

Photodynamic Therapy with Two Different Photosensitizers as a Supplement to Instrumentation/Irrigation Procedures in Promoting Intracanal reduction of *Enterococcus faecalis*

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Abstract

Introduction: This *in vitro* study aimed to investigate the antibacterial effects of photodynamic therapy (PDT) with methylene blue (MB) or toluidine blue (TB) (both at 15 µg/mL) as a supplement to instrumentation/irrigation of root canals experimentally contaminated with *Enterococcus faecalis*. **Methods:** Seventy extracted teeth had their root canals contaminated with an endodontic strain of *E. faecalis* for 7 days, instrumented with nickel-titanium instruments and irrigated either with 2.5% NaOCl or with 0.85% NaCl, and then randomly distributed into four experimental groups: MB/NaOCl (PDT with MB and NaOCl as the irrigant), TB/NaOCl (PDT with TB and NaOCl as the irrigant), MB/NaCl (PDT with MB and NaCl as the irrigant), and TB/NaCl (PDT with TB and NaCl as the irrigant). For PDT, the photosensitizer remained in the canal for 2 minutes before exposed to red light emitted from a diode laser for 4 minutes. Samples were taken before and after instrumentation/irrigation and following the specific PDT procedure for each group, plated onto Mitis-salivarius agar and the colony forming units counted. **Results:** Regardless of the irrigant used (NaOCl or NaCl), instrumentation significantly reduced bacterial counts in comparison to the baseline ($p < 0.001$). NaOCl as the irrigant was significantly more effective than NaCl, and this difference persisted after PDT, irrespective of the photosensitizer used ($p < 0.05$). PDT with either MB or TB did not significantly enhance disinfection after chemomechanical preparation using NaOCl as irrigant ($p > 0.05$). No significant differences were observed between the two photosensitizers ($p > 0.05$). **Conclusion:** These *in vitro* results suggest that PDT with either MB or TB may not exert a significant supplemental effect to instrumentation/irrigation procedures with regard to intracanal

disinfection. Further adjustments in the PDT protocol may be required to enhance predictability in bacterial elimination before clinical use is recommended. (*J Endod* 2009; ■:1–5)

Key Words

Endodontic treatment, *Enterococcus faecalis*, methylene blue, photodynamic therapy, toluidine blue

Apical periodontitis is a disease caused by bacteria, and, consequently, successful treatment of this condition is dependent on the effective elimination of intracanal bacterial populations. Although complete eradication of the infection in the entire root canal system is the ideal goal to be reached, maximum reduction in bacterial counts to levels that are compatible with periradicular tissue healing is the attainable goal in current clinical endodontics (1). Studies have revealed that instrumentation and irrigation with antimicrobial irrigants, such as NaOCl, although more effective than saline solution, do not suffice to predictably render root canals free of bacteria, with about 40% to 60% of the canals still containing cultivable bacteria after chemomechanical preparation (2-7). The use of an interappointment antibacterial medication with a calcium hydroxide paste has been shown to significantly increase the incidence of negative cultures (4, 8-10).

The issue as to whether or not the root canal treatment of teeth with apical periodontitis should be concluded in one or two visits is one of the greatest controversies in endodontics nowadays (11, 12). The establishment of treatment protocols that can predictably disinfect the root canals in one visit has the potential to help smooth this discussion. In this regard, the idea of speeding up root canal disinfection while maintaining efficacy sounds interesting and should be pursued. In this regard, the use of laser technology arises as a possibility in endodontic therapy (9-12).

