

CLSM ASSESSMENT OF TUBULE PENETRATION AND BACTERIAL LEAKAGE EVALUATION OF TWO RESIN-BASED SEALER

ABSTRACT

AIM: The aims of this study were to assess the penetration of two endodontic sealers (salicylate and epoxy resin-based sealers) into dentinal tubules using CLSM; and to evaluate the bacterial leakage of roots filled with the same sealers associated with gutta-percha. **MATERIAL AND METHODS:** For sealer penetrability assessment, thirty bovine roots were instrumented and divided into three groups: AHP: EDTA + filling with AH Plus and gutta-percha (n=10), MTAF: EDTA + filling with MTA Fillapex and gutta-percha (n=10), control group: canals were not irrigated with EDTA and were filled with gutta-percha and AH Plus (n=5) or MTA Fillapex (n=5). Rhodamine B was added to the sealers in order to provide adequate fluorescence. The roots were transversely sectioned 3mm from the apex to enable CLSM analysis. Leakage was evaluated for turbidity of the broth in a split chamber model system for 30 days, using *Enterococcus faecalis* as a microbial marker. Thirty roots were instrumented and divided in four grupos: AHP: filling with AH Plus and gutta-percha (n=10); MTAF: filling with MTA Fillapex and gutta-percha (n=10); positive control: filling with gutta-percha without sealer (n = 5); negative control: sealing with cyanoacrylate to test the seal of the system (n = 5). **RESULTS:** The medians for dentinal tubule penetration were 6.8% (AHP) and 6.6% (MTAF) (P = 0.82). The average time for bacterial leakage was 8 days in both experimental groups (P = 0.79). **CONCLUSION:** MTA Fillapex and AH Plus presented similar behavior regarding dentinal tubule penetration and bacterial leakage.

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INTRODUCTION

A successful endodontic therapy is strongly associated with adequate chemomechanical preparation, as well the tridimensional filling of the root canal system. Endodontic sealer's creates a physical barrier to avoid bacterial and oral and tissue fluids leakage, contributing to root canal treatment failure.¹

Gutta-percha is not capable to provide adequate sealing of the apical and coronal portion of the roots.¹ Therefore, an endodontic sealer must fill the root canal system laterally and apically because gutta-percha is incapable to fill accessory and lateral canals. Endodontic sealer must also have great adaptation to the root canal walls, to attach accessory and main gutta-percha cones, and to adhere the filling mass to dentin walls mechanically.²

Entombing viable microorganisms within the dentinal tubules by isolating them from potential nutrient sources has been reported in the current literature.² Previous reports have discussed the importance of sealer penetration into dentinal tubules.³⁻⁵ However, recent studies have indicated no correlation between their dentine tubule penetration and sealing ability.³

Epoxy resin-based sealers have greater dimensional stability, low rates of solubility, great radiopacity⁶ and optimal adhesiveness to the root dentin than others endodontic sealers.⁷ Salicylate resin -based sealers has

biocompatibility,⁸ antibacterial activity,⁹ adhesiveness,¹⁰ and solubility.¹¹ These sealers present adequate radiopacity, easy handling, great working time, and expansion during setting.

The aims of this study were: to compare the penetration patterns of a salicylate and an epoxy resin-based sealers into dentinal tubules through CLSM (Confocal Laser Scanning Microscopy), and to evaluate the apical bacterial leakage on teeth filled with these sealers and gutta-percha. The experimental hypotheses were: 1 - that the epoxy resin-based sealer will present higher dentinal tubule penetration and, 2 - lower apical bacterial leakage.

MATERIAL AND METHODS

Sixty bovine teeth were selected for sealer penetrability (n = 30) and bacterial leakage (n = 30) assessment. The crowns were removed and the root length was standardized (15 mm).

The working length was established as being 1 mm shorter than the apical foramen. Canals with apical diameter of less than 0.25 mm were excluded from this study. Root canal preparation was performed by the same operator (R. B.) using hand files up to the #40 K-file (Dentsply Maillefer, Ballaigues, Switzerland). Between each instrument change, all specimens were irrigated with 2mL

of 2.5% sodium hypochlorite (Farmácia Marcela, Porto Alegre, RS, Brazil).

