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## Microbial leakage of MTA, Portland cement, Sealapex and zinc oxide-eugenol as root-end filling materials

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### Abstract

**Objective:** The aim of this study was to compare the microbial leakage of mineral trioxide aggregate (MTA), Portland cement (PC), Sealapex and zinc oxide-eugenol (ZOE) as root-end filling materials.

**Study design:** An in vitro microbial leakage test (MLT) with a split chamber was used in this study. A mixture of facultative bacteria and one yeast (*S. aureus* + *E. faecalis* + *P. aeruginosa* + *B. subtilis* + *C. albicans*) was placed in the upper chamber and it could only reach the lower chamber containing Brain Heart Infusion broth by way of leakage through the root-end filling. Microbial leakage was observed daily for 60 days. Sixty maxillary anterior human teeth were randomly assigned to different groups - MTA and PC (gray and white), Sealapex + zinc oxide and ZOE, control groups and subgroups to evaluate the influence of EDTA for smear layer removal. These materials were further evaluated by an agar diffusion test (ADT) to verify their antimicrobial efficacy. Data were analyzed statistically by Kruskal-Wallis and Mann-Whitney test.

**Results:** In the MLT, Sealapex + zinc oxide and ZOE did not show evidence of microbial leakage over the 60-day experimental period. The other materials showed leakage from the 15th day. The presence of smear layer influenced microbial leakage. Microbial inhibition zones were not observed in all samples tested by ADT.

**Conclusion:** Sealapex + zinc oxide and ZOE did not show microbial leakage over the experimental period, whereas it was verified within 15 to 45 days in MTA and Portland cement.

**Key words:** Mineral trioxide aggregate (MTA), Portland cement, Sealapex, bacterial leakage, root-end filling.

## Introduction

Endodontic surgery is a therapeutic option in cases of failed endodontic retreatment. The effectiveness of the apical seal obtained by root-end filling materials is of paramount importance. The ideal properties of root-end filling include optimal apical seal, biocompatibility, adhesiveness to dentine walls and microbial control.

Several materials have been recommended for root-end filling, including gutta-percha, amalgam, ZOE cement, Cavit, composite resins, glass ionomers, Super-EBA and mineral trioxide aggregate (MTA) (1-5).

Mineral trioxide aggregate, developed at Loma Linda University, USA, has been indicated for sealing off the pathways of communication between the root canal system and the outer surface of the tooth (1, 7). This material seems to allow significantly less dye and bacterial leakage and better adaptation than amalgam, Super-EBA and IRM (8). MTA has also been shown to promote hard tissue deposition and biocompatibility (9-11).

These substances support matrix formation in a similar manner in cultures of osteoblast-like cells and also apposition of reparative dentine when used as direct pulp capping material in rat teeth (12). Estrela et al. (13) studied the antimicrobial and chemical properties of some materials, including MTA and Portland cement. The analyses of chemical elements present in MTA and in two samples of Portland cement were performed with X-ray fluorescence spectrometer. They reported that Portland cement contains basically the same chemical elements of MTA, except for bismuth. They also reported that Portland cement has pH and antimicrobial activity similar to MTA. Holland et al. (14) observed the rat subcutaneous connective tissue reaction to implanted dentine tubes filled with MTA, Portland cement or calcium hydroxide, and found similar mechanisms of action for these materials. Saidon et al. (15) compared *in vitro* the cytotoxic effect and tissue reaction of MTA and Portland cement after bone implantation in guinea pig mandibles. There was no difference in cell reactions. Bone healing and minimal inflammatory response adjacent to MTA and Portland cement implants were observed during the experimental period, suggesting that both materials were well tolerated. The results of these studies show that MTA and Portland cement have similar *in vitro* and *in vivo* biocompatibility. These findings also suggest that Portland cement might be used as a less expensive root-end filling material.

There is a commercially available MTA, called Pro-Root MTA (Dentsply, Tulsa, OK, USA), that has a gray powder. Holland et al. (16) analyzed the reaction of rat subcutaneous connective tissue to the implantation of dentine tubes filled with white MTA. The results indicated that the mechanisms of action of white and gray MTA are similar.

Thus, the purpose of this study was to compare the mi-

crobial leakage of MTA and Portland cement in two presentation forms (gray and white), Sealapex + zinc oxide and ZOE as root-end filling materials.

## Materials and Methods

### -Test organisms

This experiment used a mixture of five microorganisms – four reference bacterial strains and one yeast strain, obtained from the American Type Culture Collection. Facultative bacteria included were *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), and *Bacillus subtilis* (ATCC 6633). The yeast used was *Candida albicans* (ATCC 10231).

