UNIVERSITY OF BIRMINGHAM
University of Birmingham Research at Birmingham

The presence of smear layer affects the antimicrobial action of root canal sealers

Zancan, R F; Di Maio, A; Tomson, P L; Duarte, M A H; Camilleri, J

DOI:
10.1111/iej.13522

License:
Other (please specify with Rights Statement)

Document Version
Peer reviewed version

Citation for published version (Harvard):
Zancan, RF, Di Maio, A, Tomson, PL, Duarte, MAH & Camilleri, J 2021, 'The presence of smear layer affects the antimicrobial action of root canal sealers', *International Endodontic Journal*, vol. 54, no. 8, pp. 1369-1382. https://doi.org/10.1111/iej.13522

Link to publication on Research at Birmingham portal

Publisher Rights Statement:
This is the peer reviewed version of the following article: Zancan, RF, Di Maio, A, Tomson, PL, Duarte, MAH, Camilleri, J. The presence of smear layer affects the antimicrobial action of root canal sealers. International Endodontic Journal, which has been published in final form at: https://doi.org/10.1111/iej.13522. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

General rights
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.
•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
•User may use extracts from the document in line with the concept of ‘fair dealing’ under the Copyright, Designs and Patents Act 1988 (?)
•Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 27. Apr. 2024

The presence of smear-layer affects the antimicrobial action of root canal sealers

Journal:	*International Endodontic Journal*
Manuscript ID	IEJ-20-00776.R3
Wiley - Manuscript Type:	Original Scientific Article
Keywords:	antimicrobial activity, AH Plus, BioRoot, MTA Fillapex, smear layer, TotalFill BC sealer

SCHOLARONE™
Manuscripts

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The presence of smear-layer affects the antimicrobial action of root canal sealers

[1]Zancan RF, [2]Di Maio A, [3]Tomson PL, [1]Duarte MAH, [3]Camilleri J

[1] Bauru School of Dentistry, Department of Restorative Dentistry, Dental Materials and Endodontics, University of São Paulo, Bauru, São Paulo, Brazil; [2]School of Biosciences, University of Birmingham, Birmingham, U.K, [3]School of Dentistry, College of Medical and Dental Sciences, University of Birmingham, Birmingham, U.K

Key words: antimicrobial activity, AH Plus, BioRoot, MTA Fillapex, smear layer, TotalFill BC Sealer.

Running title: Smear-layer on dentine affects sealer properties

Acknowledgement

The authors thank Dr. Sarah Kuehne for reviewing the antimicrobial testing protocol and Chuta Kooanantkul for his constant and valuable support. This study was supported by the State of São Paulo Research Foundation, FAPESP (2018/03554-0).

Correspondence

Dr J. Camilleri, Institute of Clinical Sciences, College of Medical and Dental Sciences, School of Dentistry, 5, Mill Pool Way, Edgbaston, Birmingham, B5 7EG, U.K. J.Camilleri@bham.ac.uk

For Peer Review

ABSTRACT

Aim To assess the chemical and microstructural characteristics of dentine after the use of two irrigation protocols and correlate this with the antimicrobial properties of HCSC and changes to the dentine structure / chemistry after sealer placement.

Methodology Two irrigation protocols - Protocol A using 2% NaOCl used ~~in~~ 5mL/5 min and Protocol B with 2% NaOCl (5mL/5 min) followed by 17% EDTA (5mL/3 min) were used to prepare dentine. The chemical and microstructural changes following irrigation were assessed by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and Fourier transform infrared (FT-IR) spectroscopy (n=5) on dentine obtained from the mid-root and coronal parts of extracted human teeth. Four sealers (AH Plus, BioRoot, MTA Fillapex, TotalFill) were characterized by SEM/EDS (n=3). The ability of the sealers to eradicate intratubular *Enterococcus faecalis* biofilms was assessed by live/dead dye and confocal laser scanning microscopy to measure the percentage of living cells. The effect of combined irrigation and root filling on the dentine was assessed by SEM and EDS analysis (n=5). Statistical analysis was undertaken using one-way ANOVA and a number of post hoc tests to detect intergroup differences. The F test was used for comparison of variances in the microbiology testing.