Photodynamic therapy (PDT), or photoactivated disinfection, uses light of a specific wavelength to activate a nontoxic photoactive dye (photosensitizer) in the presence of oxygen. The transfer of energy from the activated photosensitizer to available oxygen gives rise to the formation of highly reactive oxygen species, such as singlet oxygen and free radicals, which can kill microorganisms by damaging essential cellular molecules, including proteins, membrane lipids, and nucleic acids (13). *In vitro* (14-22) and *in vivo* (23, 24) studies using PDT have shown that this approach has the potential to maximize root canal disinfection. However, while disclosing and

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confirming the excellent antibacterial potential of PDT, none of these studies have consistently examined the effectiveness of this procedure in supplementing bacterial elimination after chemomechanical procedures, which is the greatest potential use of this technology with regard to root canal disinfection. Moreover, to the best of the authors' knowledge, there is so far no study comparing the effects of two widely used photosensitizers, methylene blue and toluidine blue, in promoting root canal disinfection. Therefore, the aim of this *in vitro* study was to evaluate the additional antibacterial effects of PDT using methylene blue or toluidine blue after instrumentation/irrigation of root canals experimentally contaminated with an endodontic strain of *Enterococcus faecalis*.

Material and Methods

Specimen Preparation and Contamination

The study protocol was approved by the Ethics Committee of the Estácio de Sá University. Seventy single-rooted and single-canal human extracted teeth were selected for this study. The presence of a single canal was determined by radiographs taken both in the mesiodistal and buccolingual directions and confirmed after conventional access preparation, which was completed using round burs and Endo-Z burs (Dentsply/Maillefer, Ballaigues, Switzerland). The working length (WL) was established by introducing a K-file #10 in the canal until its tip was visualized at the apical foramen through a stereoscopic microscope. In order to standardize the apical constriction size, root canals were instrumented 1 mm beyond the apical foramen with K-files up to size 20 under irrigation with running water. Afterward, the apical foramen was sealed with a fast set epoxy resin, and the teeth were mounted vertically up to the cervical region in plaster blocks. The blocks containing the teeth were sterilized in autoclave for 15 minutes at 121 °C.

An endodontic strain of *E. faecalis* (MB 35), which was isolated from a treated root canal in the study by Zoletti et al (25), was used in this experiment. Bacterial suspension was prepared by adding 1 mL of a pure culture of this *E. faecalis* strain, grown in trypticase soy broth (Difco, Detroit, MI) for 24 hours, to 5 mL of fresh trypticase soy broth. Each root canal was completely filled with the *E. faecalis* suspension using sterile 1-mL insulin syringes without overflowing. Sterile K-files #15 were used to carry the bacterial suspension to the entire root canal length. Blocks were then placed inside a metallic box and incubated at 37 °C for 7 days. Fresh culture medium was added to the canal at 1, 4, and 6 days after the initial inoculum. After 7 days of experimental contamination, 68 teeth followed in the study, whereas 2 teeth were subjected to scanning electron microscopy to allow visualization of the pattern of colonization. These two teeth were fixed in 10% buffered formalin, longitudinally split, and then dried in ascending ethanol concentrations. After dehydration to their critical point in CO₂ (Cressington Sputter Coater 108, Watford, England) and sputter-coating with gold under vacuum, the root canal walls of the two teeth were examined for bacterial colonization using a scanning electron microscope (JSM-5800LV; JEOL, Tokyo, Japan).

Testing Procedures

Teeth were randomly divided into four experimental groups according to the postinstrumentation procedures. Groups were as follows: (1) MB/NaOCl: PDT with 15 µg/mL methylene blue (irrigated with 2.5% NaOCl during instrumentation), (2) TB/NaOCl: PDT with 15 µg/mL toluidine blue (irrigated with 2.5% NaOCl during instrumentation), (3) MB/NaCl: PDT with 15 µg/mL methylene blue (irrigated with 0.85% NaCl during instrumentation), and (4) TB/NaCl: PDT

with 15 µg/mL toluidine blue (irrigated with 0.85% NaCl during instrumentation).

Root canals from all groups were instrumented as follows. The coronal and middle segments of the canal were prepared by using a LA Axxess bur #35 (SybronEndo, Glendora, CA). Then, rotary ProTaper Universal instruments (Dentsply/Maillefer) were used to prepare the canal up to the WL.

