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Monitoring the effectiveness of photodynamic therapy with periodic renewal of the photosensitizer on intracanal *Enterococcus faecalis* biofilms

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ABSTRACT

Background and objective: Photodynamic therapy (PDT) can eliminate microorganisms in a root canal. However, the parameters for disinfection remain undefined. This study assessed the effectiveness of a PDT protocol against intracanal *Enterococcus faecalis* biofilms.
Materials and methods: Root canals were contaminated with *E. faecalis* for 21 days. The instrumentation was associated to irrigation with 0.85% saline or an alternate irrigation (AI) with 5.25% NaOCl and 17% EDTA. Complementary treatments included saline/PDT and AI/PDT. Four PDT cycles were performed using a diode laser (660 nm, 40 mW) delivered through a tapered optical fiber. In each cycle, the root canal was filled with 1.56 μM/mL methylene blue and irradiated for 150 s. Microbiological samples were collected before (S1) and after (S2) instrumentation; after PDT (S3); and daily over the course of 14 days (S4–S17). Colony-forming units (CFUs) were counted, positive cultures verified, and data subjected to parametric and proportion's tests.
Results: The highest bacterial load reduction was observed in S2. In regard to S3, Saline/PDT reduced 1.3 $\log^{10}$ CFU counts ($p = 0.000$ for S2) and no CFUs were recovered after AI/PDT treatment. All canals were CFU-free on the 14th day for saline/PDT, AI and AI/PDT. Positive cultures were observed in 60% of saline-irrigated canals on the 14th day, whereas the saline/PDT, AI and AI/PDT treatments resulted in germ-free canals after 10, 5 and 2 days, respectively.
Conclusion: Our findings suggest immediate and delayed antibacterial effects using the PDT protocol tested.

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1. Introduction

Root canal disinfection is one of the main goals of endodontic treatment [1–5]. Although chemomechanical preparation (CMP) has been shown to significantly reduce bacterial content [2,3], the use of an interappointment intracanal medication has been recommended to eliminate the remaining bacteria [6,7]. However, this approach extends the duration of treatment sessions [6–8], does not alter the histomicrobiological status [9,10] or fully eliminate the microorganisms in the root canal system [11,12]. Because proper intracanal disinfection increases the clinical and radiographic success in teeth with apical periodontitis [13–16], more effective disinfection protocols can be useful to further improve endodontic outcomes.

Photodynamic therapy (PDT) is a method that can potentially be used to help in root canal disinfection [17]. This therapy employs a dye, termed a photosensitizer, with a resonance at a specific wavelength of a low-intensity, visible-light laser. The photosensitizers undergo a chemical reaction when activated by light, which generate free radicals or superoxide ions via hydrogen or electron transfer (Type I process). They can also be excited to a short-lived

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excited state that then converts to a long-lived triplet state generating singlet oxygen molecules ($^1O_2$; Type II process), which can rapidly eradicate microorganisms [18].

In vitro [18–24] and clinical [25] studies have supported the use of PDT as a supplement to CMP, but there is no consensus regarding optimal treatment parameters for bacterial elimination [17–26], particularly of *Enterococcus faecalis,* a multiple drug resistance microorganism [22] often associated with endodontic treatment failure [2]. Thus, the aim of this *ex vivo* study was to assess the effectiveness of an alternative PDT protocol based on cyclic application of a photosensitizer on intracanal *E. faecalis* biofilms, following CMP. The null hypothesis tested was that there is no difference among the different protocols used in reducing artificial intracanal infection.

2. Materials and methods

2.1. Sample preparation and root canal contamination

After local ethics committee approval, forty extracted human canines were selected for this study. The teeth were horizontally sectioned, resulting in a 16-mm remaining root. After apical patency was established with a #15 K-file (Dentsply Maillefer, Ballaigues, Switzerland), the canal was prepared up to a #25 K-file under irrigation with 5.25% NaOCl (Lenza Farmacêutica Ltda, Belo Horizonte, MG, Brazil). The smear layer was removed using 17% EDTA for 3 min. Each tooth was fixed in the center of a glass tube that was covered with rubber and autoclaved. The apical foramen was sealed with two layers of cyanoacrylate (Super Glue, Loctite, Itapeva, SP, Brazil), and each canal was filled with approximately 20 μL of a suspension containing *E. faecalis* (ATCC 19433) at 10^9 colony-forming units (CFU)/mL. The experimental units were placed in screw-cap plastic vials and incubated at 37 °C, and the reinoculation was performed every 48 h for 21 days [27].