Results The use of NaOCl alone left the smear layer seemingly intact, with traces of chlorine remaining on dentine. The use of BioRoot sealer restored the calcium ion levels of dentine which are depleted by the irrigation with EDTA. BioRoot exhibited antimicrobial properties against intratubular bacteria even in the presence of smear layer (Protocol A). The smear layer removal improved the bactericidal effect of all sealers and Ca^{2+} leaching. The use of a chelating agent was important for the intratubular sealer penetration for AH Plus but not the other sealers.

Conclusion The removal of smear layer was associated with greater penetration of AH Plus into the dentinal tubules but not for the penetration of HCSC sealers. ~~removal of smear layer~~

For Peer Review

~~is necessary for AH Plus but not for the HCSC sealers.~~ BioRoot was a more effective sealer in reducing the bacterial load in the dentinal tubules than the other materials tested and the presence of smear layer did not affect its activity.

For Peer Review

Introduction

The use of sodium hypochlorite (NaOCl) as a root canal irrigant provides broad spectrum antimicrobial action and dissolution of necrotic tissue (Zehnder 2006, Wong & Cheung 2014). Bacteria can spread to a depth of approximately >500 µm into the dentinal tubules (Ghorbanzadeh *et al.* 2016), while penetration of NaOCl might be limited to the first 77 to 300 µm layer (Vahdaty *et al.* 1993, Zou *et al.* 2010, Ghorbanzadeh *et al.* 2016) and also have more limited penetration in the apical parts of the root canal (Virdee *et al.* 2020). Furthermore, the complexity of the root canal system also results in areas that are inaccessible to disinfection by NaOCl.

The use of calcium chelators such as ethylene diamine tetracetic acid (EDTA) for smear layer removal enables bacteria that might be trapped within dentinal tubules to be more accessible to ~~the~~ irrigants. The bacteria remaining in the dentinal tubules are either removed by the further antimicrobial action of NaOCl or are entombed by sealer penetration. Although AH Plus possess low antimicrobial properties (Bailon-Sanchez *et al.* 2014, Arias-Moliz & Camilleri 2016) it bonds to collagen (Neelakantan *et al.* 2015) which is exposed by the chelating action of EDTA and has been reported to provide an adequate dentine seal (Viapiana *et al.* 2016).

For Peer Review

Newer root filling techniques such as the use of single cones are more dependent on sealer properties since the root filling has a high proportion of sealer. Furthermore, the wide range of chemical compositions of different types of sealers require distinct dentine pretreatments for optimal performance (Saleh 2002). Unlike resin-based sealers, the hydraulic calcium silicate cement (HCSC) sealers do not rely on the chelation and widening of dentinal tubules to produce a good dentine seal (Zancan *et al.* 2021).

HCSCs set through a hydration reaction forming calcium hydroxide (Camilleri 2017, Camilleri *et al.* 2013) which is responsible for their antimicrobial activity (Arias-Moliz & Camilleri 2016, Koutroulis *et al.* 2019) and chemical bond to dentine (Tay *et al.* 2007, Reyes-Carmona *et al.* 2009). The moisture present in the smear layer is required to enhance the interaction of the

HCSC with their surroundings resulting in the improvement of the biological properties of the materials (Yildirim *et al.* 2008, 2010). EDTA's ability to chelate calcium ions has been shown to disrupt HCSC hydration (Lee *et al.* 2007) compromising their hardness, flexural and bond strength (Yan *et al.* 2006, Yildirim *et al.* 2010, Aggarwal *et al.* 2011). The interaction of the HCSC with the dentine and their enhanced antimicrobial characteristics following hydration has led to a paradigm shift in the use of chelators prior to root canal filling.

