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MTA Obturation of Pulpless Teeth with Open Apices: Bacterial Leakage as Detected by Polymerase Chain Reaction Assay

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Polymerase chain reaction (PCR) followed by reverse dot blot was used to detect *Enterococcus faecalis* leakage through mineral trioxide aggregate (MTA) apical obturations of pulpless teeth with open apices. Prepared root canals of 34 extracted teeth were given a standard apical foramen opening and received orthograde apical obturation with MTA; three groups had 1-, 2-, or 3-mm thickness. Sterilized specimens were inoculated with *E. faecalis* and incubated in sterile medium. DNA extracted from the specimens was amplified by polymerase chain reaction, which yielded a specific segment of *E. faecalis* 16S rDNA. On day 10 of incubation, no specimens were contaminated. On day 50, almost 17% of specimens were contaminated, with no statistically significant difference between groups (Chi-square = 0.48; *df* = 2; p = 0.787). Therefore, MTA provides an adequate seal even in cases of orthograde apical obturation of pulpless teeth with open apices.

Three-dimensional obturation of the root-canal system to the canal apex is fundamental for successful endodontic therapy (1). Inadequate apical seal is a major cause of failure in nonsurgical endodontic treatment (2). Apical seal is better in the traditional Schilder obturation technique and in System B and Thermafil techniques than in lateral condensation or the single cone technique (3). However, in vertical condensation methods using warm gutta-percha, increased apex diameter is linked to an increased risk of overextension and apical leakage (4, 5). In pulpless teeth with open apices, divergent apex architecture makes both complete debridement and control of obturation uncertain (6). To avoid the disadvantages of apexification in adults with calcium hydroxide, several methods have been proposed to obturate open apices without first inducing a natural apical barrier (7–12). Mineral trioxide aggregate (MTA) is biocompatible (13, 14), bacteriostatic (15), and has better sealing ability than amalgam, Intermediate Restorative Material (IRM), or Super EBA used as root-end filling material (16); it has cement-conductive properties (17) and there is neoformation of bone and periodontal ligaments in direct contact with it (18). MTA is ideal to form an apical barrier against which to condense gutta-percha in pulpless teeth with open apices (19, 20). The purpose of this in vitro study was to evaluate bacterial microinfiltration of 1-, 2-, and 3-mm thick apical MTA obturations, using polymerase chain reaction (PCR) followed by reverse dot blot.

MATERIALS AND METHODS

Specimen Preparation

Specimens were 34 extracted, human, single-root teeth that had not undergone endodontic treatment. Specimen preparation was as follows: the root surface was debrided and the tooth was immersed in 5% sodium hypochlorite solution (Niclor 5, OGNA, Italy) for 1 h and then stored in physiological solution; the root canal was preflared using K-Flexofiles (Dentsply Maillefer, Switzerland) up to size 20, then shaped using ProTaper S1-S2-F1-F2 (Dentsply Maillefer) to the working length. The working length was established when the instrument was visible at the apical foramen. Using a syringe with 22-gauge needle, the root canal was irrigated with 33 ml of 5% sodium hypochlorite (Niclor 5, Ogna, Italy) at 50°C, alternating with 2 ml of 10% EDTA (Tubuliclean, Ogna, Italy); total irrigation time: 11 min/specimen. The apical foramen was shaped to 80 ISO by extruding ProTaper F2 exactly 9 mm from the apex. The root canal was dried with paper points, and the integrity and shape of apical foramen and canal cleansing were microscopically checked (Carl Zeiss Pro Magis, Germany; magnification, ×10). The tooth was sectioned to obtain a 13-mm root residue. The specimens were randomly subdivided into 3 groups of 10, plus 4 controls. The specimens from each group were obturated in their apical portion with MTA as follows: group I, 1-mm thickness; group II, 2-mm thickness; group III, 3-mm thickness; two negative controls (numbers 3 and 4), approximately 2-mm thickness. Two positive controls (numbers 1 and 2) were not obturated. The obturation thickness of MTA was determined from the most coronal part of the apical foramen to the coronal margin of the obturation; white MTA (ProRoot, Dentsply Maillefer) was positioned with an MTA Endo Gun (Dentsply Maillefer) under microscopic vision (×10) and compacted with a plugger; unifor-

mity of the obturation was radiologically checked. A damp paper point was inserted in each canal and the specimens were placed in a sealed container under 100 relative humidity; after 48 h, the paper points were removed and the hardening of MTA was checked. A pipette tip was placed in each canal and cemented to the dentine with cyanoacrylic cement. Surfaces were sealed with nail polish to cover 3 mm of the pipette tip adjacent to the root and the root walls from the pipette tip to the coronal margin of the MTA obturation (1 mm from the apical foramen for group I; 2 mm for group II; 3 mm for group III). Negative controls were completely sealed, also covering the apex (numbers 3 and 4); positive controls were sealed to 3 mm from the apex (numbers 1 and 2). Each specimen was fixed with cyanoacrylic cement on to a steel wire support (diameter, 0.8 mm), and this was placed in a 50-ml Falcon tube with screw stopper in a stable, vertical position. The canal apex corresponded to the 25-ml notch on the tube. The tubes were placed in envelopes and sterilized in ethylene oxide (Fig. 1).