SEALER PENETRABILITY ASSESSMENT:

Thirty roots were randomly divided in three groups: (1) AHP – canals were irrigated with 2 mL of 17% EDTA (Farmácia Marcela) for smear layer removal and were filled with AH Plus (Dentsply-Maillefer) (n = 10); (2) MTAF – canals were irrigated with EDTA (Farmácia Marcela) and were filled with MTA Fillapex (Angelus, Londrina, PR, Brazil) (n = 10); (3) CG (control group) – canals were not irrigated with EDTA (Farmácia Marcela) and were filled with AH Plus (n = 5) and MTA Fillapex (n = 5).

The root canals were filled with a #40 gutta-percha master cone (Tanariman Industrial Ltda., Manacapuru, AM, Brazil) and B7 accessory gutta-percha cones (Tanariman Industrial Ltda.) using the lateral compaction technique. Rhodamine B dye in a ratio of 0.1% (4) was added to the sealers in order to provide adequate fluorescence to enable the CLSM assessment.

For CLSM assessment, the apical 3 mm were excluded and 2 mm-thick slices were obtained using a double-sided diamond disc under water-cooling in a cutting machine (Extec Labcut 1010, Enfield, USA). Next, surfaces were polished with Arotec paste (Arotec, Cotia, SP, Brazil) in order to remove

dentin debris eventually generated during the cutting procedures.

Slices were assessed from coronal to the apex surfaces with the Olympus FluoView Confocal Laser 1000 Microscope (Olympus Corporation, Tokyo, Japan). The respective absorption and emission wavelengths for rhodamine B was 540/590 nm. Dentin samples were analyzed using the ×10 lens. The sealer penetration area into the dentinal tubules was measured using Adobe Photoshop CS6 (Adobe Systems, San Jose, USA) as described by Kok et al.⁵

BACTERIAL LEAKAGE ASSESSMENT:

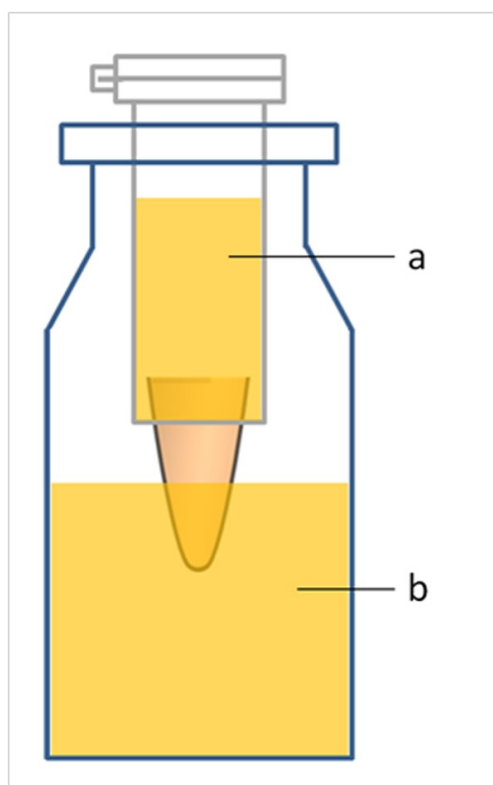
Thirty roots were randomly divided into four groups: (1) AHP – roots filled with AH Plus (Dentsply-Maillefer) (n=10); (2) MTAF – roots filled with MTA Fillapex (Angelus) (n=10); (3) PCG (positive control group) – root canals were filled with gutta-percha and no sealer (n = 5); (4) NCG (negative control group) – roots were filled and completely sealed with cyanoacrylate to guarantee the complete seal between the chambers, avoiding pathways of communication (n = 5).

The external surfaces of AHP, MTAF, and PCG samples were covered with two layers of nail polish (L'Oréal Brasil Comercial de Cosméticos Ltda., Rio de Janeiro, RJ, Brazil), except for the area 1 mm around the apical foramen. Specimens from NCG were completely covered with two layers of nail

polish (L'Oréal Brasil Comercial de Cosméticos Ltda.).