HCSCs are affected by variations in pH, temperature and humidity (Prati & Gandolfi 2015, Khalil *et al.* 2016), therefore experimental models should be chosen with care. Agar diffusion tests (ADTs), direct contact tests (DCTs) and dentine block models are commonly employed to assess the antimicrobial activity of endodontic sealers (Zhang *et al.* 2009b, Andrade *et al.* 2015, Arias-Moliz & Camilleri 2016, Candeiro *et al.* 2016). The buffering capacity of the agar plate and its possible interaction with the disinfecting agents invalidate ADTs (Editorial-Board 2007). Despite being a widely accepted technique, it`s substitute DCTs (Editorial-Board 2007) may affect the chemistry and hydration of HCSCs by bacteriological media. The use of dentine block models provides a reliable method to assess the complex interactions between dentine, biofilm and sealer (Wang *et al.* 2014).

For Peer Review

To date the effect of the irrigation protocol used prior to root filling has not been investigated in depth. The antimicrobial activity of HCSC sealers is well established and several studies have evaluated the effect of the final irrigant on properties of the sealer (Lee *et al.* 2007, Arias Moliz & Camilleri 2016). However, no studies have evaluated the effect of the disinfection and smear layer removal protocols on the antimicrobial properties of HCSC sealers and the best irrigation protocol that enhances the sealer properties and also how sealers with a different chemistry interact with dentine after placement.

The null hypotheses tested are that: 1. irrigated dentine does not influence the antimicrobial activity of ~~the~~ HCSC and epoxy resin sealers against *Enterococcus faecalis* (*E. faecalis*) intratubular infection in dentine, taking into consideration the presence of smear layer for the non-chelator protocol; 2. sealers with a different chemistry interact in the same way with

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

dentine; 3. dentine modified by the irrigation protocols interacts with sealers in the same way as unmodified dentine.

Material and Methods

The following irrigation protocols were used:

- Protocol A: 2% NaOCl (Cerkamed, Stalowa Wola, Poland) - 5 mL/5 minutes;
- Protocol B: 2% NaOCl - 5 mL/5 minutes - followed by 17% EDTA (Sigma, Stoinhem, Germany) – 5 mL/3 minutes.

Microstructural and chemical analysis of the dentine

For Peer Review

Extracted human maxillary incisors were obtained from the Birmingham Dental School Tissue Bank – School of Dentistry, University of Birmingham, Birmingham, U.K. (ethical approval number: 14/EM/2811- BCHCDent397), embedded in auto polymerizing epoxy resin (Epoxyfix; Struers GmbH, Ballerup, Denmark) and sectioned through their root long axis using a hard tissue microtome (Isomet, Buhler, Lake Buff, IL, USA). The resulting halves were then ground using progressively finer diamond discs (Stuers ApS, Ballerup, Denmark) and diamond-impregnated solutions (Stuers ApS) on an automatic polishing machine (Buehler Phoenix Beta Grinder/Polisher, Dusseldorf, Germany), finishing with a silicon suspension of 1 µm.

The dentine specimens were treated using the chemical irrigation Protocols A or B, after which they were dried in a vacuum desiccator, attached to aluminum stubs and sputter coated with a conductive layer of gold using a TK8842 Gold Target (Emitech Limited, Ashford, U.K.). The smear layer removal on the dentine ultrastructure after each stage of the irrigation protocol was assessed by scanning electron microscopy (SEM; Zeiss MERLIN Field Emission SEM; Carl Zeiss NTS GmbH, Oberkochen, Germany) at 2K× magnification (*n* = *5*). For Energy Dispersive Spectroscopy (EDS) analyses, the following parameters were used, EHT= 20 kV, Iprobe = 1000 pA and WD= 8.5 mm for a 35° take off (elevation angle). Alterations to the dentine surfaces by the different irrigation protocols were measured by monitoring changes in the calcium, phosphorus and chlorine.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Fourier transform infrared spectroscopy (FT-IR) was used to determine compositional changes of human dentine ($n = 5$) after each irrigation protocol. The mean of three separate acquisitions of spectra data was obtained for each sample using a Nicolet 6700 FTIR machine (Thermo Scientific Instruments Corp., Madison, WI, USA) and Omnic 8 software suite (Thermo Scientific Instruments Corp.) within the mid-IR spectrum (range: 1600–750 cm^{-1}) at a resolution of 0.482 cm^{-1} and 32 scans. After scanning, the baseline tracing was performed, and the areas under the infrared bands amide III (1298–1216 cm^{-1}), phosphate (1170–780 cm^{-1}) and carbonate (888–816 cm^{-1}) were calculated using Microsoft Excel (Seattle, WA, USA). Subsequently, the ratio of the amide III/phosphate was determined indicating the organic components of dentine. The inorganic components were calculated by the carbonate/phosphate band area ratios. As the interpositions of the bands of carbonate and phosphate, the latter was subtracted from the former to obtain the real value of the phosphate band.