The microorganisms were inoculated in 7 mL of Brain Heart Infusion (BHI; Difco Laboratories, Detroit, MI, USA) broth and incubated at 37°C for 24 h. The experimental suspensions were prepared by cultivation of the biological indicators on the surface of Brain Heart Infusion Agar (BHIA; Difco Laboratories, Detroit, MI, USA), following the same incubation conditions; microbial cells were resuspended in saline to give a final concentration of about 3x10<sup>8</sup> cells/mL, adjusted to No. 1 MacFarland turbidity standard. One milliliter of each of these pure suspensions was used to obtain a mixture of the test microorganisms.

### -Microbial Leakage Test

#### Tooth preparation

Sixty maxillary anterior human teeth, extracted for different reasons, were selected for this study. Preoperative mesiodistal and buccolingual radiographs of each root were taken to verify the existence of a single canal, absence of internal or external resorption or calcification and a fully formed apex. The teeth were removed from storage in 0.2% thymol solution and were immersed in 5% sodium hypochlorite (NaOCl; Fitofarma, Lt. 20442, Goiânia, GO, Brazil) for 30 min to remove organic tissue. The crowns were removed and tooth length was standardized to 16 mm (from root apex to coronal border).

After initial radiographs, standard access cavities were prepared and the cervical third of the canals was enlarged with ISO size 70 to ISO size 110 Gates-Glidden drills (Dentsply/Maillefer, Ballaigues, Switzerland). The teeth were prepared up to an ISO size 50 K-File (Dentsply/Maillefer) 1 mm short of the apical foramen. During instrumentation, the root canals were irrigated with 3 mL of 1% NaOCl (Fitofarma) at each change of file. Root canals were dried and filled with 17% EDTA (pH 7.2) (Biodinâmica, Ibiporã, PR, Brazil) for 3 min for smear layer removal.

Thereafter, under a continuous air/water spray, the apical 3 mm of each root was cut off perpendicular to the long axis of the tooth with a fissure bur in a high-speed handpiece. A 3-mm-deep root-end cavity was prepared

with ultrasonic tips powered by an ultrasonic unit (Nac, Adiel, Ribeirão Preto, SP, Brazil).

Half of the specimens (30) had their apical portion filled with 17% EDTA pH 7.2 and this was left for 3 min to allow smear layer removal. After cleaning and shaping, the root canals were steam sterilized for 30 min at 121°C, with the components of the leakage apparatus. Thus, a perfect microbial control can be obtained without changing the dental structure or damaging the leakage apparatus. The other specimens (30) were not filled with EDTA.

#### *-Experimental Groups*

The teeth were randomly assigned to 10 groups of 6 roots each and two control groups, according to the materials tested and use or not of EDTA (Table 1).

To standardize root-end fillings, gutta-percha points were adapted 3 mm short of the apex in all specimens. The materials were prepared according to the manufacturer's directions (except for Groups 6 and 7, in which a consistent mixture was obtained by adding 0.0633g of each material to 0.1015g zinc oxide) and the root-end cavities were filled. Six specimens were filled with Gray Portland cement and totally impermeabilized (negative control) and 6 specimens were not root-end filled (positive control). The teeth were wrapped in wet gauze and placed in an incubator at 37°C for 24 h to allow complete set of the root-end filling materials.

#### *-Study Design*

In the experimental model, a split platform (upper and lower chamber) was used. In the upper chamber, there was a microbial suspension with the biological markers while the lower chamber contained a culture medium. The microbial mixture could only reach the lower chamber by leaking through the root-end filling. The coronal portion of the root canal of each tooth

was connected to the cut end of a 1.5 mL polypropylene Eppendorf tube (Cral, São Paulo, SP, Brazil) using a cyanoacrylate adhesive (Super Bonder, Itapevi, SP, Brazil) and epoxy resin (Durepoxi, São Paulo, SP, Brazil) to prevent leakage at the connection. The tooth-tube connections were entirely coated with two layers of nail polish (Max Factor, Cosmetics and Fragrances, Los Angeles, CA, USA), except for the apical 3 mm of the root. The teeth used as negative controls were completely coated with two layers of nail varnish including the apical portion of the tube. The specimens (teeth coupled to the polypropylene tubes) were sterilized in 5% NaOCl for 30 min and then rinsed with sterile water for 30 min.