Specimens were then mounted to an apparatus with polypropylene tubes (Eppendorf do Brasil Ltda, São Paulo, SP, Brazil) (upper chamber) and 10 mL glass flasks (lower chamber) (Figure 1). The coronal portion of the root was adapted in the tube with epoxy resin (Durepoxi; Henkel Ltda, Itapevi, SP, Brazil). Two layers of cyanoacrylate were applied on the interface teeth/epoxy resin and epoxy resin/ tube to prevent bacterial penetration through these interfaces. Each chamber was placed in 10 mL glass flasks. The tube/ root set and the glass flask was sterilized separately by ethylene oxide gas.

Figure 1. Apparatus with polypropylene tubes and glass flasks. (a) Upper chamber with 500 μ L of the BHI with *E. faecalis* inoculum. (b) Lower chamber with fresh BHI broth.



For the coronal leakage assays, a standard strain of *E. faecalis* (ATCC 29212) was employed. The *E. faecalis* cells were suspended in BHI broth to and adjusted in a spectrophotometer (absorbance equal to 0.032, and 800 nm wavelength) to match the turbidity of a 0.5 McFarland scale. In a laminar flow cabinet, the glass flasks (lower chamber) were filled with a quantity of BHI (Brain Heart Infusion, Difco Laboratories-Becton Dickinson and Company, Franklin Lakes, USA) enough to allow the immersion of the apical portion of the roots. In order to assess the bacterial leakage, 1 mL of the inoculum was transferred to the inner portion of the upper chamber, in direct contact to the coronal portion of the root canal filling. The interface of the tubes with the glass flasks was sealed with a film (Parafilm M; Saint Louis, USA).

The apparatus was incubated at 37°C in a CO₂ chamber. During the experimental period, 500 μ L of the BHI with *E. faecalis* inoculum was replaced every 48h with a new 500 μ L aliquot of sterile BHI. Leakage was assessed by checking the turbidity of the BHI broth in the glass flasks until the last apparatus presented turbidity of the BHI. Purity of growth was checked by cultivation of 100 μ L of BHI broth with 5% of defibrinated sheep blood (Newprov Produtos para Laboratório, Pinhais, PR, Brazil). Purity of the cultures were checked by colonies morphology and cellular characteristics, by using a phase-contrast

microscope (Leitz Dialux 22, Leica, Basel, Switzerland) at $\times 1000$ magnification. Additionally, Gram stain test, catalase test were used to confirm the purity of the growth.

The statistical analysis was performed by using the software BioEstat 5.3 (Instituto de Desenvolvimento Sustentável Mamirauá, Belém, PA, Brazil). The sealer penetration area and the bacterial leakage were assessed using Kruskal-Wallis and Mann-Whitney tests, respectively. For all statistical tests the level of significance was set at 5%.

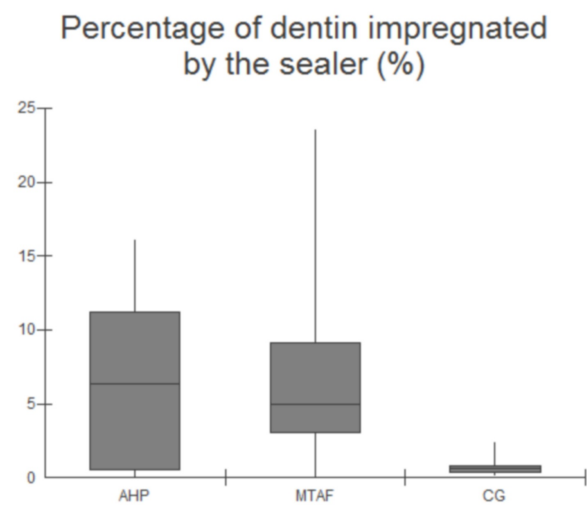
RESULTS

There was no difference between the percentage of sealer penetration into dentinal tubules for the specimens from AHP and MTAF groups ($P > 0.05$). The median of percentage of dentin impregnated by the sealer was 6.8% and 6.6% for the AHP and MTAF groups, respectively (Figure 2). Lower values for sealer impregnation were observed in the CG (equal to 0.6%) when compared to the test groups ($P < 0.05$). Figure 3 shows CLSM images of sealer penetration into dentin tubules in each experimental group.