Sealer characterization

The following sealers were assessed:

- AH Plus (AH; Dentsply DeTrey GmbH, Konstanz, Germany);
- BioRoot (BR; Septodont, Saint Maur-des-Fosses, France);
- MTA Fillapex (MF; Angelus Dental Solutions, Londrina, SP, Brazil);
- TotalFill BC Sealer (TF; FKG Dentaire, La-Chaux-de-Fonds, Switzerland).

The sealers were mixed according to the manufacturer's instructions, allowed to set at 37°C and 100% humidity after which they were embedded in cold cure epoxy-resin. Samples were polished, mounted on an aluminum stub and coated with gold ($n = 3$). They were characterized using scanning electron microscopy (SEM) in back scatter mode and energy dispersive spectroscopy (EDS). The sealer composition was assessed by the monitoring changes in the aluminum, calcium, carbon, oxygen, silicon, tungstate and zirconium.

Antimicrobial activity test

For Peer Review

Caries-free maxillary human incisors obtained from the Birmingham Dental School Tissue Bank – School of Dentistry, University of Birmingham, Birmingham, U.K. (ethical approval number: 14/EM/2811- BCHCDent397), were prepared following previously described protocols (Ma *et al.* 2011) with some modifications. The teeth were sectioned horizontally using a hard tissue microtome (Isomet; Buhler Ltd.) at 1 and 5 mm below the cementoenamel junction, thereby obtaining cylindrical root segments standardized to a length of 4 mm. Subsequently, the root canal was enlarged with a size 6 Gates-Glidden bur (1.5 in diameter; Tulsa Dentsply, Tulsa, OK, USA) under water cooling. Each root segment was sectioned into two semi cylindrical halves using a hard tissue microtome (Isomet; Buhler Ltd.). The outer cementum was ground with 600-grit silicon paper (Carbine; Buehler Ltd.) and the specimen size was adjusted to 4 × 4 × 2 mm to fit the inner wall of a 2 mL filter tube (Pall Corporation, Ann Arbor, MI, USA). The samples were then autoclave sterilized at 121°C for 20 min. After this the specimens were treated with the irrigating solutions as per Protocol A or Protocol B. The antimicrobial activity tests were conducted under aseptic conditions in a laminar flow chamber (Guardian MSC1200; Monmouth Scientific, Bridgewater, U.K.). A standard strain of *E. faecalis* (American Type Culture Collection [ATCC 29212]) was used for intratubular infection. After confirmation of the strain purity by Gram staining and colony morphology, cells were grown in sterile brain-heart-infusion (BHI; Oxoid, Basingstoke, UK) broth and incubated at 37ºC overnight. Subsequently, a spectrophotometer (Jenway 7315, Stone, UK) was used to adjust the bacterial density to 10^7 cells/mL in BHI broth, at an optical density of 1 at 600 nm according to the 0.5 MacFarland standard.

