Antibacterial activity of two MTA-based sealers root canal sealers

Morgental RD, Vier-Pelisser FV, Oliveira SD, Antunes FC, Cogo DM, Kopper PMP. Antibacterial activity of two MTA-based sealers root canal sealers. Int Endod J 2011; 44:1128-33.

PMID: 21895702 [PubMed - indexed for MEDLINE]

http://dx.doi.org/10.1111/j.1365-2591.2011.01931.x.

International Endodontic Journal (ISSN: 0143-2885)

Antibacterial activity of two MTA-based sealers root canal sealers

R. D. Morgental[1]

F. V. Vier-Pelisser[1]

S. D. Oliveira[2]

F. C. Antunes[2]

D. M. Cogo[1]

P. M. P. Kopper[3]

[1]Department of Endodontics, School of Dentistry, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil;

[2]Laboratory of Immunology and Microbiology, Faculty of Biosciences, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil;

[3]Department of Endodontics, School of Dentistry, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

Address Correspondence to:

Profa. Dra. Fabiana Vieira Vier-Pelisser

E-mail: endopelisser@gmail.com

Abstract

Aim This laboratory study evaluated the pH and antibacterial activity of Endo CPM Sealer and MTA Fillapex by two different methods, using White MTA and Endofill as references for comparison.

Methodology Antibacterial activity was evaluated against *Enterococcus faecalis* (ATCC 29212). The agar diffusion test (ADT) was performed to evaluate the effect before setting. The materials were placed in four equidistant wells made in ten agar plates. After incubation at 37ºC for 48 h, the inhibition zones were measured using a digital paquimeter. The direct contact test (DCT) was performed to assess the antibacterial effect after setting. Suspensions of crushed materials were prepared and mixed with *E. faecalis*. After different periods of time (1, 6, 15 and 60 min), the survival of bacteria was assessed by using 10-fold serial dilution and cultivated on agar plates in triplicate. Colony-forming units (CFU)/mL were calculated after incubation. pH values were also measured in triplicate. Comparison between sealers in the ADT and DCT were performed by the Kruskal-Wallis test.

Results In the ADT, inhibition zones were found with MTA Fillapex and Endofill. They were similar to each other and greater than the other sealers ($P<0.05$). None of the tested sealers demonstrated antibacterial activity in the DCT, thus all sealers had similar bacterial counts compared to the negative control group ($P>0.05$). White MTA and Endo CPM Sealer suspensions had pH values greater than 11, while MTA Fillapex and Endofill had lower values.

Conclusions MTA Fillapex and Endofill had an antibacterial effect against *E. faecalis* before setting, but none of the sealers maintained antibacterial activity after setting, despite the high pH of the MTA-based materials.

Key Words: antibacterial activity; Endo CPM Sealer; Endofill; MTA Fillapex; White MTA.

Introduction

Mineral trioxide aggregate (MTA) is a biomaterial that has been investigated for endodontic applications since the early 1990s (Roberts et al. 2008). First, it was suggested to treat root perforations and in root-end fillings (Lee et al. 1993, Torabinejad et al. 1993). Currently, it is being used also in conservative pulpal treatments, repair of root resorption and apexification procedures (Menezes et al. 2004b, Jacobovitz & Lima 2008, 2009). MTA is widely accepted for its biocompatibility and excellent sealing capacity (Torabinejad & Chivian 1999, Scarparo et al. 2010).

However, despite favorable characteristics, MTA has physical properties that hinder its use for root canal filling (Roberts et al. 2008). The need for a biocompatible material that induces the formation of mineralized tissue, and also has suitable flow rate and manipulation, led to the development of MTA-based root canal sealers. Thus, a new formulation was created: Endo CPM Sealer (EGEO, Buenos Aires, Argentina). The powder consists of fine hydrophilic particles that form a colloidal gel in the presence of moisture, similar to the original MTA (Orosco et al. 2008, Gomes-Filho et al. 2009).