Regarding the bacterial leakage, the specimens from MTAF group had similar behavior in comparison to those from AHP group ($P > 0.05$). The median number of days for turbidity of the BHI was 8 days for both groups. The minimum and maximum period of leakage in AHP and MTAF groups were 3 and

30 days. Specimens from PCG presented turbidity at the first assessment (24 h after the beginning of the assay), while the NCG presented absence of turbidity at the end of the experiment (i.e. 30 days). Figure 4 represents bacterial leakage for AHP and MTAF groups.

Figure 2 - Box-plot presenting median, 25th percentile, 75th percentile, minimal and maximal values of percentage of dentin impregnated by the sealers.



DISCUSSION

The apical third is the most critical portion of the root canal for cleaning and shaping procedures.¹² The anatomical complexity of the apical third combined with the limited access of the endodontic instruments and irrigants to this region contributes to the difficulty of debris, smear layer, and microorganisms removal. Therefore, the apical third was chosen to assess the sealer penetrability into the dentin.

Wu and Wesselink¹³ showed that regardless of the technique used for root canal preparation, the apical third presented greater

amount of debris when compared with middle and cervical third. The enlargement of the apical third up to #40 K-file was performed in order to achieve satisfactory cleaning of this region and promote great adaptation of the filling material. Fornari et al.¹⁴ stated that great enlargement of the apical third must lead to a

better cleanness of this region. Additionally, according to Brunson et al.¹⁵ an apical enlargement to ISO #40 with a 0.04 taper will allow for tooth structure preservation and maximum volume of irrigation at the apical third.

Figure 3 - CLSM ($\times 10$) of sealer penetration into dentin tubules of the MTA (A), AHP (B) and CG (C) groups.

