Comparison of antibacterial activities of root-end filling materials by an agar diffusion assay and Alamar blue assay

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Abstract  Background/purpose: The purpose of this in vitro study was to evaluate antibacterial activities of the leachable components of root-end filling materials (calcium silicate cement). Materials and methods: Root-end filling materials were mixed according to the manufacturers’ directions and then placed into 6-mm-diameter Teflon rings with mixed materials; they were allowed to set for 24 hours and then tested. Antibacterial activities of the root-end filling materials were evaluated against Streptococcus sanguinis, Streptococcus mutans and Escherichia coli. The antibacterial activity of the root-end filling materials tested was determined by measuring the diameter of each zone of inhibition (on an agar diffusion test). An Alamar blue assay was used to detect bacterial growth. Statistical analysis was conducted using one-way analysis of variance. Results: Zones of inhibition were observed in the zinc oxide-eugenol cement (IRM) group treating S. sanguinis and E. coli agar plates in the agar diffusion test. Cultures of S. sanguinis and E. coli showed the lowest absorbances with the IRM group at different times of observation (1 hour, 3 hours, 6 hours and 12 hours) (P < 0.05). The growth of S. mutans showed no significant difference between controls and any tested materials (P > 0.05). Conclusion: We concluded that both the agar diffusion test and Alamar blue assay gave comparable findings of assessing the antimicrobial activity present in root-end filling materials. No antimicrobial activity was detected for mineral trioxide aggregate, calcium silicate cement, or amalgam after coming into contact with S. mutans, S. sanguinis and E. coli. IRM showed high antimicrobial activity against both S. sanguinis and E. coli.

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Introduction

Pulpal and periradicular diseases may require an apicoectomy due to undesirable treatment outcomes. Many materials have been proposed for use as root-end fillings, including amalgam, zinc oxide–eugenol cements, composite resins, polycarboxylate cements, glass ionomer cements, copolymers, resin-modified glass ionomers, calcium- and silicate-based (CS) cements, and mineral trioxide aggregate (MTA). It is important to seal effectively the root-end or perforation site to prevent future contamination. Bacteria present in deeper layers of the infected root dentine may remain even after conventional root canal treatment and may cause periapical complications. Therefore, bacteria should be eliminated to ensure successful outcomes for patients. The prognosis of root treatment depends on successfully eliminating associated microorganisms and infected tissues.

MTA and CS are calcium silicate compounds. MTA cement is made of hydrophilic particles and contains mineral oxides, including tricalcium silicate, tricalcium aluminate, tricalcium oxide, and silicon oxide, which set in the presence of moisture. CS cement contains additives that exhibit acceptable physical and biological properties compared to MTA. For ideal sealing ability and biocompatibility, root-end filling materials should have antibacterial activity. Although many reports have been published on the antibacterial and antifungal properties of MTA, controversy exists in the literature regarding the antimicrobial activity of MTA. In one study, MTA was shown to delay or inhibit the growth of Enterococcus faecalis compared to Fill Canal or Sealapex. In contrast, another study comparing the effects of MTA and Portland Cement on Candida albicans, Staphylococcus aureus and Escherichia coli showed no antimicrobial effects for MTA. Streptococcus spp. were the predominantly isolated microbial genera in infected dental root canals, followed by Streptococcus spp. (14.2%), Porphyromonas spp. (12.2%), Ent. faecalis (9.6%), Staphylococcus salivarius (8.6%), Prevotella spp. (8.1%), Lactobacillus spp. (7.1%), Actinomyces spp. (7.1%), C. albicans (4.1%), Fusobacterium spp. (3.6%) Veillonella spp. (2.5%), Eubacterium spp. (2.5%), Bacillus spp. (2.0%), and E. coli (1.6%).

The antimicrobial activity of root-end filling materials varies with different study conditions, test methods, microbial species examined, and materials used. The most common methods of assessing antimicrobial activity are the agar diffusion test (ADT) and the direct-contact test (DCT). Results of the ADT are highly influenced by the solubility and diffusibility of the test agent in agar; thus, this test is not suitable to assay water-insoluble materials. The DCT is a quantitative and reproducible assay that allows for the testing of water-insoluble materials; it can be used to test materials in various stages of setting. Alamar blue (AB) is a tetrazolium-based dye, that incorporates resazurin and resorufin as oxidation–reduction indicators that yield colorimetric changes and a fluorescent signal in response to metabolic activity; the blue nonfluorescent oxidized form becomes pink and fluorescent upon reduction. The AB assay is a simple, rapid, low-cost, appropriate technology that does not require expensive instrumentation to determine bacterial growth. The AB assay incorporates a colorimetric and fluorometric growth indicator that changes based on the detection of metabolic activity in cells. Currently, there is an absence of reports assessing the antimicrobial activity of root-end filling materials using the AB assay.