Dentine specimens were placed inside the filter tubes with the internal root canal surface facing outwards, followed by addition of 500 μL of the adjusted bacterial culture. The tubes were centrifuged at 1400*g*, 2000*g*, 3600*g*, and 5000*g* sequentially, twice for each sample, for 5 minutes. After each centrifugation, the supernatant was replaced with fresh 500 μL culture. The samples were then incubated at 37°C in BHI broth and in air. After every 48 hours the procedure was repeated. The incubation lasted for 5 days.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

The dentine specimens were removed from each tube, followed by rinsing in sterile water for 1 minute and air drying. The specimens were randomly divided into four experimental groups according to the sealer applied and a control group that received no treatment. Another group was prepared and was not infiltrated with bacteria. The root canal walls were dried with sterile absorbent paper points (Dentsply) and the sealers were applied into it surface using a cavity liner applicator achieving an approximate film thickness of 0.5 mm measured with a digital pachymeter (Barasti, Amsterdam, Netherlands). The samples were set in an oven at 37°C in 100% relative humidity for 7 days.

For Confocal Laser Scanning Microscopy (CLSM) analysis, bacterial viability was analyzed using the SYTO 9/propidium iodide technique (Live/Dead BacLight Viability Kit; Molecular Probes, Eugene, OR, USA). Dentine discs were washed with 1000 µL phosphate-buffered saline (PBS) to eliminate sealer residues and samples were subsequently stained with 30 µL of the dye, in a light free environment, for 10 minutes. The samples were then placed in a 35 mm petri dish, covered with water and examined with an inverted Leica TCS-SP8 confocal system equipped with an DM6 upright microscope and a 40x/0.80 HCX water immersion lens (Leica Microsystems GmbH, Mannheim, Baden-Württemberg, Germany). A blank internal control comprising of a root filled tooth without bacterial contamination was used to set the threshold for the CLSM. Four confocal “stacks” of random areas were obtained for each sample at 0.5-µm step size. In total, there were 5 samples per group, thus a total of 20 operative fields per group. For quantification Image J software (https://imagej.net/Fiji) was used to calculate the percentage of green (live cells) and red (dead cells) found after the sealer application.

Microstructural and chemical analysis after sealer placement

Extracted human single rooted teeth were obtained from the Birmingham Dental School Tissue Bank – School of Dentistry, University of Birmingham, Birmingham, U.K. (ethical approval number: 14/EM/2811- BCHCDent397), The teeth were stored in water. After coronal

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

access, the working length (1 mm short of the apical foramen) was established by inserting a size 10 K-file (Dentsply) until its tip was observed in the foramen through a stereomicroscope (Stemi 2000C; Carl Zeiss, Jena, Germany).The root canals were chemo-mechanically prepared up to the working length with ProTaper Gold system (Dentsply) in the following sequence of files: SX, S1, S2, F1, F2 and F3. After the use of each instrument, each root canal was irrigated with either 5 mL NaOCl (Protocol A) in a 1mL/10 seconds flow rate. In Protocol B, NaOCl was followed by 5 mL of 17% EDTA for 3 minutes. The irrigants were delivered into the root canal through a plastic syringe and capillary tip cannula (Ultradent, Southlake, TX, USA) using an in-and-out pecking motion. The canals were then dried with paper points and root filled using single cone technique with a matched gutta-percha cone and a test sealer. The teeth were stored in an oven at 37°C for 7 days to completely set. Samples were then embedded in auto polymerizing epoxy resin (Epoxyfix; Struers GmbH), sectioned longitudinally using a hard tissue microtome and polished using an automatic polishing machine. The sealer to dentine interface was imaged at 500 X magnification (*n = 5*). EDS analysis of the dentine was performed for calcium, phosphorus and chlorine at a distance of 100 μm from the interface. This was compared to control dentine that had been irrigated using either Protocol A or B but not obturated.

Statistical analysis

All statistics were performed using SPSS software version 23 (IBM, North Castle, NY, USA). A Kolmogorov−Smirnov test was used to test the normality of the data. Statistical analysis was undertaken using one-way ANOVA to detect differences between the groups. The Holm-Sidak's multiple comparisons test were used to compare the EDS analysis data while the Kruskal-Wallis, and Dunn's post hoc tests detected intergroup differences for the FT-IR analysis and the microbiology and the Tukey for the EDS analysis. The F test was used for comparison of variances in the microbiology testing.