In group MB/NaOCl, 16 teeth had their canals instrumented at the WL up to ProTaper F4 (subgroup A) and another 10 teeth up to ProTaper F2 (subgroup B). In group TB/NaOCl, the canals of 10 teeth were instrumented up to ProTaper F2. After each instrument was used, the canal was irrigated with 2 mL of 2.5% NaOCl using a 30-G NavTip needle (Ultradent, South Jordan, UT) adapted to a disposable plastic syringe. The needle was placed up to 5 mm short of the WL. At the end of root canal preparation, all teeth were irrigated with 5 mL of 17% EDTA, which was left in the canal for 3 minutes. After a final irrigation with 5 mL of 2.5% NaOCl, 1 mL of 5% sodium thiosulfate was used to neutralize the NaOCl. The canal was then rinsed with 1 mL of sterile NaCl solution. The canals from the MB/NaOCl group were filled with methylene blue, and the canals from the TB/NaOCl group were filled with toluidine blue using a 30-G NavTip needle adapted to a disposable plastic syringe. The solutions were agitated with a K-file #15 and left undisturbed in the canal for 2 minutes as a preirradiation time.

The irradiation source was a diode laser (MMOptics, São Carlos, SP, Brazil) with a total power of 40 mW and 660 nm of wavelength. The system was coupled to an optical fiber with a diameter of 300 µm. The optical fiber was initially placed up to the WL, and spiral movements, from apical to cervical, were performed to allow for adequate distribution of the light throughout the root canal. Total irradiation time was 4 minutes.

Groups MB/NaCl and TB/NaCl were each composed of 16 teeth that had their canals instrumented at the WL up to the ProTaper F2 instrument. The canals were irrigated with 0.85% NaCl instead of NaOCl, with the same total volume used in both groups MB/NaOCl and TB/NaOCl. PDT was performed as described previously using methylene blue as the photosensitizer for the MB/NaCl group and toluidine blue for the TB/NaCl group.

Sampling Procedures

Root canals were sampled before (S1) and after (S2) the instrumentation/irrigation procedures and after the specific PDT approach for each group (S3). Canals were filled with sterile 0.85% NaCl solution, and the sample was taken by the sequential use of three paper points placed to the WL. Paper points were transferred to tubes containing 1 mL of 0.85% NaCl solution and agitated in vortex for 1 minute. After tenfold serial dilutions in saline, aliquots of 0.1 mL were plated onto Mitis-Salivarius agar plates (Difco) and incubated at 37 °C for 48 hours. The colony forming units (CFUs) grown were counted and then transformed into actual counts based on the known dilution factors.

Statistical Analysis

The Mann-Whitney *U* test was used for intragroup analysis comparing the reduction in the number of CFU counts from S1 to S2, S1 to S3, and S2 to S3 in order to evaluate the efficacy of each procedure. Because comparisons of baseline samples (S1) between groups revealed no significant differences, S3 data were used for the intergroup comparative analyses using the Mann-Whitney *U* test. To compare the effects of preparation using ProTaper files F4 (MB/NaOCl subgroup A) or F2 (MB/NaOCl subgroup B), S2 data were analyzed using the Mann-Whitney *U* test. Group MB/NaOCl was compared with the other groups using the overall S3 data or only S3 data from subgroup B.