The purpose of this in vitro study was to evaluate the antibacterial activity of leachable components of root-end filling materials [zinc oxide–eugenol (IRM), amalgam, MTA, and CS] against three different types of microorganisms utilizing both the ADT and AB assays.

Materials and methods

Material preparation

The root-end filling materials (amalgam, IRM, white MTA, and CS) used in this study and their manufacturers are shown in Table 1. All materials were mixed according to the manufacturers’ directions and placed into 6-mm-diameter Teflon rings. Materials were kept at 37°C and 100% humidity and allowed to set for 24 hours before testing.

Microbial cultures

Antibacterial activities of the root-end filling materials were evaluated using S. sanguinis (ATCC 10556), S. mutans (UA159), and E. coli (ATCC 6538); all from American Type Culture Collection (Manassas, VA, USA). Bacteria were grown aerobically from frozen stock cultures to the late logarithmic or early stationary phase in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA) at 37°C. In all tests, 18–20-hour cultures were used. Cells were harvested by centrifugation and resuspended in fresh BHI broth. The optical density (OD) of the bacterial cultures was assayed using a spectrophotometer, and bacterial numbers were standardized at an OD at 600 nm = 0.35.

Table 1 Root-end filling materials tested and their constituents.

<table>
<thead>
<tr>
<th>Material</th>
<th>Manufacturer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRM</td>
<td>Caulk Dentsply, Milford, DE</td>
<td>Polymethylmethacrylate reinforced zinc-oxide eugenol cement 75% Portland cement, 5% calcium, 20% bismuth oxide</td>
</tr>
<tr>
<td>MTA</td>
<td>Dentsply, Tulsa Dental, Germany</td>
<td>CaO, SiO2, and AI2O3 as matrices and ZnO, MgO, and Fe2O3</td>
</tr>
<tr>
<td>CS</td>
<td>Chung Shan, Taiwan</td>
<td>69.6% Ag, 17.7% Sn, 11.8% Cu, 0.67% Zn</td>
</tr>
<tr>
<td>Amalgam</td>
<td>Disperalloy (Johnson and Johnson Inc. Montreal, Canada)</td>
<td></td>
</tr>
</tbody>
</table>
ADT

The agar plate diffusion procedure was used to observe the antibacterial activity of the root-end filling materials. BHI agar plates were used for the ADT, and bacteria were inoculated using sterile cotton-tipped swab application. The set root-end filling materials were placed on the surface of the bacterial culture plates and incubated under aerobic conditions for 1 week. Positive control plates were streaked with bacteria, but no root-end filling material was used. Afterwards, the antibacterial activity of each root-end filling material was determined by measuring the diameter of the zones of inhibition. Five agar plates were used for each bacterial strain tested. All assays were repeated three times to ensure reproducibility. Statistical analysis was conducted using one-way analysis of variance. Tests of differences between the treatments were analyzed by Student–Newman–Kuels test, and a value of $P < 0.05$ was considered statistically significant.

AB assay

The AB assay was performed by a method originally reported by Shiloh et al.\textsuperscript{20} and modified by Kuda and Yano.\textsuperscript{21} S. sanguinis, S. mutans and E. coli were separately cultured in 2 mL BHI broth in the presence or absence of root-end filling discs. At 1 hour, 3 hours, 6 hours and 12 hours, AB dye (Biosource, Camarillo, CA, USA) was added to the bacterial cultures. Aliquots of 0.1 mL from each serial 10-fold dilution were placed into four 96-well flat-bottom polystyrene microplates. Additionally, 0.1 mL phosphate-buffered saline was used as the reagent blank. Next, 0.05 mL 3TYG/A (30 g/L tryptone, 15 g/L yeast extract, and 3 g/L glucose) was added. Each 96-well plate was immediately placed in a model 550 microplate reader (Bio-Rad, Hercules, CA, USA), and the OD at 570–600 nm was recorded for each well. OD data from each well and time point were compiled in a computer after subtracting the OD of the reagent blank (AB-REDOX value). Statistical analysis was conducted using one-way analysis of variance. Tests of differences between treatments were analyzed by the Student–Newman–Kuels test, and a value of $P < 0.05$ was considered statistically significant.

Results

Zone of inhibition (Figs. 1 and 2)

In the E. coli culture plates, the IRM group had the largest zones of inhibition ($P < 0.05$) on day 1, day 3 and day 7 of testing. In contrast, amalgam, MTA and CS root-end filling materials generated no zones of inhibition. In the S. sanguinis culture plates, the IRM group had the largest zones of inhibition ($P < 0.05$) at each time point. Zones of inhibition were not observed for amalgam, MTA or CS root-end filling materials. The S. mutans culture plates failed to show a zone of inhibition for any of the root-end filling materials tested.
diseases, and alterations to dental restorations, and is closely related to infective endocarditis. S. mutans dominance depends on competition with S. sanguinis and is influenced by the production of antimicrobial compounds. Endodontically treated teeth are vulnerable to recontamination from the oral cavity. A recent study has demonstrated the presence of bacterial biofilms in otherwise well-executed root canal treatments. Therefore, an endodontically treated tooth is vulnerable to treatment failure due to residual infection as well as the possibility of reinfection of the root canal system. Based on these findings, the present study selected S. mutans, S. sanguinis and E. coli as test microorganisms.