Another MTA-based root canal sealer with enhanced consistency, MTA Fillapex (Angelus, Londrina, PR, Brazil) is now available. It has resinous components and its manufacturer claims that it has excellent radiopacity, easy

handling and great working time. However, there is a lack of scientific information about this new sealer.

Numerous studies have evaluated the effect of MTA on microorganisms associated with endodontic disease with divergent methodologies and results (Torabinejad et al. 1995, Estrela et al. 2000, Al-Hezaimi et al. 2009, Ribeiro et al. 2010). There are few investigations about the antimicrobial activity of Endo CPM Sealer (Tanomaru et al. 2008) and none about MTA Fillapex.

The persistence of microorganisms in dentinal tubules, lateral canals and apical ramifications after root canal treatment has been reported (Sjögren et al. 1997, Peters et al. 2001, Nair et al. 2005). If the filling provides a good seal, it will only impair the exit of bacteria entrapped in the root canal system. However, to eradicate the remaining microorganisms, the antimicrobial activity of the sealer could play an important role (Spangberg et al. 1973, Nawal et al. 2011).

The agar diffusion test (ADT) is used extensively to assess the antimicrobial effect of endodontic sealers, despite its well-known limitations (Cobankara et al. 2004). Its results are influenced by the solubility and diffusibility of the material in the culture medium. Also, this test cannot distinguish the microbiostatic and microbicidal properties of the material (Tobias 1988). On the other hand, the direct contact test (DCT) does not have these disadvantages and it can be used to assess the antimicrobial effect of water-insoluble materials, providing quantitative and reproducible results (Weiss et al. 1996, Zhang et al. 2009).

Enterococcus faecalis is often used in research which aims to evaluate the antimicrobial properties of endodontic materials. It seems to play a significant role in the aetiology of persistent periradicular lesions (Gomes et al. 2006). *E. faecalis* possesses several virulence factors that contribute to its ability to survive the effects of conventional root canal therapy (Kayaoglu & Ørstavik 2004). Besides, this Gram-positive facultative anaerobe is able to invade dentine tubules and bind to collagen (Love 2001).

The aim of this study was to evaluate the effect of two MTA-based root canal sealers (Endo CPM Sealer and MTA Fillapex) against *E. faecalis* by two different methods: the agar diffusion test (ADT) and the direct contact test (DCT) to assess the antibacterial activity before and after setting, respectively. White MTA and Endofill were used as references for comparison. The pH values were also recorded and correlated to the antibacterial activity results.

Materials and methods

Materials

White MTA, Endo CPM Sealer, MTA Fillapex and Endofill were tested and compared (Table 1). The materials were prepared in accordance to the manufacturer's recommendations.

Agar diffusion test (ADT)

The microbiological assays were carried out under aseptic conditions in a laminar flow chamber (Quimis, Diadema, SP, Brazil). The antibacterial activity was evaluated using a standard strain of *E. faecalis* (ATCC 29212). The microorganisms were cultivated in Brain Heart Infusion – BHI broth (Merck, Darmstadt, Germany) at 37ºC for 18 h. Then, a bacterial suspension was prepared with 0.85% saline solution to match the turbidity equivalent to 1.0 McFarland standard tube, corresponding to 3 X 10^8 CFU/mL.

Ten replica plates containing BHI agar were spread with 0.1 mL of the bacterial suspension, using a Drigalsky's loop. Thereafter, four wells of 6 mm in diameter and 4 mm in depth (one for each material) were made with a punch by removing the agar at equidistant points and then filled immediately with the materials to be evaluated. Two plates did not receive the bacterial suspension; one did not receive the sealers and aimed to control the sterilization of the culture medium, while the other received the sealers and aimed to control their contamination.

All plates were maintained at room temperature for 2 h for prediffusion of the materials, and then incubated at 37ºC for 48 h under aerobic conditions. The inhibition zones around each one of the wells were then measured in millimetres using a digital paquimeter (Digimess, São Paulo, SP, Brazil).

