address: grasielalimoeiro@gmail.com
0099-2399/$ - see front matter

https://doi.org/10.1016/j.joen.2023.05.007

Photodynamic therapy (PDT) is used as an adjunctive therapy to endodontic treatment to eliminate microorganisms that are persistent to chemical-mechanical preparation. It is a good option to maximize disinfection of the root canal system[6].

To the best of our knowledge, there are only a few studies investigating the efficacy of PDT against *E. faecalis* in dentinal tubules using confocal laser scanning microscopy (CLSM). Furthermore, there are few studies that have evaluated whether the use of PDT after ultrasonic agitation of endodontic irrigants increases the antimicrobial efficacy, thus representing a gap in the literature. The aim of the present study was to investigate the antibacterial effect of PDT on dentinal tubules in the apical 5 mm of mandibular premolars contaminated with *E. faecalis* in 3 different groups using CLSM. The null hypothesis was that there would be no significant difference in the reduction of *E. faecalis* in the 3 groups.

MATERIALS AND METHODS

This study was approved by the local research ethics committee under number 3,648,229. Thirty-four human mandibular premolars were used with fully developed roots, without previous endodontic treatment, and free of fractures and calcifications and extracted for periodontal reasons. The teeth were stored in 0.1% thymol (Ags Química) until the beginning of the experiment.

A sample calculation was performed (G Power 3.1.9.4, Franz Faul) using an alpha error of 0.05 and a beta error of 0.80. The minimum number of samples calculated for each group was 10[7]. Roots were washed for 1 hour to remove all residues of the thymol solution and then dried with gauze and an air jet. Teeth were standardized to a size of 20 mm by grinding the occlusal surfaces with a diamond disk (Buehler). The working length (WL) was determined by introducing a K-file #10 (Dentsply Maillefer) into the canal until its tip was visualized at the apical foramen. The WL was established 1 mm short of the apical foramen. All canals were instrumented at the WL with the K-file #20 instrument (Dentsply Maillefer), and the irrigation was performed with 1 mL 0.9% saline solution (Laboratório Farmacêutico Arboreto). Subsequently, all samples were immersed in the following solutions: 10 minutes in 1% sodium hypochlorite (NaOCl) solution (Asfer Indústria Química Ltda) and 10 minutes in 17% EDTA (Asfer Indústria Química Ltda). Subsequently, all root apexes were sealed with a 7 wax (Imodonto) to prevent leakage of the irrigation solutions. Each tooth was placed in a plastic cylinder 2 cm high and 2 cm in diameter filled with condensation silicone (Zhermack SpA) and then sterilized with ethylene oxide (Acecil).

The reference microorganism was *E. faecalis* (ATCC 29212, American Type Culture Collection). The strain was thawed, and 25 μL of it was transferred to a tube containing sterile brain-heart infusion (BHI) broth, which was then kept in an incubator at 37°C for 24 hours. After this time, the broth was turbid, which was compared with the 1.5 × 108 colony-forming unit/mL Mac Farland scale[8]. Each sample was contaminated using a micropipette. The teeth were placed in sterile glass containers and placed in a 10% CO_2 oven at 37°C for 21 days[8]. All samples were hydrated with sterile BHI broth daily. To check the viability of the contamination, sterile paper tips were inserted into the root canals for 1 minute every 3 days. Then, the same paper tips were placed in tubes containing sterile BHI broth, which were stored in a CO_2 oven at 35°C/37°C for 24 hours. If the BHI broth remained turbid, contamination was confirmed.

The 34 teeth were randomly allocated (www.randon.org) into 3 different experimental groups ($n = 10$): the PDT group (instrumented canals and PDT), the PUI group (instrumented canals and PUI), and the PUI-PDT group (instrumented canals, PUI, and PDT), along with a control group ($n = 4$) (noninstrumented canals).

In all the canals of the experimental groups, the pathway was confirmed with manual K-files #10, #15, and #20 (Dentsply Maillefer). The irrigation was performed using 1 mL 2.5% NaOCl between each change of files. Subsequently, all root canals of these same groups were instrumented with ProTaper Next up to X3 (Dentsply Maillefer) using the X-Smart plus motor (Dentsply Maillefer) at 300 rpm and a torque of 3 Ncm. At each file change, the root canals were irrigated with 2 mL 2.5% NaOCl using Ultradent syringes (Ultradent Products) and Navitips (Ultradent Products) for a total of 6 mL 2.5% NaOCl.