The polypropylene tubes were attached to a rubber cover that was placed into a 10-mL sterile glass flask containing the culture medium. The flasks were filled with 8 mL BHI broth (Difco, Detroit, MI, USA) in such way that 3 mm of the root apex were immersed in the broth. The specimens were placed into the culture medium (BHI) and, to ensure sterilization, the testing apparatus was incubated at 37°C for 24 h. No growth was observed after this period.

#### *-Bacterial Inoculation*

The whole apparatus was incubated at 37°C. Fresh overnight cultures of organisms were added to the tubes at 7-day intervals. Figure 1 shows a schematic presentation of the MLT apparatus. Microbial leakage was assessed daily for 60 days, having as a reference the turbidity of the culture medium, which was considered an indicator of microbial contamination. Positive BHI tubes were selected and inocula were spread on BHI agar surface under identical incubation conditions. Gram stains of the BHI growth and from colonies growing on BHI agar were carried out.

**Table 1.** Experimental groups, according to the materials tested.

<b>Experimental Groups</b>	<b>Material tested</b>
Group 1	White Portland Cement - Non Structural (Colatex, Anápolis, GO, Brazil)
Group 2	ProRoot (MTA, Dentsply Tulsa Dental, Tulsa, Ok, USA)
Group 3	White MTA (Angelus, Soluções odontológicas, Londrina, PR, Brazil)
Group 4	Gray MTA (Angelus, Soluções odontológicas, Londrina, PR, Brazil)
Group 5	Gray Portland Cement (Cimento Goiás, CPII, F32, Goiânia, GO, Brazil)
Group 6	White Portland Cement - Structural (Estrutural, Votoran Branco, cimento Rio Branco SA, RJ, Brazil)
Group 7	Sealapex (Sybron Kerr, Romulus, MI, USA) + zinc oxide (SS White, Artigos Dentários, Rio de Janeiro, RJ, Brazil)
Group 8	Zinc oxide-eugenol cement (SS White, Artigos Dentários, Rio de Janeiro, RJ, Brazil)
Group 9	Positive Control
Group 10	Negative Control

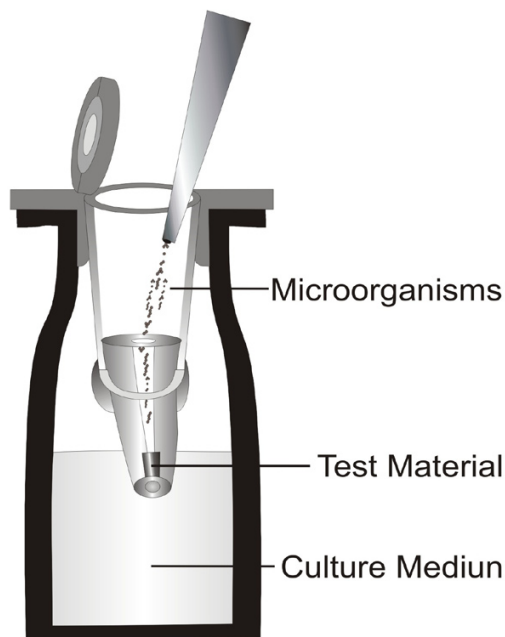


Fig. 1. Schematic presentation of the microbial leakage test.

*-Agar Diffusion Test*

Forty Petri plates containing 20 mL BHI agar were inoculated with 0.1 mL of the experimental suspensions using sterile swabs that were brushed across the medium, obtaining confluent growth. Five cavities (4 mm in depth; 4 mm in diameter) were prepared in each of two agar plates (total - 10) with a copper coil and completely filled with the freshly mixed test materials. The plates were pre-incubated for 1 h at environmental temperature followed by incubation at 37°C for 48 h. The diameters of the zones of microbial inhibition

were measured. Positive and negative controls were obtained by maintaining the plates either with or without inoculum, for the same period and under identical incubation conditions. Another positive control group was obtained with 9-mm-diameter paper disks immersed in 2% chlorhexidine for 1 min and then placed over the BHI agar surface. This control had the same number of samples as the other controls. All assays were carried out under aseptic conditions. Samples from the culture medium surrounding halos were extracted and immersed in 7 mL BHI and incubated at 37°C for 24 to 48 h. The inhibition zone around each well was recorded in millimeters.

Data were analyzed statistically by the Kruskal-Wallis test to reveal significant differences among the materials and the Mann-Whitney test was used to compare the variations factors within the groups (influence of presence or absence of smear layer).

**Results**

The minimum and maximum periods (days) in which microbial leakage occurred and the mean rank of comparison of the test materials are shown in table 2.