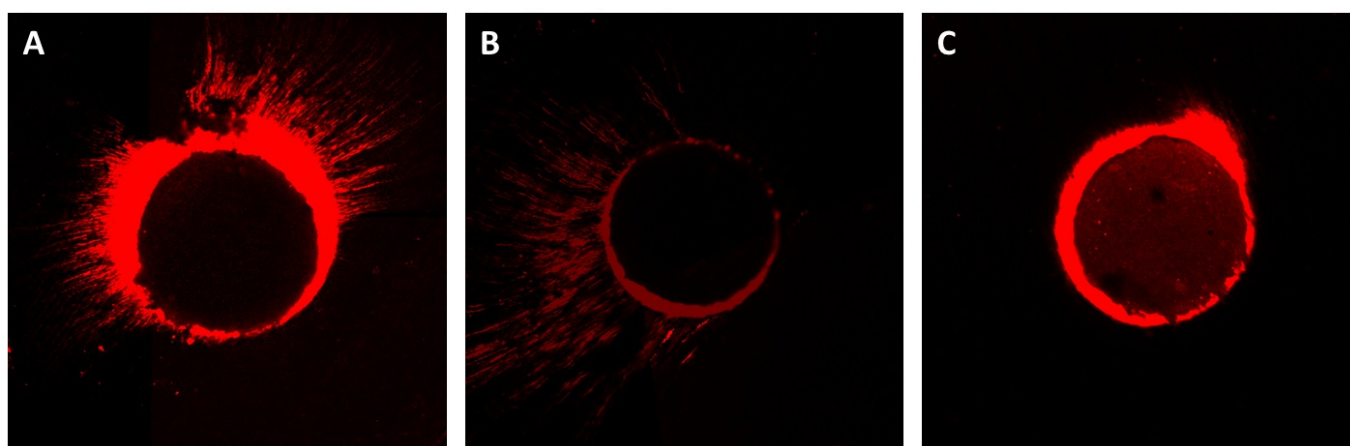
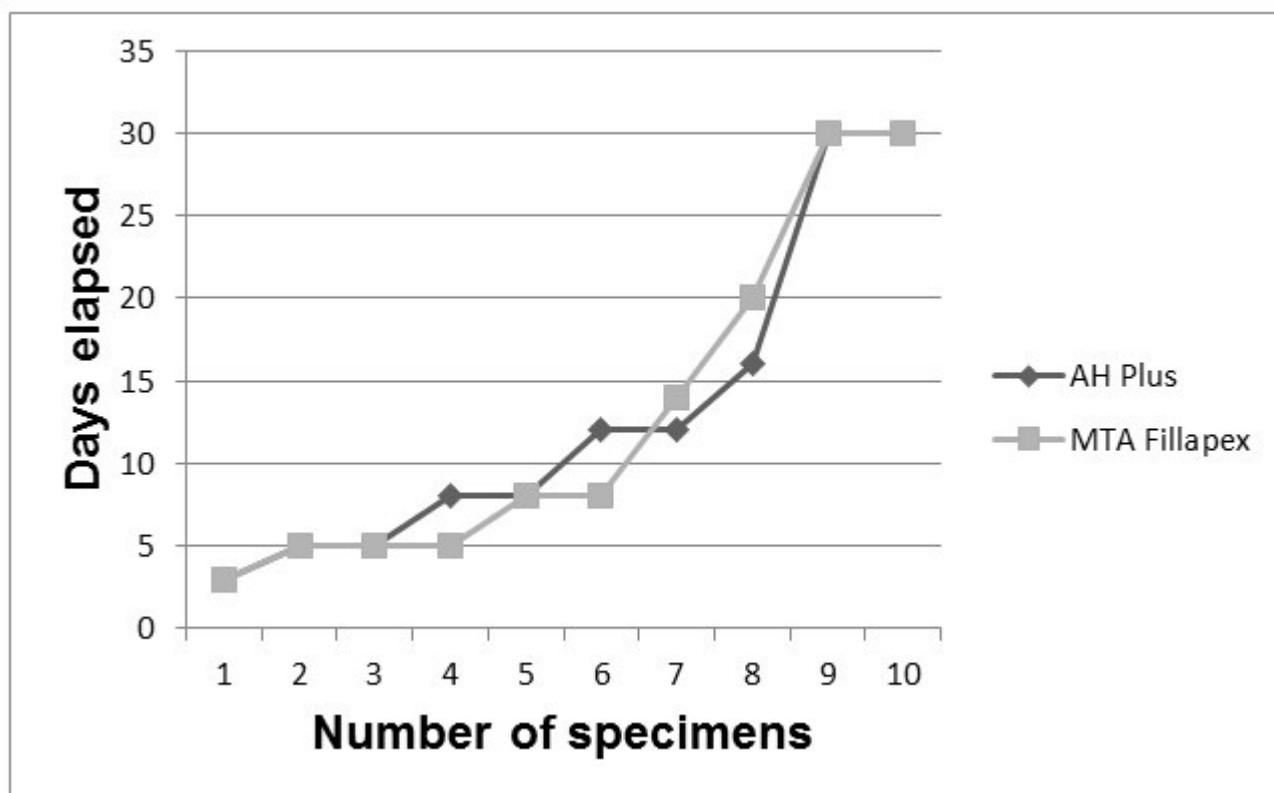


Figure 4 - Graph representation of bacterial leakage for the two experimental groups.



In this study, the root canals were filled using lateral compaction technique, a widespread technique. In addition, Kok et al.⁴ showed that different filling techniques promote similar sealer penetration into dentinal tubules when an epoxy resin -based sealer was used for root canal filling.

Previous studies have reported differences regarding the flow properties of endodontic sealers.^{16,17} The flowability of an endodontic sealer consists in the ability to penetrate into the irregularities and accessory canals of the root canal system.¹⁸ Differences in flowability among various endodontic sealers may be partially explained by their different chemical compositions. In this study, the similarity observed between tubule penetration presented by AHP and MTA-F can be explained by the fact that both sealers are resinous. Although MTA Fillapex presents MTA on its composition, there is a predominance of resin components (i.e. salicylate resin). MTA Fillapex and AH Plus have flow values above those required by ISO 6876:2001.^{16,17} Silva et al.¹⁶ observed better flowing of MTA Fillapex in comparison with AH Plus. On the other hand, Vitti et al.¹⁷ showed opposite results. Despite the absence of consensus in previous studies for the flow tests, the results of the present study demonstrated that MTA Fillapex and AH Plus had similar tubule penetration into the dentin tubules ($P > 0.05$), similar to those

results obtained by Kok et al.⁵ So, the first experimental hypothesis was rejected.