Results

Microstructural and chemical analysis of the dentine

For Peer Review

1
2
3 The scanning electron micrographs of the dentine irrigated with Protocols A or B with untreated
4
5 dentine used as control are shown in Figure 1a and the elemental analysis for calcium,
6
7 phosphorus and chlorine is shown in Table 1a. Smear layer was still present after 2% NaOCl
8
9 irrigation. When NaOCl was followed by EDTA widening of the dentinal tubules was observed
10
11 (Figure 1). EDS analysis revealed deposition of chlorine in the dentine for both irrigation
12
13 protocols, with higher values ($p<0.05$) following 2% NaOCl final flush (Table 1). The lowest
14
15 values of calcium and phosphate resulted after EDTA rinse.
16
17
18 The results of dentine treatment with Protocols A and B on the amide III/phosphate and
19
20 carbonate/phosphate ratio are presented in Table 2. Dentine surfaces before (initial) and after
21
22 immersion in the irrigation solutions were not significant in terms of organic and inorganic
23
24 components.
25
26
27
28 *Sealer characterization*
29
30 The scanning electron micrographs (Figure 2a) revealed a microstructure with particles of
31
32 various sizes and opacity for all the test sealers. The shiny particles (marked as red arrows)
33
34 were rounded for AH Plus, and were evenly distributed across the sample. BioRoot, MTA
35
36 Fillapex and TotalFill had non-uniformly distributed particles, with no shape standardization.
37
38 The latter exhibited particles with lower levels of brightness occurring in smaller quantities.
39
40
41
42 EDS scans are shown in Figure 2b. AH Plus and MTA Fillapex exhibited shiny particles which
43
44 were rich in calcium (Ca) and tungsten (W) and the former also had particles composed of
45
46 zirconium (Zr) and oxygen (O). BioRoot and TotalFill also contained zirconium whilst presence
47
48 of chlorine (Cl) was found in BioRoot. MTA Fillapex, BioRoot and TotalFill all included calcium
49
50 (Ca), silicon (Si) and oxygen (O) while the MTA Fillapex included also aluminium and traces
51
52 of titanium.
53
54
55 *Antimicrobial activity test*
56
57
58
59
60

For Peer Review

Table 3 shows the percentage of live cells in the dentinal tubules after contact with different sealers after the dentine was irrigated using either Protocol A or B. Representative confocal laser scanning microscope images of the groups described in Table 3 are shown in Figure 3.

After root canal dentine was irrigated with 2% sodium hypochlorite only (Protocol A), BioRoot eliminated more bacteria than the other sealers ($p < 0.05$). AH Plus and TotalFill were not statistically significant ($p > 0.05$) and were similar to the control group (unirrigated and unfilled teeth) thus ineffective against *E. faecalis*. When EDTA was used in conjunction with NaOCl as in Protocol B, BioRoot and AH Plus both showed a reduction in viable bacteria in the dentinal tubules ($p < 0.05$).

Microstructural analysis after sealer placement

The scanning electron micrographs of the dentine to sealer interface for all sealers using both irrigation protocols are shown in Figure 4. Protocol A was associated with interfaces with poor sealer adaption to dentine for AH Plus and MTA Fillapex (marked as red arrows). For AH Plus the removal of smear layer allowed the penetration of the sealer in the dentinal tubules when using irrigation Protocol B. MTA Fillapex also exhibited tubular penetration but this was not as marked as the AH Plus (marked as white arrows). The BioRoot and TotalFill sealers ~~showed adequate~~had sealer to dentine interfaces without gaps using both protocols.

The EDS analyses for calcium, phosphorus and chlorine performed on the dentine 100 μm away from the sealer-dentine interface after root canal filling are shown in Table 4. After sealer placement the levels of calcium in dentine increased for HCSC groups for both irrigation protocols with the highest values for BioRoot ($p<0.05$). When the dentine was irrigated with both sodium hypochlorite and EDTA (Protocol B), TotalFill exhibited lower values of phosphorus when compared with the other sealers ($p<0.05$), while in Protocol A, no differences occurred between them ($p>0.05$). The control (unfilled root canal) had higher values of chlorine than dentine obtained from root-filled teeth.