In the PUI and PUI-PDT groups, the following final irrigation protocol was performed: 1 mL EDTA 17% for 10 seconds and activation for 20 seconds repeated 3 times for a total volume of 3 mL EDTA 17%, followed by 2 mL NaOCl 2.5% for 10 seconds and activation for 20 seconds repeated 3 times for a total volume of 6 mL 2.5% NaOCl. Ultrasonic activation (Gnatus Equipamentos Médico Odontológicos Ltda) was performed with the Irrisonic E1 tip (Helse).

In the groups using PDT, a 0.01% methylene blue solution (Chimiolux, DMC) was fully inserted into the root canals using the same type of syringe and tip used for irrigation. The dye was agitated with a K-file #15 and remained in the canal for 5 minutes (preirradiation time). A laser diode (DMC Importação e Exportação de Equipamentos Ltda) with an energy of 4 J, a fluence of 320 J/cm, and a wavelength of 660 nm with a fiber optic coupling of 1-mm diameter penetrated the entire root canal for 40 seconds. The photosensitizing agent was removed from inside the canal using sterile paper tips (Dentsply Indústria e Comércio Ltda).

Analysis of the Sample

A cross section was performed using a 102 mm × 0.3 mm × 12.7 mm diamond cutting disc (11C, Erios Equipamentos Ltda) at a 5-mm root apical height. Each cross section was subjected to the following procedure: 5 minutes in EDTA 17%, removal of the excess with saline solution, and subsequent drying with gauze. Then, the dye was applied for 10 minutes with the help of a dispensing pipette using 1.5 μL LIVE/DEAD Syto 9 (Baclight Bacterial Viability Kit, Life Technologies Corporation). Each sample section was placed on a glass slide, and the cross section was fixed on it to be analyzed by CLSM using the LEICA TCS SP2 MVLC (Microsystems GmbH) so that the amount of alive and dead bacteria in the dentinal tubules could be visualized. Four images of random areas of each sample were taken with an oil immersion objective at 40× magnification. Each image represented an area of 275 × 275 mm^2. Leica Confocal LAS AX software (Microsystems GmbH) was used for quantification. The parameters evaluated in each group were the percentage of the green population (ie, live cells). The results were subjected to the Shapiro-Wilk normality test and showed nonnormal behavior. The nonparametric Kruskal-Wallis (Dunn) test was performed with a significance level of 5%.

RESULTS

There was a lower percentage of live bacteria in the PDT + PUI association (Fig. 1), with a significant difference compared with the control group and PDT ($P < .05$). There was no significant difference in the percentage of live bacteria between PUI + PDT and PUI ($P > .05$) (Table 1).

DISCUSSION

This study investigated the antibacterial effect of 3 methods for disinfecting the dentinal tubules of mandibular premolars contaminated with *E. faecalis*: PDT, PUI, and PUI-PDT. PUI with 2.5% NaOCl in association with PDT resulted in a greater reduction of *E. faecalis* from the dentinal tubules compared with the control and PDT groups, so the null hypothesis was rejected.

E. faecalis was used because it is commonly associated with cases of endodontic failure because of its ability to form a biofilm and penetrate the dentinal tubules, which protects it from chemical irrigants[9]. After an incubation period of 3 weeks, *E. faecalis* can form a biofilm that adheres to dentin walls[10]. The images obtained in this study using CLSM demonstrate the ability of *E. faecalis* to invade dentinal tubules after 21 days of contamination and incubation.

NaOCl 2.5% was chosen as the irrigation solution because of its ability to denature bacterial proteins and dissolve organic tissues and because it has been used at this concentration in previous studies[11,12]. Conventional irrigation with a syringe and needle, although widely used, does not meet some basic requirements of contemporary endodontics, including the ability to deliver the irrigation solution along the entire length of the main canal and lateral canals. PUI was used in this study because it is more effective in cleaning root canals compared with conventional irrigation[5,13].

