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Endodontic photodynamic therapy *ex vivo*

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Abstract

Objective—To evaluate the anti-microbial effects of photodynamic therapy (PDT) on infected human teeth *ex vivo*.

Materials and Methods—Fifty-two freshly extracted teeth with pulpal necrosis and associated periradicular radiolucencies were obtained from 34 subjects. Twenty-six teeth with 49 canals received chemomechanical debridement (CMD) with 6% NaOCl and twenty-six teeth with 52 canals received CMD plus PDT. For PDT, root canal systems were incubated with methylene blue (MB) at concentration of 50 µg/ml for 5 minutes followed by exposure to red light at 665 nm with an energy fluence of 30 J/cm². The contents of root canals were sampled by flushing the canals at baseline and following CMD alone or CMD+PDT and were serially diluted and cultured on blood agar. Survival fractions were calculated by counting colony-forming units (CFU). Partial characterization of root canal species at baseline and following CMD alone or CMD+PDT was performed using DNA probes to a panel of 39 endodontic species in the checkerboard assay.

Results—The Mantel-Haenszel chi-square test for treatment effects demonstrated the better performance of CMD+PDT over CMD ($P=0.026$). CMD+PDT significantly reduced the frequency of positive canals relative to CMD alone ($P=0.0003$). Following CMD+PDT, 45 of 52 canals (86.5%) had no CFU as compared to 24 of 49 canals (49%) treated with CMD (canal flush samples). The CFU reductions were similar when teeth or canals were treated as independent entities. Post-treatment detection levels for all species were markedly lower for canals treated by CMD+PDT than were for those treated by CMD alone. Bacterial species within dentinal tubules

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were detected in 17/22 (77.3%) and 15/29 (51.7%) of canals in the CMD and CMD+PDT group, respectively ($P = 0.034$).

Conclusion—Data indicate that PDT significantly reduces residual bacteria within the root canal system, and that PDT, if further enhanced by technical improvements, holds substantial promise as an adjunct to CMD.

Keywords

Photodynamic therapy; methylene blue; endodontic disinfection; *ex vivo*

INTRODUCTION

Endodontic treatment is the clinical management of a microbiological problem (1) and the main target of treatment is the microorganisms residing within the root canal system (2). However, the complexity of the root canal system makes complete debridement and removal of bacteria with instrumentation, irrigation and intracanal medicaments virtually impossible (3). In addition, current endodontic procedures require very good technical skills, and use medicaments whose effectiveness has never been definitively proven in human clinical trials. Three systematic reviews (4–6) on the outcome of primary non-surgical root canal treatment summarized findings from longitudinal clinical studies published up to 2006, in which treatments were carried out by undergraduate students, graduate students, general dental practitioners or specialists. The estimated success reported in these studies was 75% (6) and 78% (4,5). In a recent systematic review by Ng *et al.* (2010) that included fourteen studies published between 1993 and 2007, the pooled proportion of teeth surviving over 2–10 years following root canal treatment was found to range between 86% and 93% (7). However, Wu *et al.* (2009) reported several factors that contribute to the overestimation of successful outcomes after primary root canal treatment: A high percentage of cases confirmed healthy by periapical radiography reveal apical periodontitis on cone beam computed tomography and by histology; extractions and retreatments were rarely recorded as failures; and the recall rate was often < 50% in longitudinal clinical studies (8). General dentists perform about 75% of root canal procedures (9), and thus it might be anticipated that failure rates are even greater in general practice (6). When strict radiographic criteria were used, the success rates were approximately 66%, 75%, 77% and 85% for treatments carried out by general dental practitioners, undergraduate students, graduate students and specialists, respectively (6). Given that more than 20 million root canals are performed yearly in the U.S. (10), approximately 2 million endodontic failures could be avoided by better disinfection procedures. The development of adjunctive antibacterial therapeutic strategies to CMD therefore becomes important in the evolution of methods to target residual microorganisms in the root canal system.