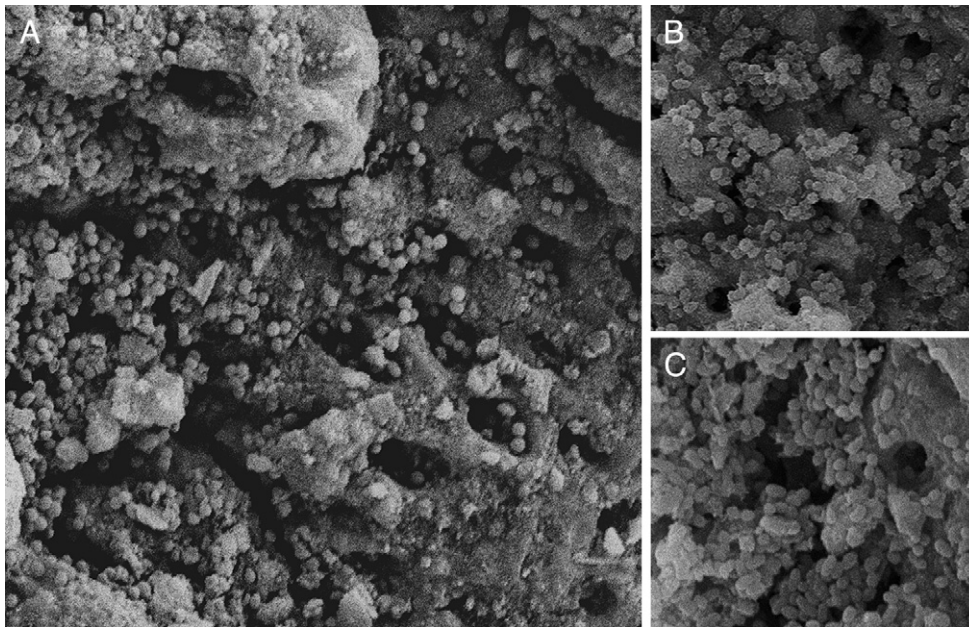


Figure 1. Scanning electron micrographs showing the pattern of colonization of the root canal walls by the endodontic strain of *Enterococcus faecalis* MB 35 (A, original magnification, $\times 2,700$; B, $\times 1,500$; and C, $\times 4,000$).

The incidence of negative cultures was compared between PDT with the two photosensitizers using the Fisher exact test. The significance level for all analyses was set at $p < 0.05$.

Results

Scanning electron microscopy revealed that the root canal walls of the two specimens analyzed were densely colonized by cells of the endodontic strain of *E. faecalis* (Fig. 1). In several regions, cells were organized in biofilms, and, in some instances, they were also seen penetrating the dentinal tubules. Effective colonization of the root canals was further confirmed by the fact that all cases had high bacterial counts in S1.

Table 1 depicts the mean, median, range, and percent reduction of CFUs observed for all groups. Intragroup analyses were performed to investigate the ability of each procedure of reducing the bacterial counts when compared with the previous conditions. A reduction in the number of CFUs from S1 to S2 as well as from S1 to S3 was statistically significant for all groups ($p < 0.001$ for all groups). A reduction from S2 to S3 was observed but not to the point of reaching statistical significance ($p = 0.22$ for the overall MB/NaOCl group, $p = 0.67$ for the MB/NaOCl subgroup B, $p = 0.73$ for TB/NaOCl, and $p = 0.09$ for MB/NaCl), except for group TB/NaCl ($p = 0.005$).

The Mann-Whitney *U* test did not show significant differences when comparing S2 samples from MB/NaOCl subgroups A and B ($p = 0.22$). This made all S3 samples from group MB/NaOCl available for comparisons as a whole, increasing statistics robustness. Even so, to avoid potentially undetected biases, S3 data from subgroup B were used for comparisons with the other groups, which were all instrumented up to ProTaper F2.

Intergroup comparisons failed to disclose significant differences between the groups of PDT with methylene blue and PDT with toluidine blue instrumented with ProTaper F2 and irrigated with either NaOCl ($p = 0.27$) or NaCl ($p = 0.17$). However, the group of PDT with methylene blue irrigated with NaOCl was significantly more effective in eliminating bacteria from the root canals when compared with the group of PDT

with methylene blue irrigated with NaCl ($p < 0.001$). The same was observed for PDT with toluidine blue ($p = 0.02$).

Data related to the incidence of negative cultures in S2 and S3 samples are shown in Table 2. No significant differences were observed for intragroup (S2 vs S3) or intergroup (S3 data from MB/NaOCl subgroup B vs TB/NaOCl, MB/NaCl vs TB/NaCl) analyses.