Inoculation

Under a laminar flow hood, each tube was removed from its envelope and filled with 30 ml of sterile culture medium (YG broth) prepared with 10 g/l of yeast extract (Difco Laboratories, Detroit, MI) and 10 g/l of glucose, completely immersing the root apex in medium. With a long-needle insulin syringe, 50 μl of medium containing alpha-hemolytic *Enterococcus faecalis* (ATCC 29212) plus 100 μl of culture medium were placed in each tip; the tubes were closed and incubated at 37°C. The culture medium was checked for contamination in each specimen at 10, 20, 30, 40, and 50 days of incubation via optical examination and PCR followed by reverse dot blot.

FIG 1. Experimental model used.

DNA Extraction, PCR, and Reverse Dot Blot

DNA was extracted with a QIAamp spin column (Qiagen, USA) and was eluted in a 100-μl volume. The sequences of the PCR primers, specific for *E. faecalis* 16S/23S rDNA, were: 5'-GAT-TAG-ATA-CCC-TGG-TAG-TCC-AC-3' (forward primer) and 5'-CCC-GGG-AAC-GTA-TTC-ACC-G-3' (reverse primer). The forward primer was biotinilated at the 5' end. The reported amplicon length was 600 bp. Ten-microliter aliquots of extracted DNA were amplified using the specific primers (Analitica, Italy) in a reaction volume of 50 μl, consisting of 10 μl of DNA sample and 40 μl of reaction mixture. The mixture contained 1× PCR buffer (Sigma Chemical Co., St. Louis, MO) 1.5 units of Taq DNA polymerase (Sigma), 1.5 mM MgCl2 (Sigma), 0.2 mM of each dNTP (Sigma), and 20 pmol/μl of each primer. PCR cycling conditions applied were: an initial denaturation step of 10 min at 95°C; 20 cycles comprising: denaturation (25 s at 95°C), primer annealing (40 s at 53°C), extension (40 s at 70°C); a final extension step at 70°C for 8 min. PCR cycling was performed in a DNA thermocycler (PCR sprint, Hybaid, USA). PCR was checked by running samples on a 1% agarose gel stained with ethidium bromide (Fig. 2). Amplicons were then analyzed by reverse dot blot in an automated instrument (Profi-Blot II T, Tecam, Switzerland) using the Reverse Dot Blot Method (Symbiosis, Italy). After denaturation, the amplified products were hybridized to specific DNA probes immobilized on nitrocellulose strips. The hybrids were detected by NBT/BCIP colorimetric reaction using streptavidin-conjugated alkaline phosphatase (Fig. 3).

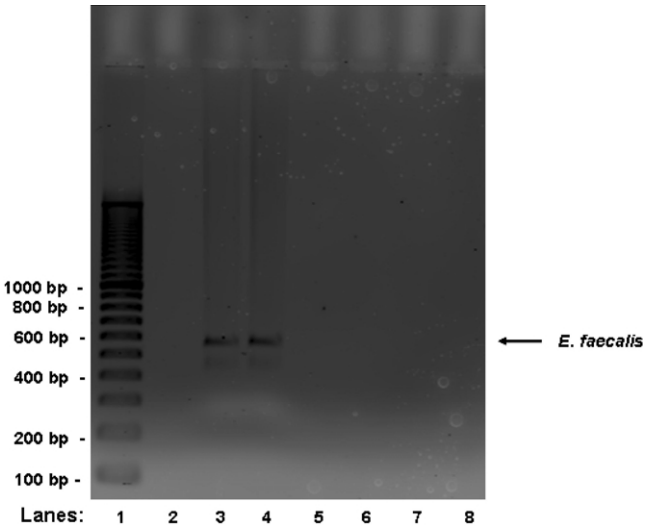

FIG 2. 1% agarose gel stained with ethidium bromide. Lane 1: 100 bp DNA ladder. Lane 2: PCR mix (no target). Lane 3: positive control (number 1). Lane 4: positive sample (from group I). Lane 5: negative control (number 3). Lane 6: negative control (number 4). Lane 7: negative sample (from group I). Lane 8: negative sample (from group I).

RESULTS

PCR amplification and reverse dot blot using an *E. faecalis*-specific primer pair detected this bacterial species in specimens 1 and 2 (positive controls) after 24 h. Specimens 3 and 4 (negative controls) were not contaminated at 50 days. Other results are shown in Table 1. Data were analyzed with the Chi-squared test ($p < 0.05$), revealing no statistically significant difference among the three groups (Chi-squared = 0.48; $df = 2$; $p = 0.787$).