Photodynamic therapy (PDT) was developed as a therapy for cancer and is based on the concept that a non-toxic photosensitizing agent, known as photosensitizer, can be preferentially localized in premalignant and malignant tissues and subsequently activated by light of the appropriate wavelength to generate singlet oxygen and free radicals that are cytotoxic to cells of the target tissue (11). In recent years, PDT has been employed to target microorganisms in root canals *in vitro* (12–28) and *in vivo* (29–32) suggesting its usefulness as an adjunct to current endodontic disinfection techniques. Methylene blue (MB) is a well-established photosensitizer that has been used in PDT for targeting various gram-positive and gram-negative oral bacteria (33) and was previously employed to study the effect of PDT on endodontic disinfection (14,19,20,22,25,26). MB has been used as a photosensitizing agent for almost nine decades (34). It has been used for the detection of mucosal premalignant lesions (35) and as a marker dye in surgery (36). The hydrophilicity

of MB (37), along with its low molecular weight and positive charge allows passage across the porin-protein channels in the outer membrane of gram-negative bacteria (38). MB, whose intravenous administration is FDA approved for methemoglobinemia, predominantly interacts with the anionic macromolecule lipopolysaccharide and results in the generation of MB dimers (38), which participate in the photosensitization process (38).

The objective of the present study was to evaluate the antimicrobial effects of MB-mediated PDT in a stringent and clinically relevant evaluation using naturally human infected teeth *ex vivo* treated immediately upon their extraction. Teeth with radiographic evidence of periradicular lesions were chosen because they were guaranteed to be grossly infected, which mimics the clinical situation that leads to higher failure rates (2). The use of naturally-infected teeth, which contain a much broader range of pathogens and deeper penetration into tubules than any *in vitro* model system provides an excellent test of the potential of PDT in achieving root canal disinfection.

MATERIALS AND METHODS

Collection of teeth and groups

Fifty-two freshly extracted teeth with pulpal necrosis and radiographic evidence of periradicular lesions were obtained from 34 subjects in the Department of Oral and Maxillofacial Surgery, Massachusetts General Hospital, Boston. Permission to collect extracted teeth was authorized by Institutional Review Board-approved informed consent. Patients had no systemic disease and had not taken any antibiotics in the previous 3 months. Following extraction, teeth were placed into individual sterile vials and transferred within 30 minutes to the Applied Molecular Photomedicine Laboratory at The Forsyth Institute for preparation and experimentation. The external surface of each tooth was cleaned with 10% povidone-iodine. After 5 min, the disinfectant was removed from the surface with isopropyl alcohol and the tooth was decoronated apical to the roof of the pulp chamber with a sterile rotating diamond saw (#911H, Brasseler USA, Savannah, GA) set at 20,000 rpm. Teeth were assigned to two groups. The first group comprised 26 teeth (5 incisors, 3 canines, 6 premolars, 12 molars) with 49 canals that received only chemomechanical debridement (CMD group) (Fig. 1). The second group comprised 26 teeth (4 incisors, 2 canines, 6 premolars, 14 molars) with 52 canals that received CMD followed by PDT (CMD+PDT group) (Fig. 1). In eight subjects, more than one tooth was obtained (22 teeth). In these cases, teeth were randomly allocated to one of the above groups.

Baseline bacterial samplings

In both groups, a baseline microbial sample of the root canal was taken. The canal was completely filled with pre-reduced anaerobically sterilized (PRAS) Ringer's solution using a sterile Monoject tuberculin syringe with 27-G X 1/2-in. detachable needle (Sherwood Medical, St. Louis, MO). A sample was collected by introducing an ISO size 10 K-type file to a working length of 0.5 mm short of the apical foramen and then agitated in the canal solution in the canal for 60 seconds. The file was then removed and the file handle was cut off under aseptic conditions and put in a 1.5 ml microcentrifuge tube containing 1 ml PRAS Ringer's solution. The canal contents were aspirated using the same syringe as above and added in the tube containing the file.

Root canal treatment

Chemomechanical debridement was performed in teeth of both groups using standard K files and 0.04 mm/mm taper number 7 series 29 Ni-Ti rotary Profiles™ (Dentsply Maillefer, Tulsa, OK) to achieve a master apical file size of .465 (ISO equivalent) for distal canals of mandibular molars, palatal canals of maxillary molars, and all single rooted teeth. Number 6

0.04 mm/mm taper Ni-Ti rotary Profiles™ were used to achieve a master apical file size of .360 (ISO equivalent) for buccal canals of maxillary molars and mesial canals of mandibular molars. RC Prep® (Premium Products, Plymouth Meeting, PA) was used as a lubricant during instrumentation and canals were irrigated with 10 cc of 6% sodium hypochlorite (NaOCl) throughout the instrumentation sequence. All irrigants used were dispensed using a 30 gauge Max-I-Probe (Dentsply Maillefer). After canal preparation an aliquot of 1 ml of 17% ethylenediaminetetraacetic acid (EDTA) solution was left *in situ* for 3 min for smear layer removal, and was replaced by 1 ml of 6% NaOCl for 3 minutes.