All positive control teeth showed microbial leakage and none of the negative controls leaked. The best results were observed for Sealapex + zinc oxide and ZOE groups. Smear layer removal was statistically significant under the test conditions (p = 0.016).

In the agar diffusion test (ADT), the results showed absence of microbial inhibition zones in all samples. Bacteria were viable in the positive control group, while the negative control group was free of microorganisms under the experimental conditions. The positive control group containing 2% chlorhexidine showed inhibition zones of 18 mm in diameter, on average.

Table 2. Minimum and maximum periods (days) at which microbial leakage occurred and mean rank of comparison among the materials tested.

Materials	n	Minimum (days)	Maximum (days)	Mean rank	p
G1 – White Portland Cement® NE	6	15	15	8.00 (A)	0.000 (a)
G2 - Pro Root Dentsply®	6	15	30	13.25 (AB)	
G3 – White MTA Angelus®	6	15	60	20.00 (BC)	
G4 – Gray MTA Angelus®	6	30	45	22.25 (BC)	
G5 – Gray Portland Cement®	6	15	>60	24.50 (C)	
G6 – White Portland Cement® E	6	45	45	26.00 (C)	
G7 - Sealapex®	6	> 60	> 60	41.00 (D)	
G8 - ZOE®	6	> 60	> 60	41.00 (D)	
With EDTA	24	15	> 60	19.81 (A)	0.016 (b)
Without EDTA	24	15	> 60	29.19 (B)	

The greater the values on minimum, maximum and mean rank, the lesser the microbial microleakage (a – Kruskal-Wallis test, b – Mann-Whitney test).

\* Capital letters (A, B, C and D) indicate statistically significant differences

## Discussion

Leakage of microorganisms is an important factor in endodontic treatment failure. Considering that root-end filling materials are placed in small cavities (3-mm deep), their properties should ideally include good adhesion to dentine walls and biocompatibility (6).

The methodology employed in this study was based on that of previous investigations using the *in vitro* microbial leakage model (2, 4, 5). Despite the restraint of leakage study methodology (5), dye and radioisotope leakage models have limitations such as the molecular size of most dye particles being smaller than that of bacteria and these models do not reproduce the interactions between microbial and non-microbial tracers (17). Therefore, several studies have pointed out that microbial leakage tests should preferably be used (18).

A possible limitation of current investigation on MLT was the lack of comparison of the number of microorganisms that penetrated the test materials into the culture medium. However, while considering microbial leakage in clinical conditions, it is important to understand that the time of contamination and the ideal conditions of the microenvironment for survival and adaptation of microorganisms associated to host response are decisive aspects for determining different characteristics of periapical pathology.

Thus, the purpose of the present study was just to verify the presence or absence of microbial contamination by way of turbidity of culture medium, and not to determine the number of microorganisms that penetrated the test materials in the 60-day period. The experimental model used compared *in vitro* the sealing capacity of the root-end filling materials. However, it must be clinically considered the importance of the number of contaminant microorganisms. In this study, the turbidity of the culture medium was used as reference and an indicator of microbial microleakage (a qualitative microbial analyze than quantitative). Chailertvanitkul et al. (19) reported that in one qualitative analyze the microleakage is evidenced as positive only when occurred the turbidity on culture medium, indicating complete penetration of microorganisms through the restorative and root canal filling materials. Considering identical explanation, they reported that the number of microorganisms required to cause apical periodontitis until is unknown. Regardless of the presentation form (gray or white), MTA and Portland cement showed similar behaviour under the test conditions. Microbial leakage in these materials was observed within 15 to 45 days. All samples that had the apical 3 mm filled with Sealapex + zinc oxide and ZOE did not show any microbial microleakage throughout the 60-day experiment. The influence of EDTA applied to root-end preparation was statistically significant ( $p = 0.016$ ) (Table 2).

The findings of this study differ from those of a previous

investigation that also used an *in vitro* microbial leakage model (8). These authors compared the ability of MTA, amalgam, IRM and Super-EBA to prevent *S. epidermidis* leakage in root-end filling. Most samples that had the apical 3 mm filled with amalgam, super-EBA or IRM started to show leakage within 6 to 57 days. Most specimens root-end filled with MTA did not show any leakage throughout the experimental period (90 days). Different results are observed depending on the experimental model (20). Wu et al. (21) compared the long-term leakage of root-end filling materials (amalgam, Super-EBA, MTA and Fuji II and Hi Dense glass ionomer cements) during a 1-year period using a fluid transport model. At 24 h, MTA leaked more than amalgam, Hi Dense, and Super-EBA. After 3-6 months, Fuji II and MTA showed statistically better results. After 12 months, MTA, Fuji II and Hi Dense leaked less than Super-EBA and amalgam. Adamo et al. (3) assessed the performance of MTA, Super-EBA, composite and amalgam as root-end filling materials using a bacterial microleakage model. Analysis of the data showed no statistically significant differences in the microleakage rate among the tested groups at 4-, 8- and 12-week intervals.