Previous *in vitro* studies[14-17] have demonstrated the bactericidal efficacy of PDT. The use of a photosensitizer with high

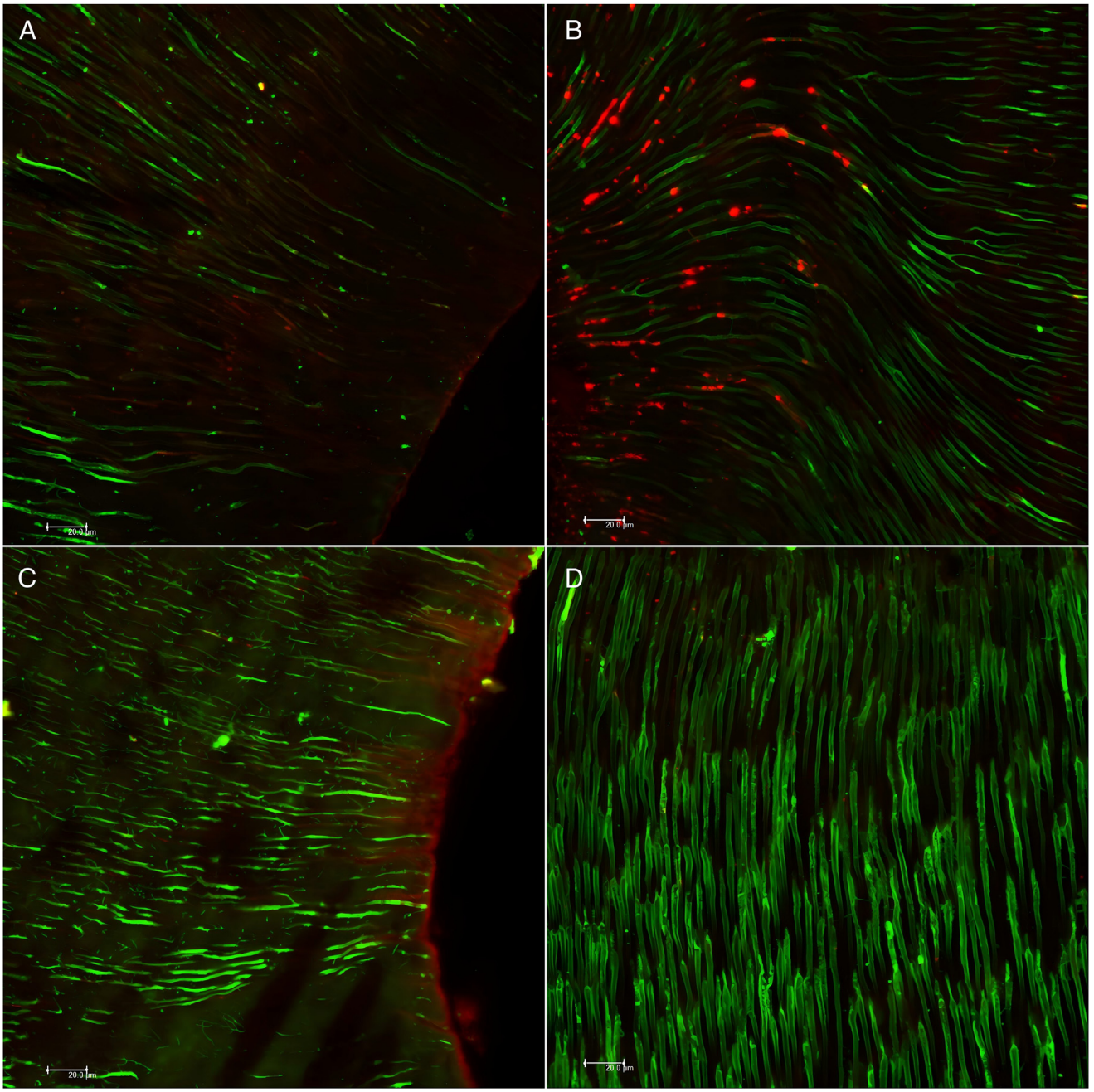

FIGURE 1 – Images acquired using CLSM showing cell viability and live (*green*) and dead (*red*) bacteria in each group. (*A*) PUI-PDT, (*B*) PUI, (*C*) PDT, and (*D*) control.