Post-SET bacterial sampling

Immediately after chemomechanical disinfection, each specimen in the CMD group was aseptically mounted on a rubber dam attached to a rack. The contents of root canals were sampled by flushing the root canals with a coronal application of 1-ml of sterile phosphate buffered saline (PBS) with a Pro Rinse® 30 gauge irrigation needle (Dentsply Maillefer) (Fig. 2). The bacterial suspension was collected in a 1.5 ml microcentrifuge tube positioned below the apical foramen and bacterial yielding was measured spectrophotometrically for each sample. After vortexing for 20 seconds, serial dilutions were prepared and 100 µl aliquots were inoculated onto blood agar and incubated anaerobically for 7 days.

Photodynamic therapy

Following CMD, specimens in the CMD+PDT group were treated by MB-mediated PDT. Methylene blue (Sigma, St Louis, MO) was dissolved in sterile PBS and filter-sterilized immediately prior to use. The final concentration used was 50 µg/ml (134 µM). The ultraviolet-visible absorption spectra of MB in PBS were recorded from 200 to 800 nm using quartz cuvettes with 1 cm path length on a diode-array spectrophotometer and were characterized by a long-wavelength maximum at 665 nm as shown previously (14).

All individual specimens were aseptically mounted on a rubber dam, with the rubber dam frame attached to a rack. Then the canals were filled to the level of the access cavity with MB solution using a Pro Rinse® 30 gauge irrigation needle (Dentsply Maillefer) for 5 minutes. Following incubation, the canal was dried with a paper cone. Light was then applied in the root canal system of the specimens in appropriate groups for 2.5 minutes followed by a break of 2.5 minutes and a second light exposure for 2.5 minutes. The irradiation source was a diode laser (BWTEK Inc., Newark, DE) with an output power of 1 Watt and a central wavelength of 665 nm. The system was coupled to a 250-µm diameter optical fiber (22) that was mechanically notched over a one-centimeter length at approximately one-millimeter intervals (Schoelly Imaging Inc., Worcester, MA). The fiber was able to uniformly distribute light at 36° within the root canal. The power density was 100 mW/cm² and the total energy fluence dose was 30 J/cm². The fiber optic was wiped with ethanol after the completion of each light exposure.

Post-PDT bacterial sampling

The contents of root canals were sampled by flushing the root canals as described above. Serial dilutions were prepared and 100 µl aliquots were inoculated onto blood agar and incubated anaerobically for 7 days.

Dentinal Shavings

Following flushing of tooth specimens, intracanal dentinal shavings were removed from the CMD group (9 teeth with 22 canals) and CMD+PDT group (12 teeth with 29 canals) and gathered in an microcentrifuge tube containing 1.5 ml of BHI. Briefly, a 21 mm length nickel-titanium rotary file (#25, Sequence, Brasseler, Savannah, Georgia) with a tip diameter

of 0.25 mm and a taper of 0.06 mm/mm was inserted to length in each 12 and 14 mm tooth specimen (i.e. at length, the 21 mm Sequence file protruded 9 mm and 7 mm beyond each 12 mm and 14 mm tooth specimen root tip respectively). This generated a circumferential dentinal tubule penetration of 205 to 455 μ or 205 to 485 μ for each 12 mm or 14 mm tooth specimen respectively measured from each root tip to coronal level.

Microbial analysis

The microbial composition of root canals before and after treatment was assayed using a whole genomic probe assay as described previously (22). Tris-EDTA buffer (1.5 ml) was added to the plates and the bacterial colonies were harvested using glass rods. The cell suspensions were placed into individual Eppendorf tubes and sonicated for 10 sec to break up clumps. The optical density (OD) of each suspension was adjusted to a final OD of 1.0, which corresponded to approximately 10^9 cells. Ten μ l of the suspension (10^7 cells) was removed and placed in another Eppendorf tube with 140 μ l of TE buffer and 150 μ l of 0.5M NaOH. The samples were lysed and the DNA was placed in lanes on a positively charged nylon membrane using a Minislot device (Immunelectrics, Cambridge, MA, USA). After fixation of the DNA to the membrane, the membrane was placed in Miniblotter 45 (Immunelectrics) with the lanes of DNA perpendicular to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes against 39 species found in endodontic infections (39) were hybridized in individual lanes of the Miniblotter. After hybridization, the membranes were washed at high stringency and the DNA probes were detected using antibody to digoxigenin conjugated with alkaline phosphatase for chemifluorescence detection. Signals were detected using AttoPhos substrate (Amersham Life Science, Arlington Heights, IL, USA) and were scanned using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA, USA). Computer-generated images were analyzed to determine the fluorescence intensity associated with each sample and probe. Two lanes in each membrane contained DNA standards with 1 ng (10^5 bacteria) and 10 ng (10^6 bacteria) of each species. The sensitivity of the assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. The measured fluorescence intensities were converted to absolute counts by comparison with the standards on the same membrane. Failure to detect a signal was recorded as zero.