Direct contact test (DCT)

The methodology used was adapted from Zhang et al. (2009). The endodontic sealers were manipulated according to manufacturers' instructions and inserted in a glass device with four orifices of 5 mm in diameter and 5 mm in depth (one for each material). They were allowed to set at 37ºC in 100% humidity for 7 days. Next, the blocks of set sealers were crushed to powder with ceramic mortar and pestle (CoorsTek, Golden, CO, USA). The powder was sterilized by ethylene oxide gas (Esteriliplus LTDA, Porto Alegre, RS, Brazil). Suspensions of each crushed material were prepared with saline solution at concentrations of 50 mg/mL.

Bacterial suspensions were prepared as described for the ADT and mixed with sealers suspensions in equal volumes (500 µL) inside polypropylene microtubes. Saline solution without sealers served as a negative control. After incubation at room temperature for 1, 6, 15 and 60 min, the survival of the bacteria in the solutions was assessed by 10-fold serial dilutions to 10^{-9} and culture on BHI agar plates. After incubation at 37°C for 48 h, colonies on the plates were counted and CFU/mL was determined. All experiments were made in triplicate.

Measurements of pH

Suspensions of each crushed material were prepared with deionized water at concentrations of 50 mg/mL. The pH of the supernatant of each sealer suspension was measured with a pH meter (Digimed, São Paulo, SP, Brazil)

previously calibrated at room temperature (25ºC). Before the measurements, the suspensions were mixed by vortexing for 30 s and centrifuged for 30 s to allow measurement of the clear supernatant. The pH values were evaluated 1, 6, 15 and 60 min after preparing the suspensions. Deionized water was used as a control. All experiments were performed in triplicate. The mean values of pH with the standard deviation were calculated.

Statistical analysis

To analyze data obtained in the ADT and for comparisons among sealers at each experimental period in the DCT, Kruskal-Wallis and Dunn tests were applied. For comparisons between experimental periods at each sealer in the DCT, Friedman test was applied. The level of significance was established at 5%. Data from pH analysis were submitted to descriptive statistics. Statistical analysis was performed with the software BioEstat 5.0 (CNPq, Brasília, DF, Brazil).

Results

Table 2 shows data obtained in the ADT for each sealer. Endofill (positive control) had the largest inhibition zone (Figure 1a,c), similar to MTA Fillapex (Figure 1a) and greater than the other sealers ($P<0.05$). White MTA (Figure 1a,b) and Endo CPM Sealer (Figure 1a) themselves resulted in diffusion in the agar, but they were not able to inhibit *E. faecalis*. There was no bacterial growth on the two control plates.

The results of the DCT are showed in Figure 2. None of the set materials had antimicrobial activity against *E. faecalis*. There was a significant difference between the bacterial counts of White MTA and Endofill in the first minute period ($P<0.05$). However, the bacterial counts of all sealers were similar to the negative control group in all experimental periods ($P>0.05$). Moreover, there were no significant differences throughout the experimental periods for any sealers ($P>0.05$).

The mean pH values for the sealers suspensions are described in Table 3. White MTA and Endo CPM Sealer suspensions presented pH values greater than 11 at all experimental periods. MTA Fillapex and Endofill had lower but also alkaline values. Deionized water had a neutral pH.

Discussion

This study investigated the pH and antibacterial activity of different endodontic sealers against *E. faecalis*. In the ADT, it was possible to observe inhibition zones with MTA Fillapex and Endofill. This fact could be explained by the presence of resin and eugenol, respectively. In regard to MTA Fillapex, there is no other data available about its antimicrobial effect. On the contrary, zinc oxide and eugenol-based sealers, such as Endofill, have been investigated extensively and have been used as positive controls in antimicrobial activity assays (Gomes et al. 2004, Tanomaru et al. 2008, Pinheiro et al. 2009).

White MTA and Endo CPM Sealer did not have inhibition zones against *E. faecalis*. Similar results were reported previously by Estrela et al. (2000), who used gray MTA. On the other hand, Tanomaru et al. (2008) verified that white MTA and Endo CPM Sealer had inhibition zones of 15 and 12 mm against *E. faecalis*, respectively. These two sealers showed visible diffusion in the agar medium, which could lead to misinterpretation of their antibacterial activity.