Discussion

E. faecalis has been the most frequent species associated with post-treatment apical periodontitis (26). In the present study, root canals were experimentally contaminated with a strain of *E. faecalis* originally isolated from a root canal-treated tooth with post-treatment disease (25). Although this species has been recently questioned as to its importance in the etiology of treatment failures (25, 27), such a role cannot be surely disregarded in the light of current evidence. Moreover, *E. faecalis* has been widely used as a valuable microbiological marker for *in vitro* studies because it has been shown to be able to successfully colonize the root canal in a biofilm-like style, invade dentinal tubules, and resist to some endodontic treatment procedures (28-30). The wild strain used in this study effectively colonized the root canal walls after 7 days of incubation, forming biofilm structures in several regions, as shown by scanning electron microscopy analysis and confirmed by CFU counting of S1 samples.

PDT has been suggested as a good option to maximize root canal disinfection (16-17, 19), which confers it with a potential to be used to predictably disinfect canals in one visit. However, a protocol of PDT to be used as an effective antibacterial supplement to chemomechanical therapy remains to be established. There are many variables to be taken into account when developing a PDT protocol, including light parameters, photosensitizers, and light delivery techniques (17, 20, 31, 32). For instance, the photosensitizer should ideally present low levels of dark toxicity and exhibit selective toxicity against the target cells/tissue after activation (13, 33, 34). The peak of absorption of the photosensitizer should match the wavelength of the light used for irradiation in order to promote formation of singlet oxygen, a very reactive oxygen

TABLE 1. Counts of *Enterococcus faecalis* Colony-forming Units before (S1) and after (S2) Instrumentation/Irrigation Procedures, and following Photodynamic Therapy Using Either of Two Photosensitizing Agents (S3)

Groups	S1			S2			S3			% reduction Median		
	Mean	Median	Range	Mean	Median	Range	Mean	Median	Range	S1-S2	S1-S3	S2-S3
MB*/NaOCl	4.19 × 10 ⁶	2.90 × 10 ⁶	1.60 × 10 ⁵ – 1.58 × 10 ⁷	2.13 × 10 ³	1.20 × 10 ²	0 – 2.37 × 10 ⁴	2.06 × 10 ²	7.5 × 10 ¹	0 – 1.34 × 10 ³	99.99	99.99	12.28
TB**/NaOCl	6.50 × 10 ⁶	6.04 × 10 ⁶	1.68 × 10 ⁶ – 1.20 × 10 ⁷	1.63 × 10 ³	2.10 × 10 ²	0 – 8.04 × 10 ³	3.66 × 10 ²	1.20 × 10 ²	0 – 1.46 × 10 ³	99.99	99.99	0***
MB/NaCl	1.40 × 10 ⁶	1.00 × 10 ⁶	1.10 × 10 ⁵ – 4.60 × 10 ⁶	4.45 × 10 ³	1.21 × 10 ³	1.40 × 10 ² – 3.20 × 10 ⁴	1.53 × 10 ³	5.05 × 10 ²	1.10 × 10 ² – 1.15 × 10 ⁴	99.84	99.93	62
TB/NaCl	2.47 × 10 ⁶	1.74 × 10 ⁶	1.60 × 10 ⁵ – 6.00 × 10 ⁶	1.35 × 10 ⁴	6.80 × 10 ³	1.00 × 10 ¹ – 4.09 × 10 ⁴	3.09 × 10 ³	1.18 × 10 ³	0 – 1.95 × 10 ⁴	99.48	99.94	80.46

*MB, methylene blue

**TB, toluidine blue

***% of reduction of 0 (zero) was only observed when both S2 and S3 were culture negative

TABLE 2. Incidence of Negative Cultures after Instrumentation/Irrigation Procedures (S2) and following Photodynamic Therapy Using Either of Two Photosensitizing Agents (S3)

Groups	S2	S3
MB*/NaOCl (subgroup B)	2/10***	4/10
TB**/NaOCl	2/10	3/10
MB/NaCl	0/16	0/16
TB/NaCl	0/16	1/16

*MB, methylene blue

**TB, toluidine blue

***number of cases with negative culture/number of cases examined

species mostly responsible for PDT-mediated bacterial killing (13, 24). Both photosensitizers used in the present study, methylene blue and toluidine blue, are phenothiazine dyes that have a maximum wavelength absorption of 656 nm and 625 nm, respectively (35). Antibacterial effectiveness of PDT may also depend on the photosensitizer concentration (13). For endodontic disinfection, photosensitizers have been tested at concentrations ranging from 6.25 µg/mL to 25 µg/mL for methylene blue (17, 20, 31) and from 10 µg/mL to 100 µg/mL for toluidine blue (18, 19, 21, 24, 36). In the present study, for the sake of direct comparison between their antibacterial efficacies, the same concentration of 15 µg/mL for both photosensitizers was used. Other parameters and the protocol for PDT application were based on Garcez et al (16, 23).