Statistical Analysis

The principal endpoint calculated for each canal was the residual level of colony-forming units (CFUs) following treatment relative to the pretreatment CFU level (residual %CFUs). For multi-rooted teeth the value for each tooth was the average of the canal values. Treatment effects were evaluated in a logistic model using generalized estimating equations (GEE) to account for correlations between canals from the same tooth. An indicator (0/1) variable was included to estimate and adjust for single-rooted/multi-rooted teeth. Similar analyses were done for teeth stratified as single/multi-rooted using Mantel-Haenszel analysis.

RESULTS

Figure 1 shows the distribution of 52 teeth in the CMD (26 teeth) and CMD+PDT (26 teeth) groups. The number of canals from each tooth that received either treatment is also provided. These numbers are highlighted in bold when teeth were incompletely disinfected following treatment and the number of positive canals is given in parentheses. The results clearly demonstrated the better performance of CMD+PDT over CMD. The summary Mantel-Haenszel chi-square test for treatment effects was significant ($P=0.026$). Overall, 13 of 26 teeth (50%) were positive following CMD, whereas 6 of 20 teeth (30%) were positive following CMD+PDT. Among single-rooted teeth, 5 of 14 teeth (35.7%) were positive

following CMD, whereas only 1 of 12 teeth (8.3%) was positive following CMD+PDT. Among multi-rooted teeth, 8 of 12 teeth (66.7%) were positive following CMD and 5 of 14 teeth (35.7%) were positive following CMD +PDT.

CMD+PDT significantly reduced the frequency of positive canals relative to CMD alone ($P=0.0003$) (Table 1). Canals from single-rooted teeth were less likely to be positive post-treatment than canals from multi-rooted teeth ($P=0.10$). Following CMD+PDT, 45 of 52 canals (86.5%) had no CFU as compared to 24 of 49 canals (49%) treated with CMD (Table 1) (canal flush samples). Post-treatment microbial levels were low as a percent of pretreatment levels (%CFU). For CMD+PDT only 7 of 52 canals (13.5%) were positive and all had %CFU less than 0.1% of pretreatment CFU levels. However, following CMD 25 of 49 canals were positive (51%) and 22.4% of canals had post-treatment values greater than 0.1% of pretreatment levels (Table 1). The CFU reductions were similar when teeth or canals were treated as independent entities. Analysis stratified by tooth type indicated that post treatment %CFU values were more often positive and also at higher levels of infection in canals that received CMD relative to canals that have received CMD+PDT ($P<0.0001$).

The microbial composition of canal biofilms (canal flush samples) was studied by checkerboard DNA-DNA hybridization. Pre- and post-treatment frequencies (+/-) were obtained for 39 species found in endodontic infections with whole genomic probes for 45 canals that received CMD+PDT and 44 that received CMD alone (Fig. 3). The number of canals positive for each species pretreatment was quite high and the pattern was similar for both treatments. Post-treatment detection levels for all species were systematically and markedly lower for canals treated by CMD+PDT than for those treated by CMD alone. Key endodontic pathogens resisting intracanal disinfection procedures (40) were dramatically reduced (Fig. 3, highlighted in grey).

The frequency of dentinal infection (after debridement up to 485 μ m) was also evaluated (Table 2). In the CMD group, tubules from 17/22 canals (77.3%) were positive after treatment, while in the CMD+PDT group tubules from 15/29 (51.7%) canals were positive ($P=0.034$).

DISCUSSION

The present study was built on the interdependent foundations of: 1) developing an *in vitro* model for testing PDT (14,20,22); 2) the utilization of an FDA approved drug – methylene blue (MB) – as the photosensitizer (14,20,22); 3) the development of a novel light delivery system that maximizes the distribution of light within the entire anatomy of the root canal system (22); 4) the ongoing refinement of light and drug dosimetry (14,20,22,41); and 5) the assessment of PDT safety (41). The hypothesis of this study was that near complete elimination of residual root canal bacteria could be achieved using PDT as an adjunctive procedure to SET in chronically-infected extracted human teeth *ex vivo*. Our findings show that MB-mediated PDT significantly enhanced the effect of CMD. Four *in vivo* studies have also suggested the potential of PDT as an adjunctive technique to eliminate residual root canal bacteria after CMD (29–32). Toluidine blue-mediated PDT offered a means of destroying microorganisms remaining after using sodium hypochlorite alone (29) or citric acid and sodium hypochlorite as co-irrigants (30). PDT significantly enhanced the effect of CMD in teeth with necrotic pulps using a conjugate between polyethyleneimine chlorine ₆₆ conjugate (31) and toluidine blue (32).