TABLE 1 - Median, Minimum, and Maximum Values of the Percentages of Live *Enterococcus faecalis* Biofilm Cells after the Different Protocols

	Live cells
Groups	Median (minimum-maximum)
Control	74.86 (57.37-87.22)^{A}
PDT + PUI	4.3 (0.29-30.69)^{B}
PUI	18.95 (7.18-35.03)^{B}
PDT	40.71 (23.31-69.57)^{A}

PDT, photodynamic therapy; PUI, passive ultrasonic irrigation.
Different superscript capital letters in the vertical direction indicate statistically significant differences ($P < .05$).

permeability in the dentinal tubules is necessary, and methylene blue showed its success in relation to the amount of singlet oxygen produced when excited by light. The PDT group showed no significant reduction in *E. faecalis*. The energy used was 4 J[12,18-20], which demonstrated significant success in bacterial reduction when only PDT was used. One way to achieve better results with PDT would be to increase the energy to 9 J, as is currently used[21]. It is worth noting that in this experiment, CLSM was used to evaluate and quantify the presence of live intratubular bacteria (green color)[22,23]. Other studies used bacterial cultures[14] or scanning microscopy[17], which do not quantify but rather only assess the presence or absence of bacteria in the dentinal tubules. Wang and Huang[9] compared the same groups as in this experiment, and the PUI-PDT group showed significant bacterial reduction compared with the PUI group. However, in the study by Wang and Huang, the colony-forming unit counting method was used, which concerns only the main canal, and electron microscopy was used to analyze whether there were morphologic changes in *E. faecalis* in the dentinal tubules or not.

The combination of PUI and PDT was the most efficient[21], probably because of the ability of ultrasonic cavitation and the generation of sonic current, which allowed deep penetration of 2.5% NaOCl into hard-to-reach areas such as ramifications and dentinal tubules, destroying biofilm clusters. This destruction may have favored the penetration of methylene blue into the dentinal tubules, enhancing the effect of PDT. In C-shaped molars, the use of PDT as an adjunct to endodontic treatment contributed to the reduction of *E. faecalis*[24,25].

It is important to emphasize that despite the efficacy of PDT in conjunction with PUI using NaOCl 2.5%, complete elimination of intratubular bacteria was not possible[26], as evidenced by confocal laser scanning microscopic images. Modern mechanized instrumental techniques (rotary and reciprocal) accelerate the clinical time of endodontic treatments and make the use of auxiliary procedures in intratubular bacterial disinfection essential. New studies in this field should be strengthened, especially *in vivo*, to achieve more satisfactory results in eliminating biofilm.

Considering the strengths and limitations of this *ex vivo* study, the results are very encouraging for future studies in this area. It would be interesting to investigate if the use of full-strength NaOCl and chlorhexidine with/without PUI + PDT contributes to better disinfection.

The results of this experiment show that PUI with NaOCl 2.5% combined with PDT produced a greater intratubular reduction of *E. faecalis* compared with the control group and PDT.

ACKNOWLEDGMENTS

The authors deny any conflicts of interest related to this study.

REFERENCES

1. Safavi KE, Spangberg LS, Langeland K. Root canal dentinal tubule disinfection. J Endod 1990;16:207–10.
2. Brändle N, Zehnder M, Weiger R, Waltimo T. Impact of growth conditions on susceptibility of five microbial species to alkaline stress. J Endod 2008;34:579–82.
3. Chivatxaranukul P, Dashper SG, Messer HH. Dentinal tubule invasion and adherence by Enterococcus faecalis. Int Endod J 2008;41:873–82.
4. Zhao D, Shen Y, Peng B, Haapasalo M. Root canal preparation of mandibular molars with 3 nickel-titanium rotary instruments: a micro-computed tomographic study. J Endod 2014;40:1860–4.
5. van der Sluis LWMM, Versluis M, Wu MK, Wesselink PR. Passive ultrasonic irrigation of the root canal: a review of the literature. Int Endod J 2007;40:415–26.
6. Garcez AS, Nuñez SC, Hamblim MR, et al. Photodynamic therapy associated with conventional endodontic treatment in patients with antibiotic-resistant microflora: a preliminary report. J Endod 2010;36:1463–6.
7. Ahangari Z, Asnaashari M, Akbarian Rad N, et al. Investigating the antibacterial effect of passive ultrasonic irrigation, photodynamic therapy and their combination on root canal disinfection. J Lasers Med Sci 2021;12:e81.
8. Dornelles-Morgental R, Guerreiro-Tanomaru JM, de Faria-Júnior NB, et al. Antibacterial efficacy of endodontic irrigating solutions and their combinations in root canals contaminated with Enterococcus faecalis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:396–400.
9. Wang Y, Huang X. Comparative antibacterial efficacy of photodynamic therapy and ultrasonic irrigation against Enterococcus faecalis *in vitro*. Photochem Photobiol 2014;90:1084–8.