Many factors affect the size of the zone of inhibition by an antibacterial substance on agar plates. It depends on the toxicity of the substance for the particular bacterium and the diffusibility of the substance in the test medium being used. The diffusibility of an agent is a function of its hydrophilicity or hydrophobicity, size, and rate of release from the insoluble matrix in which it is bound. In this study, the ADT did not appear to be affected by the confounding factors listed above.

Several investigators have examined the antibacterial activity of different types of amalgam on a variety of facultative anaerobic bacteria; they have shown that most amalgam alloys possess some antibacterial properties.

Figure 2  Zone of inhibition for zinc oxide–eugenol (IRM). Note the translucent donut shape around the IRM. Mineral trioxide aggregate, calcium- and silicate-based cement, and amalgam showed no zones of inhibition.

Figure 3  (A) Growth inhibition of Escherichia coli following exposure to root-end filling materials observed by optical density measurement at different time intervals with the Alamar blue (AB) assay. (B) Growth inhibition of Streptococcus sanguinis following exposure to root-end filling materials observed by optical density measurement at different time intervals with the AB assay. (C) Growth inhibition of Streptococcus mutans following exposure to root-end filling materials observed by optical density measurements at different time intervals with the AB assay.
In contrast, the results of our study are similar to other published work showing that amalgam appears to have no antimicrobial activity according to the ADT.\textsuperscript{5,14} The different findings could be attributed to differences in the availability of nutrients, level of oxygen tension, incubation period, methods of evaluation, and laboratory setup. Although amalgam did not show bacterial inhibition in our agar diffusion assay, some minor antimicrobial activity was observed in the AB assay (Fig. 3). The activity may be explained by its oligodynamic properties from the presence of mercury and copper in its structure.\textsuperscript{32}

The release of eugenol by IRM may be from the progressive hydrolysis of the cement surface.\textsuperscript{5,33} The rate of release of eugenol may increase with time. Furthermore, if zinc oxide—eugenol contacts wet tissue, the eugenol concentration increases.\textsuperscript{11,34,35} A previous study has shown that samples of ProRoot MTA and IRM cement exhibited complete bacterial growth inhibition of \textit{Pseudomonas aeruginosa} until the end of the experiment; however, they were not as equally effective against \textit{E. faecalis} or \textit{Staph. aureus}.\textsuperscript{6}

Different microbial species exist at various degrees of sensitivity to stimulation. The present results also showed that IRM has high antimicrobial activity against \textit{E. coli} and \textit{S. sanguinis}, but was not effective against \textit{S. mutans}.

Although the antibacterial and antifungal properties of MTA have been extensively evaluated, there are many conflicting reports with no clear consensus. The present study showed that neither MTA nor CS exhibited antimicrobial activity in either the ADT or the AB test. One previous study focusing on MTA antimicrobial activity showed that except for \textit{E. coli}, MTA and Portland Cement were effective against microorganisms.\textsuperscript{8} Our study showed similar findings and MTA and CS exhibited no antimicrobial activity against \textit{E. coli}. Many investigations have reported that MTA has limited antimicrobial effects against some microorganisms. In contrast, another study has shown other root-end filling materials (Gray MTA (GMTA), Angelus gray MTA (AGMTA), Angelus white MTA (AWMTA), White Portland cement (WPC), Gray Portland cement (GPC)) had antimicrobial activity against \textit{Micrococcus luteus}, \textit{Staph. aureus}, \textit{E. coli}, \textit{P. aeruginosa}, \textit{C. albicans} and \textit{E. faecalis}.\textsuperscript{36}

The clinical concept is that high-pH values create environmental conditions that make microbial survival difficult. It has been reported that the main chemical component released from MTA in an aqueous solution is calcium hydroxide.\textsuperscript{11} When MTA is present on an agar plate or in a tube, the increased pH, caused by the dissociation of calcium hydroxide into calcium and hydroxide ions, may have been responsible for the observed antimicrobial activity. Calcium hydroxide dissociated into calcium and hydroxide ions in the aqueous environment, therefore, it is possible that MTA increased the pH in the test culture conditions, and this increase in pH was responsible for the antimicrobial activity of MTA against \textit{E. faecalis} and \textit{S. sanguinis}.

In conclusion, the ADT and AB assay showed comparable results in terms of antimicrobial activity of root-end filling materials. Antimicrobial activity was not exhibited by MTA, CS or amalgam against \textit{S. mutans}, \textit{S. sanguinis} or \textit{E. coli}. However, IRM showed high antimicrobial activity against \textit{S. sanguinis} and \textit{E. coli}.

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### References