None of the set sealers had antibacterial activity in the DCT. White MTA and Endo CPM Sealer allowed the survival of *E. faecalis*, despite their high pH. According to McHugh et al. (2004), *E. faecalis* is unable to live at the pH of 11.5 or greater. As the pH shown by the above mentioned sealer was between 11 and 12, it can be assumed that its alkalinity was not enough to make the environment improper to the survival of that microorganism. Its proton pump is probably the key factor in its resistance to alkaline agents (Stuart et al. 2006).

These findings contrasts with those by Zhang et al. (2009), who reported a significant decreased in bacterial viability within 6 minutes of contact with grey MTA powder. White MTA powder was employed in the present study, thus the results could not be directly compared. Holt et al. (2007) reported that grey MTA showed greater *E. faecalis* growth inhibition than white MTA and this could explain the divergence.

An important goal of root canal treatment is to eliminate or prevent the introduction of microorganisms into the root canal system (Siqueira & Roças 2008). It is well known, however, that chemomechanical preparation is not able to

completely eradicate the endodontic infection (Nair et al. 2005). Residual bacteria may remain untouched by instruments, irrigants and medicaments (Sjögren et al. 1997). To prevent new bacteria growth, filling materials and sealers should have antimicrobial properties upon contact with microorganisms and biofilms especially before setting. After this period, the most important property of the endodontic sealer should be its sealing ability.

E. faecalis was chosen as the target microorganism due to its high prevalence in persistent endodontic infections, ranging from 24 to 77% (Stuart et al. 2006). *E. faecalis* can compete with other microorganisms and adapt to adverse conditions, such as nutritional deprivation (Kayaoglu & Ørstavik 2004). This microorganism is resistant to several irrigants and intracanal medicaments used in endodontics (Menezes et al. 2004a, Zehnder & Guggenheim 2009). Therefore, the antibacterial activity of endodontic sealers against *E. faecalis* is important in clinical practice.

Historically, two different assays have been used to test the antimicrobial characteristics of endodontic sealers: the ADT and the DCT. In this study, the first test was used, despite its limitations, to evaluate fresh sealers immediately after their manipulation. The second test was performed to analyze set sealers, seven days after their mixture. In the ADT, the size of the inhibition zones from a certain substance depends on its diffusibility in the culture medium used. This fact is the main disadvantage of this semi quantitative method (Nawal et al. 2011). However,

the ADT is suitable to indicate the activity of freshly mixed materials and its inclusion is interesting for comparative reasons with previous studies.

In turn, the DCT relies on direct contact between the microorganism and the tested material. This method is virtually independent of the diffusion and solubility properties of both the material and the media (Weiss et al. 1996). In contrast with the ADT, the DCT is capable of showing the antibacterial activity of insoluble components. When new materials are in test, more than one method should be employed (Nawal et al. 2011).

To improve the assessment of the antibacterial activity of root canal sealers, new methods should be developed where there is no interference from the diffusivity and solubility of the material in the culture medium.

Conclusion

MTA Fillapex and Endofill had an effect against *E. faecalis* before setting, but they did not maintain the antibacterial activity seven days after mixture. Despite their alkaline pH, White MTA and Endo CPM Sealer did not have antibacterial activity either before or after setting.

References

Al-Hezaimi K, Al-Shalan TA, Naghshbandi J, Simon JHS, Rotstein I (2009) MTA preparations from different origins may vary in their antimicrobial activity. *Oral*

Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology **107**, 85-8.

Cobankara FK, Altinöz HC, Ergani O, Kav K, Belli S (2004) In vitro antibacterial activities of root-canal sealers by using two different methods. *Journal of Endodontics* **30**, 57-60.

Estrela C, Baummann LL, Estrela CRA, Silva RS, Pécora JD (2000) Antimicrobial and chemical study of MTA, Portland cement, calcium hydroxide paste, Sealapex and Dycal. *Brazilian Dental Journal* **11**, 3-9.

Gomes BPFA, Pedroso JA, Jacinto RC, et al. (2004) In vitro evaluation of the antimicrobial activity of five root canal sealers. *Brazilian Dental Journal* **15**, 30-5.