Tang et al. (5) investigated the ability of amalgam, IRM, Super-EBA and MTA root-end fillings to prevent endotoxin leakage. The results showed that MTA allowed less endotoxin leakage than IRM and amalgam at 1, 2, 6 and 12 weeks, and leaked less than Super-EBA at 2 and 12 weeks.

The main difference between the results of Tang et al. (5) and those of the present investigation is concerned with the type of biological indicators used and their structural characteristics. Tang et al. (5) assessed leakage by means of endotoxin, in contrast to the present study, in which Gram-positive and Gram-negative bacteria were used with respective exotoxins (teichoic acids – lipoteichoic acids) and endotoxins (lipopolysaccharides).

In this study, microbial inhibition zones were not seen in any plate submitted to ADT, which is in accordance with the findings of Torabinejad et al. (7), who investigated the antibacterial effect of some root-end filling materials by ADT. MTA had no antibacterial activity against *S. faecalis*, *S. aureus* and *B. subtilis*, and no effect on any of the strict anaerobic bacteria.

Sealapex + zinc oxide and ZOE gave the best results in microbial leakage prevention as root-end fillings compared with MTA and Portland cement (both gray and white presentations) at all time intervals. In other investigation, using dye penetration in extracted human teeth, Valera et al. (22) observed no statistical differences among the levels of the marginal infiltration of Sealapex + zinc oxide, MTA and Portland cement. MTA, Portland cement and Sealapex have been shown to induce hard tissue deposition in previous studies (9, 10, 14, 23).

It is significant to note that among the properties of root-end materials, good adhesion to dentine walls and biocompatibility are relevant after root-end resection and root-end filling (24). Trope et al. (25) evaluated histologically the healing of apical periodontitis in dogs after root-end resection and root-end filling with Super-EBA, glass ionomer cement, amalgam with varnish, IRM and a light-cured composite resin. The results showed no statistical difference between IRM and Super-EBA, and these materials gave better results when compared with the others.

Smear layer removal by EDTA was also evaluated in this study and statistically significant differences were observed. The absence of smear layer was associated with a greater number of samples presenting microbial leakage. It is important to report that smear layer removal by EDTA promotes an increase in dentinal permeability, which has a positive influence on microbial leakage.

These results are in agreement with previous investigations which found that removal of smear layer did not enhance the sealing ability (26, 27). On the other hand, some studies indicated that the smear layer can act as a barrier to microbial metabolites, preventing microbial invasion into dentinal tubules (28). Drake et al. (29) analyzing *in vitro* the effect of smear layer on bacterial retention suggested that smear layer produced during root canal preparation may inhibit bacterial colonization of root canals. One possible mechanism is that smear layer may block bacterial entry into dentinal tubules.

Behrend et al. (30) found that smear layer removal during canal obturation enhanced sealability, as evidenced by increased resistance to bacterial penetration. In that study, root canals were completely filled and the experimental period lasted 21 days. It is also relevant to highlight that *in vitro* and *in vivo* microbial leakage studies can be influenced by some factors such as material-tissue interaction, culture medium or microbial suspension and different concentrations of microbial cells in the oral cavity. This study showed differing results compared to the present investigation. It is important to consider the differences between lengths of sealed cavities (3-mm deep root-end cavities).

The findings of this study reinforce the idea that any material should be tested by different methods and have its performance compared to that of other materials before its clinical use can be recommended. Furthermore, the clinical and therapeutic implications are an important point to be considered. The results of this study showed that it is essential to observe the physical and chemical properties of root-end filling materials. A material with good biological properties does not necessarily have good adherence and antimicrobial efficacy.

Although the experimental model used in this study allowed the behaviour of the tested materials to be com-

pared with each other, lack of studies investigating the microbial leakage of MTA, Portland cement and Sealapex as root-end filling materials made a literature-based discussion difficult. Moreover, *in vitro* results should not be directly extrapolated to clinical conditions. Further research is required to support a broader clinical application of the findings of this study.

Under the test conditions, Sealapex + zinc oxide and ZOE did not show microbial leakage over the 60-day experimental period, whereas it was verified within 15 to 45 days in MTA and Portland cement (both gray and white presentations).

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