10. Wang Z, Shen Y, Haapasalo M. Effectiveness of endodontic disinfecting solutions against young and old Enterococcus faecalis biofilms in dentin canals. J Endod 2012;38:1376–9.

11. Conde AJ, Estevez R, Loroño G, et al. Effect of sonic and ultrasonic activation on organic tissue dissolution from simulated grooves in root canals using sodium hypochlorite and EDTA. Int Endod J 2017;50:976–82.

12. Estevez R, Conde AJ, Valencia de Pablo O, et al. Effect of passive ultrasonic activation on organic tissue dissolution from simulated grooves in root canals using sodium hypochlorite with or without surfactants and EDTA. J Endod 2017;43:1161–5.

13. Layton G, Wu W-I, Selvaganapathy PR, et al. Fluid dynamics and biofilm removal generated by syringe-delivered and 2 ultrasonic-assisted irrigation methods: a novel experimental approach. J Endod 2015;41:884–9.

14. Nunes MR, Mello I, Franco GCN, et al. Effectiveness of photodynamic therapy against Enterococcus faecalis, with and without the use of an intracanal optical fiber: an *in vitro* study. Photomed Laser Surg 2011;29:803–8.

15. Soares JA, Soares SMCS, de Jesus Tavarez RR, et al. Exploring different photodynamic therapy parameters to optimize elimination of Enterococcus faecalis in planktonic form. Photodiagnosis Photodyn Ther 2018;22:127–31.

16. Pourhajibagher M, Raoofian R, Ghorbanzadeh R, Bahador A. An experimental study for rapid detection and quantification of endodontic microbiota following photo-activated disinfection via new multiplex real-time PCR assay. Photodiagnosis Photodyn Ther 2018;21:344–50.

17. Pourhajibagher M, Chiniforush N, Shahabi S, et al. Sub-lethal doses of photodynamic therapy affect biofilm formation ability and metabolic activity of Enterococcus faecalis. Photodiagnosis Photodyn Ther 2016;15:159–66.

18. Pinheiro ET, Gomes BPFA, Ferraz CCR, et al. Microorganisms from canals of root-filled teeth with periapical lesions. Int Endod J 2003;36:1–11.

19. Pinheiro SL, Azenha GR, Democh YM, et al. Antimicrobial activity of photodynamic therapy against enterococcus faecalis before and after reciprocating instrumentation in permanent molars. Photomed Laser Surg 2016;34:646–51.

20. Zand V, Milani AS, Amini M, et al. Antimicrobial efficacy of photodynamic therapy and sodium hypochlorite on monoculture biofilms of Enterococcus faecalis at different stages of development. Photomed Laser Surg 2014;32:245–51.

21. de Vasconcelos Neves G, Dos Santos KSA, de Souza Sales Rocha EAL, et al. Antibacterial effect of photodynamic therapy on root canal disinfection combined with different irrigation protocols. Iran Endod J 2020;15:90–5.

22. Azim AA, Aksel H, Zhuang T, et al. Efficacy of 4 irrigation protocols in killing bacteria colonized in dentinal tubules examined by a novel confocal laser scanning microscope analysis. J Endod 2016;42:928–34.

23. Morago A, Ruiz-Linares M, Ferrer-Luque CM, et al. Dentine tubule disinfection by different irrigation protocols. Microsc Res Tech 2019;82:558–63.

24. Mustafa M, Almnea R, Ajmal M, et al. Efficacy of root canal treatment in c-shaped canals with adjunctive photodynamic therapy using micro-CT. Photodiagnosis Photodyn Ther 2021;34:102257.

25. Mustafa M, Alamri HM, Almokhatieb AA, et al. Effectiveness of antimicrobial photodynamic therapy as an adjunct to mechanical instrumentation in reducing counts of Enterococcus faecalis and Candida albicans from C-shaped root canals. Photodermatol Photoimmunol Photomed 2022;38:328–33.

26. Alves DRS, Decucio DA, Alencar AHG, et al. Effect of low-power diode laser on infected root canals. Braz Dent J 2022;33:8–17.