Gomes BPFA, Pinheiro ET, Sousa ELR, et al. (2006) Enterococcus faecalis in dental root canals detected by culture and by polymerase chain reaction analysis. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **102**, 247-53.

Gomes-Filho JE, Watanabe S, Bernabe PF, Moraes Costa MT (2009) A mineral trioxide aggregate sealer stimulated mineralization. *Journal of Endodontics* **35**, 256-60.

Holt DM, Watts JD, Beeson TJ, Kirkpatrick TC, Rutledge RE (2007) The anti-microbial effect against Enterococcus faecalis and the compressive strength of two types of mineral trioxide aggregate mixed with sterile water or 2% chlorhexidine liquid. *Journal of Endodontics* **33**, 844-7.

Jacobovitz M, Lima RKP (2008) Treatment of inflammatory internal root resorption with mineral trioxide aggregate: a case report. *International Endodontic Journal* **41**, 905-12.

Jacobovitz M, Lima RKP (2009) The use of calcium hydroxide and mineral trioxide aggregate on apexification of a replanted tooth: a case report. *Dental Traumatology* **25**, e32-6.

Kayaoglu G, Ørstavik D (2004) Virulence factors of Enterococcus faecalis: relationship to endodontic disease. *Critical Reviews in Oral Biology & Medicine* **15**, 308-20.

Lee ES, Monsef M, Torabinejad M (1993) Sealing ability of a mineral trioxide aggregate for repair of lateral root perforations. *Journal of Endodontics* **19**, 541-4.

Love RM (2001) Enterococcus faecalis: a mechanism for its role in endodontic failure. *International Endodontic Journal* **34**, 399-405.

McHugh CP, Zhang P, Michalek S, Eleazer PD (2004) pH required to kill Enterococcus faecalis in vitro. *Journal of Endodontics* **30**, 218-9.

Menezes MM, Valera MC, Jorge AOC, Koga-Ito CY, Camargo CHR, Mancini MNG (2004a) In vitro evaluation of the effectiveness of irrigants and intracanal medicaments on microorganisms within root canals. *International Endodontic Journal* **37**, 311-9.

Menezes R, Bramante CM, Letra A, Carvalho VG, Garcia RB (2004b) Histologic evaluation of pulpotomies in dog using two types of mineral trioxide aggregate

and regular and white Portland cements as wound dressings. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **98**, 376-9.

Nair PN, Henry S, Cano V, Vera J (2005) Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **99**, 231-52.

Nawal RR, Parande M, Sehgal R, Naik A, Rao NR (2011) A comparative evaluation of antimicrobial efficacy and flow properties for Epiphany, Guttaflow and AH-Plus sealer. *International Endodontic Journal* **44**, 307-13.

Orosco FA, Bramante CM, Garcia RB, Bernadineli N, Moraes IG (2008) Sealing ability of gray MTA Angelus™, CPM™ and MBPc used as apical plugs. *Journal of Applied Oral Science* **16**, 50-4.

Peters LB, Wesselink PR, Bujis JF, Van Winkelhoff AJ (2001) Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *Journal of Endodontics* **27**, 76-81.

Pinheiro CR, Guinesi AS, Pizzolitto AC, Bonetti-Filho I (2009) In vitro antimicrobial activity of Acroseal, Polifil and Epiphany against Enterococcus faecalis. *Brazilian Dental Journal* **20**, 107-11.

Ribeiro CS, Scelza MF, Hirata Júnior H, Oliveira LMB (2010) The antimicrobial activity of gray-colored mineral trioxide aggregate (GMTA) and white-colored MTA (WMTA) under aerobic and anaerobic conditions. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **109**, e109-12.

Roberts HW, Toth JM, Berzins DW, Charton DG (2008) Mineral trioxide aggregate material use in endodontic treatment: a review of the literature. *Dental Materials* **24**, 149-64.

Scarparo RK, Haddad H, Acasigua GA, Fossati ACM, Fachin EVF, Grecca FS (2010) Mineral trioxide aggregate-based sealer: analysis of tissue reactions to a new endodontic material. *Journal of Endodontics* **36**, 1174-8.