The incomplete bacterial killing in dentinal tubules following PDT may be due to: a) Incomplete MB penetration in the tubules that may be related to binding interactions with dentin components; b) Failure of MB to penetrate canal biofilms; and c) Insufficient

oxygenation. We have proposed the encapsulation of MB within poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles that may offer a novel nano-platform for enhanced drug delivery and photodestruction of canal biofilms (28). These nanoparticles have a hydrophobic core part made up of PLGA (hydrophobic) and PEO-PPO (surfactant) molecules (polyethylene oxide-polypropylene oxide). Surfactant chains project outwards from the surface of the core part because of their hydrophilic end groups (hydroxyl, quarternary ammonium). This creates a gradient from the hydrophilic end groups outside to increased hydrophobicity towards the core of nanoparticles. Hydrophilic end groups provide an anchoring effect for retention of nanoparticles to negatively-charged membranes. Due to the hydrophobic-hydrophilic orientation of the surfactant molecules, they provide good wettability to enhance interaction on/within bacterial membranes. Infiltration of dentinal tubules by MB-loaded nanoparticles has recently been demonstrated (28). George and Kishen (19) dissolved MB in a mixture composed of glycerol, ethanol and water (30:20:50) and showed greater penetration of MB into dentinal tubules. Our future studies will explore the use of ultrasonic waves for enhancement of the transdentinal movement and penetration of MB in canal biofilms. It has been demonstrated that an irrigant in conjunction with ultrasonic vibration, which generates acoustic streaming and continuous movement of the irrigant, increases the effectiveness of the cleaning of root canal (42). Regarding insufficient oxygenation, the application of perfluoro-decahydro-naphthalene in the root canal system was proposed as a carrier of oxygen for enhancement of the PDT effect (26). The basic properties of perfluorocarbons and perfluorocarbon emulsions relevant to their use as oxygen delivery systems were briefly reviewed (43). A Phase III clinical trial in cardiopulmonary bypass surgery, with a protocol that included both augmented-acute normovolemic hemodilution and intraoperative autologous donation, was interrupted following the observation of adverse events. At this time point, there is not enough information concerning the toxicity of these compounds to utilize them.

The results obtained from this study are very promising. The use of naturally infected teeth, which contain a broader range of pathogens than *in vitro* model systems, provide an excellent test of the potential of PDT in achieving root canal disinfection. However, since some living bacteria were still present in dentinal tubules following PDT, further refinement and enhancement of the PDT procedure may be necessary. The effect of biophysical means and surface tension-reducing agents on the transdentinal penetration of MB as well as the effect of supplemental hyper-oxygenation should be evaluated.

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Figure 1.

Fifty-two teeth were assigned to two experimental groups (CMD and CMD+PDT). The number of canals from each tooth that received treatment is provided. Numbers highlighted in bold indicate incompletely disinfected teeth. The number of positive canals following treatment is given in parentheses.

Figure 2.
Post-flushing of tooth specimen under rubber dam.

Figure 3.

Pre- and post-treatment detection frequencies for 39 species found in endodontic infections by checkerboard DNA-DNA hybridization with whole genomic probes for 45 canals treated by CMD+PDT and 44 treated by CMD alone.

◇ CMD, baseline ◆ post-CMD ○ PDT, baseline ● post-PDT

TABLE 1

Frequency of Root Canal Infection after CMD or CMD+PDT Treatment

	<u>Survival after treatment</u>			Total no. of canals
	None	<0.1%	>0.1%	
CMD, no.	24	14	11	49
CMD, %	49.0	28.6	22.4	
CMD+PDT, no.	45	7	0	52
CMD+PDT, %	86.5	13.5	0.0	

CMD, chemomechanical debridement; PDT, photodynamic therapy.

TABLE 2Frequency of Dentinal Infection (after debridement up to 485 μ m) after CMD or CMD+PDT Treatment

	<u>Survival after treatment</u>	
	Positive	Negative
CMD	17	5
CMD+PDT	15	14

CMD, chemomechanical debridement; PDT, photodynamic therapy.