DISCUSSION

Our results, obtained using a highly sensitive molecular method, showed that an orthograde apical plug of MTA, used to treat pulpless teeth with apices that are open or large and irregular, provided an adequate seal against infiltration by *E. faecalis*. In teeth with open apex, this method is a nonsurgical alternative to conventional long-term calcium hydroxide apexification (root-end closure), the outcome of which is not fully predictable in adults (9, 10). Unless a calcified apical barrier is created through apexification, the apical seal of gutta-percha obturations cannot be guaranteed and long-term prognosis for the tooth is uncertain (2). In other studies, teeth with open apices were obturated without first inducing a natural apical barrier (7–12): these methods created an apical barrier that avoided extrusion of the filling material during condensation. Dentine and calcium hydroxide plugs (8) or dentine chips and hydroxyapatite (21) were used for compaction in the apical third. A one-appointment technique using tricalcium phosphate as immediate apical barrier against which to condense the gutta-percha gave predictable results (7). Demineralized freeze-dried bone compacted at the canal end provided a biocompatible apical matrix in a single appointment (22). MTA is reported to be ideal to promote formation of an apical barrier in a single appointment (18, 19). Cement forms around any minimum extrusion of MTA beyond the apex (12–18). The manufacturer of MTA recommends a 3- to 5-mm thickness to be placed at the apex for apexification. It has been shown that resolution of the periapical lesion is complete at 1 yr in three cases of apical obturation with 4-mm MTA (23). In an in vitro apexification model to test seal and retention, the MTA apical barrier was 1- or 4-mm thick, with or without previous calcium hydroxide medication; leakage occurred in 91% of specimens by day 10 and in all specimens by day 70. Only 20% of control specimens (MTA placed as retrograde root-end filling) were positive. The study concluded that leakage may be caused by the intracanal delivery technique rather than by the MTA itself; orthograde delivery is a more sensitive technique (24).

The delivery technique is still controversial. Aminoshariae et al. (25) obtained a more accurate adaptation of the MTA by hand method compared with ultrasonic condensation, whereas Lawley et al. (26) found that the ultrasonically placed MTA provided a better seal than hand-placed MTA. We adapted the MTA apical plug by hand method under microscopic vision (×10). In our study, no specimen was contaminated at day 10. At day 50, 5 of 30 specimens were contaminated (16.6%). The results may have been influenced positively by the use of operative microscope to check the correct position and fit of material at the apex. Thickness of apical obturation did not significantly influence seal. This is in agreement with the above study (24) and with encouraging reports of root perforation and pulp chamber repair with MTA (27) in which the thickness of the material is not always between 3 and 5 mm. Several methods are available to study the quality of apical seal provided by root-end filling materials: extent of dye, radioisotope or bacterial penetration, electrochemical means, and fluid-filtration techniques (28). Although they do not determine the ability of root-end filling materials to prevent leakage of bacterial metabolites and their by-products, bacterial leakage studies are recommended to test the suitability of root-end filling materials (28). Endotoxin studies confirm the superior sealing capability of MTA (29). This study used *E. faecalis* to test the sealing properties of MTA; this bacterial species is frequently isolated in cases of endodontic failure (30), because it can penetrate the dentine tubules and escape chemomechanical treatment of root-canal system (31).

We used PCR followed by reverse dot blot to evaluate bacterial infiltration. PCR amplification was checked by running samples on an agarose gel (Fig. 2). Reverse dot blot was performed by hybridizing each PCR sample to a separate nitrocellulose strip. The strip carries DNA probes specific for *E. faecalis* (Fig. 3). Molecular methods are superior in terms of sensitivity and specificity versus cultivation procedures (32). A protocol for PCR detection of *E. faecalis* and *E. faecium* from the root canal has shown high specificity in detecting microbiota from root-canal specimens (33).

In conclusion, orthograde positioning of MTA in the apical third provides an adequate apical seal in cases of apices that are side, resorbed, or irregular in shape. In these cases, the outcome of gutta-percha seal may be uncertain because of the apex architecture and the presence of humidity, whereas MTA might provide predictable results.

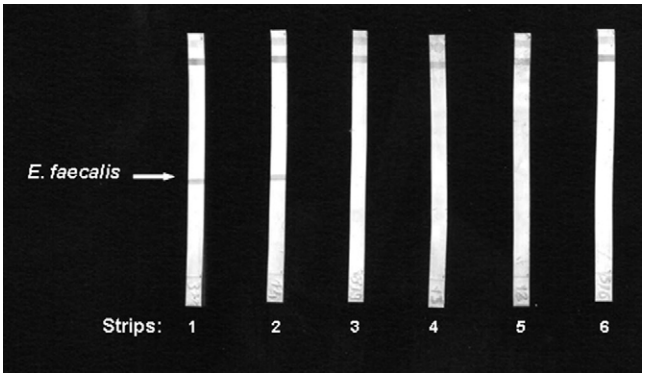

FIG 3. Strips of nitrocellulose for reverse dot blot. Strip 1: positive control (number1). Strip 2: positive sample (from group I). Strip 3: negative control (number 3). Strip 4: negative control (number 4). Strip 5: negative sample (from group I). Strip 6: negative sample (from group I) .

TABLE 1. Specimens affected by leakage in the three groups, as detected by optical examination and PCR

Day	Group I: 1-mm ($n = 10$)	Group II: 2-mm ($n = 10$)	Group III: 3-mm ($n = 10$)
10	0	0	0
20	1	0	0
30	0	0	0
40	0	1	1
50	0	1	1
Total	1	2	2

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