Siqueira JF Jr, Roças IN (2008) Clinical implications and microbiology of bacterial persistence after treatment procedures. *Journal of Endodontics* **34**, 1291-301.

Sjögren U, Figdor D, Persson S, Sundqvist G (1997) Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *International Endodontic Journal* **30**, 297-306.

Spangberg L, Engstrom B, Langeland K (1973) Biologic effects of dental materials. Toxicity and antimicrobial effect of endodontic antiseptics in vitro. *Oral Surgery, Oral Medicine, and Oral Pathology* **36**, 856-71.

Stuart CH, Schwartz AS, Beeson TJ, Owatz CB (2006) Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. *Journal of Endodontics* **32**, 93-8.

Tanomaru JMG, Tanomaru-Filho M, Hotta J, Watanabe E, Ito IY (2008) Antimicrobial activity of endodontic sealers based on calcium hydroxide and MTA. *Acta Odontologica Latinoamericana* **21,** 147-51.

Tobias RS (1988) Antibacterial properties of dental restorative materials: a review. *International Endodontic Journal* **21**, 155-60.

Torabinejad M, Chivian N (1999) Clinical applications of mineral trioxide aggregate. *Journal of Endodontics* **25**, 197-205.

Torabinejad M, Hong CU, Pitt Ford TR, Kettering JD (1995) Antibacterial effects of some root end fillings materials. *Journal of Endodontics* **21**, 403-6.

Torabinejad M, Watson TF, Pitt Ford TR (1993) Sealing ability of a mineral trioxide aggregate when used as a root end filling material. *Journal of Endodontics* **19**, 591-5.

Weiss E, Shahlav M, Fuss Z (1996) Assessment of antimicrobial activity of endodontic sealers by a direct contact test. *Endodontics & Dental Traumatology* **12**, 179-84.

Zehnder M, Guggenheim B (2009) The mysterious appearance of enterococci in filled root canals. *International Endodontic Journal* **42**, 277-87.

Zhang H, Pappen FG, Haapasalo M (2009) Dentin enhances the antibacterial effect of Mineral Trioxide Aggregate and BioAggregate. *Journal of Endodontics* **35**, 221-4.

Figures:

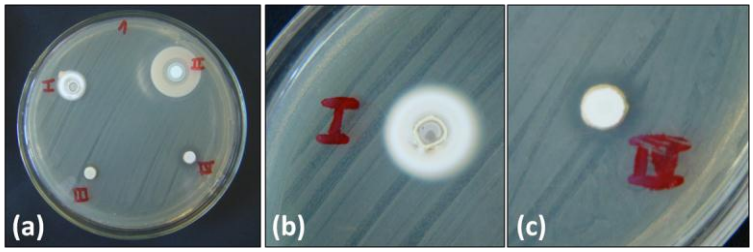

Figure 1 Agar diffusion test: (a) Diffusion of White MTA (I) and Endo CPM Sealer (II) and inhibition zones for MTA Fillapex (III) and Endofill (IV); (b) Diffusion of White MTA (I), increased image ; (c) Inhibition zone for Endofill (IV), increased image