2.2. Root canal preparation

Prior to CMP, samples (S1) were taken from each tooth. Three #25 absorbent paper points (Ind Tanari Ltda, Manacapuru, Brazil) were consecutively inserted into the root canals and after 1 min transferred to microcentrifuge tubes containing 1 mL of saline. K-files and Gates–Glidden burs (Dentsply Maillefer) were used during CMP. Each root canal was irrigated with 15 mL of solution using a plastic syringe with a 30-gauge needle (ProRinse; Dentsply Tulsa Dental, Johnson City, TN, USA) inserted at 2 mm from working length. Following coronal flaring, the root canal was prepared to the master apical file #40 using the step-back technique. Irrigation using an up-and-down motion was performed after the change of files.

The root canals were irrigated ($n = 10$) with either 0.85% saline or with an alternate irrigation (AI) regimen matching NaOCl and EDTA. AI regimen consisted of (1) irrigation with 1 mL of 5.25% NaOCl, instrumentation, an additional irrigation with 1 mL of NaOCl, intracanal aspiration (2), irrigation with approximately 200 μL of 17% EDTA, instrumentation, intracanal aspiration, and a final irrigation with 1 mL of 5.25% NaOCl. The instrumentation pattern and volume of saline and NaOCl/EDTA irrigants were the same in the two regimens. Once the instrumentation was concluded, root canals were irrigated with 2 mL of 0.85% saline, and samples were collected (S2) as described for S1.

3.1. PDT protocol

PDT was performed following root canal preparation and irrigation with either 0.85% saline (Saline/PDT, $n = 10$) or AI regimen (AI/PDT, $n = 10$). Then, the root canals were filled with methylene blue (MB) photosensitizer at 1.56 μM/mL (Chimiolux, DMC Group e Aptivalux Bioengenharia, São Paulo, Brazil) using a plastic syringe (Injex Ind. Cirúrgica Ltda, São Paulo, Brazil) (Fig. 1A and B). Following pre-irradiation for 150 s, a gallium-aluminum-arsenic (Ga-Al-As) multifunction diode laser unit (Twin Laser; MMOptics, São Paulo, Brazil) was used with a beam delivery system. This laser emits light at a wavelength of 660 nm with an output power of 40 mW. The laser light was delivered into root canals through a tapered optical fiber (Fig. 1C) with a diameter of 400 μm at the tip and a spot area of 0.0125 cm^2. A 6-J energy was deposited in each canal with spiral movements from the apical to the cervical area of the root over the course of 150 s. Following MB removal and renewal, an additional photoactivation was performed with the same irradiation parameters. This procedure was repeated twice resulting in four PDT cycles, with a total irradiation time of 600 s and 24 J. Following PDT, the root canals were irrigated with 2 mL of 0.85% saline and the third microbiological samples (S3) were taken.

3.2. Monitoring root canals and microbial processing

Microbiological samples were obtained from the root canals at 24-h intervals over the course of 14 days (S4–S17), using the same method described previously. Samples were vortexed for 30 s, followed by 10-fold serial dilutions in saline. Afterwards, 100-μL aliquots of the suspensions were spread onto brain heart infusion agar (BHI-A; Difco, Detroit, MI, USA) media in triplicate. Following incubation at 37 °C for 48 h, microbial growth was verified, and CFUs were counted. The remainder of the sample was added to brain heart infusion broth (BHI, Difco), and the frequency of positive microbial cultures was determined by observation of the turbidity of the culture media. Monoinfection by *E. faecalis* was confirmed by Gram staining and catalase testing.

3.3. Statistical methods

CFU counts were normalized by $\log^{10}$ transformation and statistically analyzed using one-way analysis of variance and Tukey tests. Positive cultures in BHI broth were statistically analyzed using Fisher's exact test. A p-value lower than 0.05 was considered statistically significant.

4. Results

Table 1 shows $\log^{10}$ CFU/mL mean values. The root canals exhibited a high number of CFU/mL in S1, whereas CMP caused a significant reduction in CFU counts in S2 sample for saline, saline/PDT, AI and AI/PDT treatments (all $p < 0.001$). Immediately following PDT, a reduction of 1.3 $\log^{10}$ of CFUs was achieved with saline/PDT treatment in S3 when compared with S2 ($p = 0.000$). Furthermore, PDT eliminated the remaining CFUs in root canals treated with AI/PDT. On the 14th day (S17) a mean of 1.92 $\log^{10}$ CFU/mL was found in the saline-irrigated canals, which was higher than that observed in the saline/PDT, AI, and AI/PDT treatments (all $p < 0.001$).

Fig. 2 exhibits the results of microbiological monitoring. On the 2nd day (S5), *E. faecalis* were observed in 50% and 0% following AI and AI PDT treatments, respectively, with significant differences between the two treatments ($p = 0.033$). On the 10th day (S13), a significant difference ($p < 0.001$) was observed for saline and saline/PDT treatments (90% and 0% of positive cultures, respectively). On the 14th day (S17), 60% of the saline-irrigated canals harbored *E. faecalis*, and the other treatments were negative for *E. faecalis*.