CLSM uses high contrast points to identify sealer distribution within dentinal tubules.⁴ When associated with epifluorescence, this methodology allows analysis of sealer distribution into dentinal tubules and its adaptation to the root canal walls. However, SEM (Scanning Electron Microscopy) allows viewing only a single plane and requires sample preparation techniques that could promote artifacts and sample distortions.¹⁹

Rhodamine B was used to promote fluorescence and allow CLSM viewing of the sealer penetration into dentinal tubules. A possible influence of the dye on endodontic sealers' chemical-mechanical properties was discarded for previous studies due the small amount of the dye (0.1%).⁴

Both groups presented similar sealer penetration, however it was not homogeneous along the entire root canal perimeter. Some portions presented deep penetration, while others were not impregnated by the endodontic sealer. Two aspects regarding these results must be highlighted. First, the action of EDTA might have been insufficient, and then, the dentinal tubules remained covered by smear layer and the sealers were not able to penetrate within them. Second, root canal walls that were not touched by the endodontic instruments (i.e. isthmuses and

flattening) and may lead some areas with dentinal tubules open, which would favor the sealer flow.

Regarding the bacterial leakage test, the second hypothesis was rejected because both AHP and MTAF groups had an equal median for the number of days until the turbidity of the BHI broth could be observed ($P > 0.05$). Two specimens from AHP and MTAF groups presented turbidity of the BHI broth only at the thirtieth day. These specimens were those that spent more time without presenting turbidity. Leakages that occur in short periods must be considered as a potential factor that might lead to endodontic failures.²⁰

Several methodologies have been employed to assess the sealing ability of filling materials and root-end filling materials such as bacterial leakage,²¹ glucose leakage,³ saliva leakage,²² some markers such as silver nitrate,²³ and dyes such as methylene blue,²⁴ and fluid transportation model.²⁵ Results of these methodologies have been unanimous in affirm that there is no technique or filling material was able to maintain adequate sealing for long periods of time.

Bacterial leakage methods are closer to the clinical reality when filling material is exposed directly to oral fluids than those that use markers of fluid transportation.¹³ However, bacterial leakage methods can't measure the amount of microorganisms that colonize the interface between filling material

and root canal wall, reaching the root apex and the periapical tissues.

The effectiveness of the method used in the present study was confirmed using the control groups. The PCG confirmed the need of an endodontic sealer to limit bacterial leakage and to cover the main gutta-percha to the accessory gutta-percha cones. All specimens of PCG group promoted turbidity of the BHI broth after 24h of the beginning of the experiment. The NCG was used to confirm the sealing between at the interfaces upper chamber/root and lower chamber/upper chamber. At the end of thirty days the BHI broth remained unchanged, that indicates that the sealing was adequate and, in experimental groups, the turbidity was caused by the leakage through the filling material. Previous studies have reported great variability on the time required for leakage, which may range of few days to three months according to the methodology used for assessment.²²

De-Deus et al.³ recently stated that there is no correlation between sealer penetrability into dentinal tubules and sealing ability. The present study did not aimed to establish any correlation between sealer penetrability into dentinal tubules and sealing ability of MTA Fillapex and AH Plus. It aimed to assess the behavior of both sealers when assessed by experimental protocols. Additionally, as two experimental groups were determined, the attempt to establish a correlation between

methodologies did not seem to be correct in a statistical standpoint.³ It can be suggested that when the aim of the study is to find some correlation between two methodologies, only one experimental group with high number of samples should be used.

The results of this study do not allow making any association between the sealer penetration into dentinal tubules and the sealing ability of endodontic fillings with MTA Fillapex and AH Plus and gutta-percha. However, it must be highlighted that a massive penetration of endodontic sealers within dentinal tubules would promote the formation of a physical barrier within the tubules and can isolate microorganisms from nutritional sources.

CONCLUSION

In conclusion, MTA Fillapex presents similar behavior to AH Plus regarding sealer penetration into dentinal tubules and similar capacity to avoid bacterial leakage when associated with gutta-percha for root canal fillings of monoradicular teeth.

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