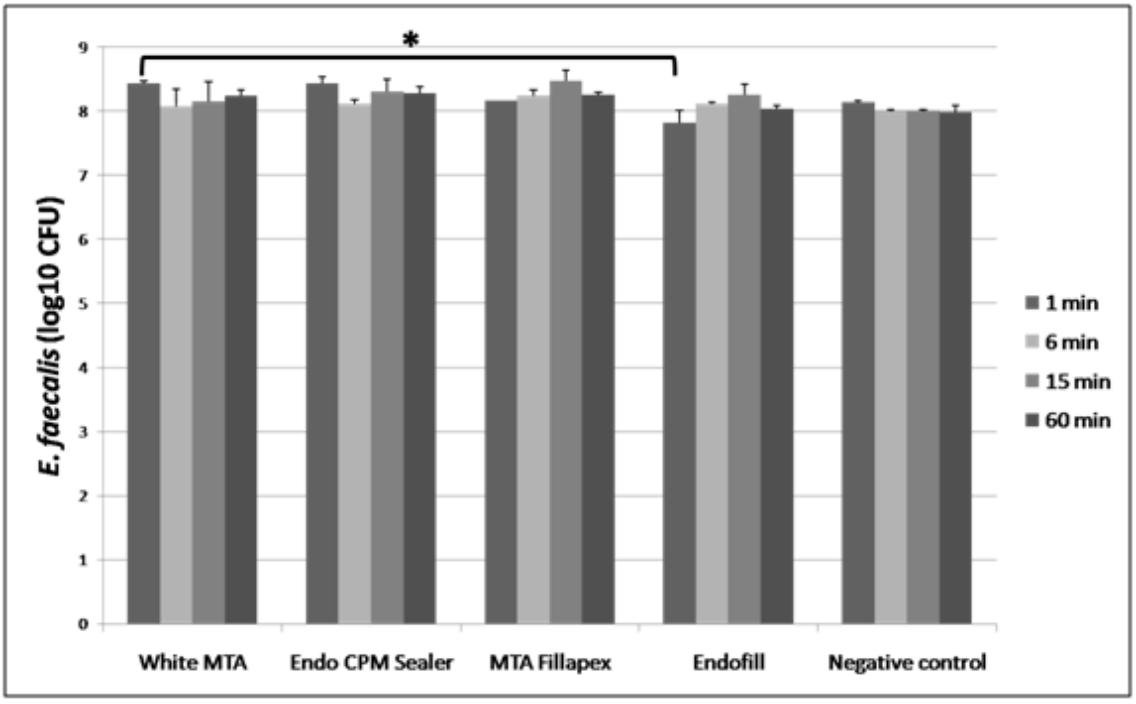

Figure 2 Survival of *E. faecalis* after incubation with sealers suspensions at different experimental periods in the DCT

Table 1 Tested materials and their composition

Materials	Composition	Manufacturer
White MTA	Powder: tricalcium silicate, tricalcium oxide, tricalcium aluminate, and other oxides Líquid: distilled water	Angelus, Londrina, PR, Brazil
Endo CPM Sealer	Powder: tricalcium silicate, tricalcium oxide, tricalcium aluminate, and other oxides Liquid: saline solution and calcium chloride	EGEO, Buenos Aires, Argentina
MTA Fillapex	Salicylate resin, diluting resin, natural resin, bismuth trioxide, nanoparticulated silica, MTA, pigments	Angelus, Londrina, PR, Brazil
Endofill (positive control)	Powder: zinc oxide, hydrogenated resin, bismuth subcarbonate, barium sulfate, sodium borate Liquid: eugenol, sweet almond oil	Dentsply, Petrópolis, RJ, Brazil

Table 2 Mean values (in mm), standard deviation and the ranks average of the bacterial inhibition zones for each sealer in the ADT

Materials	Inhibition zones	Ranks average
White MTA	0 ± 0	10.50[a]
Endo CPM Sealer	0 ± 0	10.50[a]
MTA Fillapex	7.32 ± 0.33	25.50[b]
Endofill (positive control)	9.55 ± 0.19	35.50[b]

[a, b] Different small letters indicate statistically significant difference between sealers according to the Dunn test ($P<0.05$).

Table 3 Mean values of pH for each sealer at different experimental periods

Time	White MTA	Endo CPM Sealer	MTA Fillapex	Endofill	Control
1 min	11.64 ± 0.17	11.39 ± 0.31	10.49 ± 0.07	8.22 ± 0.29	6.33 ± 0.04
6 min	11.83 ± 0.18	11.23 ± 0.12	10.5 ± 0.02	8.56 ± 0.26	7.82 ± 0.42
15 min	11.84 ± 0.2	11.36 ± 0.24	10.46 ± 0.02	8.63 ± 0.35	7.79 ± 0.54
60 min	11.84 ± 0.2	11.19 ± 0.09	10.14 ± 0.21	8.27 ± 0.54	7.07 ± 0.37