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Discussion

The three null hypotheses were rejected since: the presence of smear layer affected the antimicrobial action of the sealers against *E. faecalis* intratubular infection. The properties of HCSC and epoxy resin sealers were affected by the irrigation protocols used prior to root canal filling; the sealers affected dentine chemistry.

When used during root canal chemo-mechanical preparation, sodium hypochlorite has a wide antimicrobial spectrum and dissolves necrotic pulp tissues. Although the proteolytic action of NaOCl may cause deleterious effects on the mechanical properties of dentine (Wang *et al.* 2001), the present study found that a flush with 2% NaOCl solution caused no changes in the collagen ratios of dentine. Advocated as an adjunct irrigant to NaOCl, EDTA is a chelating agent aimed at removing the inorganic portion of the smear layer (Perez-Heredia *et al.* 2008) and enhancing sealer penetration (Lee *et al.* 2007, Yildirim *et al.* 2008, Marciano *et al.* 2015). Although the spectra obtained from FT-IR analysis revealed no differences in the degradation of the inorganic portion of dentine irrigated with EDTA followed by NaOCl, the SEM/EDS analysis revealed a reduction in the mineral content.

Root canal chemo-mechanical preparation reduces the amount of harmful bacteria, but may not reach levels compatible with healing of the periapical tissues, as instruments and NaOCl may not be able to reach bacteria in inaccessible, remote areas of the canal system (Oguntebi 1994, Nair *et al.* 2005, Wong & Cheung 2014, Versiani *et al.* 2015). The bactericidal effect of remaining chlorine ions from NaOCl flush on dentine (Table 1) (Gutiérrez *et al.* 1990) would not prevent *E. faecalis* infection, as indicated in the control groups (Figure 3). Root canal filling would compensate by having an antimicrobial effect thus eliminating the residual bacteria in anatomical complexities that are responsible for post treatment disease (Sjögren *et al.* 1997, Molander *et al.* 1998). Sealer penetration in the open dentinal tubules is related to decreased microleakage with resin-based sealers (White *et al.* 1984) as seen in Figure 1. For HCSCs, the use of a chelating agent may not be necessary, as these sealer types do not depend on smear layer removal to produce a good dentine seal, but rather on chemical interaction. As

For Peer Review

dentine-sealer hermetic seal is not easy to achieve (Tay *et al.* 2005) the release of antimicrobial compounds by sealers ~~would~~ may prevent bacterial ingress from the root canals to the periapical tissues.

Although endodontic infections are caused by multispecies biofilms, the survival and virulence factors possessed by *E. faecalis* make it the preferred single microorganism used for laboratory research to mimic persistent endodontic infections (Stuart *et al.* 2006). Furthermore, this microorganism withstands centrifuging which is an essential step of the intratubular infection process. This method provides a robust model to investigate the antimicrobial properties of materials since it takes into consideration the effect that the dentine has on material characteristics. This is particularly important when investigating materials such as HCSCs which interact chemically and physically with dentine. The use of NaOCl followed by a calcium chelator has been recommended and used in a number of studies (Ma *et al.* 2011, Andrade *et al.* 2015, Arias-Moliz & Camilleri 2016) to facilitate bacterial penetration into open dentinal tubules (Haapasalo & Orstavik 1987). By modifying a previously described and validated experimental procedure the present study verifies that bacteria are present in dentinal tubules regardless of the presence of the smear layer as shown in Figure 3.