The isolate use of PDT has been largely shown to promote adequate bacterial disinfection in infected root canals at least *in vitro* (15-17, 19, 20, 31). However, the present study, in contrast with the large majority of previous studies on the antibacterial effects of PDT in infected canals, evaluated such effects by using PDT immediately after instrumentation/irrigation procedures, which is the main proposed way to apply PDT to endodontic treatment (ie, as a supplement and not as an alternative to chemomechanical procedures) (19). The importance to test the effects of a given procedure under *in vitro* conditions simulating the clinical use cannot be overstated. Instrumentation/irrigation procedures generate an environmental stress in the root canal and may disrupt biofilm structures, events that might render bacterial community members more susceptible to the antibacterial activity of PDT. However, bacteria organized in biofilms may remain undisturbed in some areas after instrumentation, and their permanence may pose a risk to treatment outcome, making supplemental antibacterial activity a necessary approach. Hence, it would be expected that PDT be able to supplement the antibacterial effects of instrumentation/irrigation by significantly reducing or ideally eradicating residual microorganisms to the point of providing sterile or at least culture negative canals.

The present study revealed that PDT with either methylene blue or toluidine blue did not have a significant additional effect to the chemomechanical preparation using NaOCl as irrigant in the reduction of *E. faecalis* populations. When saline was deliberately used as the irrigant in order to increase the bacterial load before PDT application, the additional antibacterial effect of PDT with either photosensitizer was observed, but it was statistically significant only for toluidine blue. No significant differences were observed when comparing the two photosensitizers. The present findings indicated that the PDT protocols used may not significantly supplement the antibacterial effects of chemomechanical procedures using NaOCl as an irrigant in the main canal against a pure culture of *E. faecalis*. Further improvements in protocols are required before PDT can be indicated for use in the clinical setting.

PDT with either of the two photosensitizers did not significantly increase the number of negative cultures after instrumentation/

irrigation. In cases irrigated with NaOCl, more than one half of the specimens still presented positive cultures after PDT. Except for one case in the group of PDT with toluidine blue, no case irrigated with saline yielded negative cultures.

Even though the present findings cannot be directly extrapolated to the clinical situation, they showed that the PDT protocols used herein did not succeed in predictably eradicating or at least significantly reducing the root canal infection. One possible explanation for incomplete bacterial elimination may be the low concentration of available oxygen in the canals, especially in irregularities and in dentinal tubules. Under such conditions, the formation of cytotoxic oxygen derivatives may be precluded or minimized. In the clinical situation, conditions of low oxygen are expected to be still more critical. Also, the photosensitizer agent may have not diffused well into irregularities and dentinal tubules or even through possible bacterial biofilms persisting on untouched canal walls. Our findings suggest that the effects of PDT were probably restricted to the same root canal areas already affected by NaOCl. Thus, when adjusting the PDT protocol for enhanced activity, one should take into account the low-oxygenated environment and the diffusibility of the photosensitizer and light to be used.

In conclusion, the present study confirmed that the instrumentation/irrigation procedures significantly reduced bacterial populations in the canal. Chemomechanical preparation (instruments plus NaOCl) was confirmed to be more effective than mechanical preparation (instruments plus NaCl). Although the additional use of PDT promoted some reduction in the intracanal populations of an endodontic strain of *E. faecalis* after instrumentation/irrigation, the effects did not reach statistical significance. It is important to reinforce the concept that the PDT protocol has yet to be refined before clinical application with predictable results can ensue.

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