For Peer Review

The antimicrobial activity of HCSCs occurs after cement hydration, where Ca^{2+} ions from the calcium silicate particles of the cement reacting with the water added or present in the clinical environment are released (Camilleri 2007). Elemental migration of the experimental sealers, indicated by enhanced levels of Ca^{2+} on smear-layer-rich dentine interface, suggests the material interacts with dentine (Atmeh *et al.* 2012) which was not displayed by AH Plus. EDTA has been shown to interfere with cement hydration due its chelating ability in the absence of dentine (Lee *et al.* 2007), whereas the only source of Ca^{2+} ions would be those released by HCSCs. To mimic the clinical situation, in this study, the effect of NaOCl (Protocol A) and NaOCl + EDTA (Protocol B) was investigated on dentine, relative to the antimicrobial action of HCSCs. A 3 minutes flush with EDTA to reduce debris on the dentine in the apical third of the root was employed (Teixeira *et al.* 2005), resulting in no improvements on smear layer

scores following irrigant agitation in straight root canals (Mayer *et al.* 2002, Rödig *et al.* 2010). In the current study with the use of NaOCl only, BioRoot had the highest percentage of dead intra-tubular bacteria. TotalFill which is also a HCSC sealer had a similar response to AH Plus and the control group.

The use of NaOCl followed by EDTA resulted in fewer live bacteria for all sealers. The reason for the enhanced sealer activity could be multifactorial where the chelation preferentially affected dentine rather than the sealer (Hill 1959), and the Ca^{2+} released through the sealer surpassed the levels of Ca^{2+} extracted by EDTA. The smear-layer-free dentine and its greater wettability favoured the diffusion of hydraulic cements and fluids through dentinal tubules, increasing the ion exchange and hydration of HCSC (Tartari *et al.* 2018).

The hydration patterns of various HCSCs have been shown to be different (Camilleri *et al.* 2013, Marciano *et al.* 2016, Schembri-Wismayer & Camilleri 2017). BioRoot is mixed with water and thus the hydration is always guaranteed regardless of the environmental presence of fluids. This leads to a lower bacterial viability (Arias-Moliz & Camilleri 2016, Colombo *et al.* 2018) which has also been shown in the current study. It killed even bacteria inside the dentinal tubules without the removal of the smear layer while the TotalFill was ineffective. The hydration of HCSC significantly influences the release of calcium hydroxide. Therefore, as a water-based sealer, BioRoot RCS does not rely on OH^- ions derived from tissue fluids to form calcium hydroxide, as does MTA Fillapex which is a 2-paste system (Marciano *et al.* 2015) or TotalFill, a premixed ready-to-use injectable sealer (Loushine *et al.* 2011). Since BioRoot RCS hashigher Ca^{2+} ion leaching regardless of the dentine pre-treatment (Table 4) and thus, calcium hydroxide peaks (Xuereb *et al.* 2015), the present results show an expected higher percentage reduction of *E. faecalis* viability for both irrigation protocols. This is in agreement with previous research that reported the superiority of BioRoot BCS against intratubular infection and in the direct contact test (Arias-Moliz & Camilleri 2016, Colombo *et al.* 2018). The vehicles used in the TotalFill and the MTA Fillapex have an influence on rates of ionic dissociation and diffusion of calcium hydroxide (Estrela *et al.* 1999, Zancan *et al.* 2016).

The lesser antimicrobial effect of TotalFill against *E. faecalis* might have been influenced by insufficient levels of calcium hydroxide released. The hydration of TotalFill cannot be assumed *in vivo* and conditions of tissue fluids cannot be monitored or reproduced in laboratory settings. The TotalFill specimen discs exposed to 100% relative humidity required 10 days to achieve their final setting (Loushine *et al.* 2011), raising doubts about their complete hydration in a period of 7 days enclosed within the root canal, as demonstrated in this study. The hydration of TotalFill might be affected by the addition of calcium phosphate to tricalcium silicate, resulting in a reduction of crystalline calcium hydroxide (Loushine *et al.* 2011, Schembri-Wismayer & Camilleri 2017); its entire dependence on an aqueous environment to hydrate and set, and its presentation as a water-free paste (Xuereb *et al.* 2015). The higher susceptibility of *E. faecalis* to TotalFill achieved in previous report (Wang *et al.* 2014) might be explained by the wider dentinal tubule openings obtained by means of previous irrigation with a stronger chelating agent than the one used in the present study. This allowed better sealer penetration and enhanced antimicrobial action, while maintaining the order of susceptibility, since no differences were found between TotalFill and AH Plus in both studies. The direct contact test disregarded the dentine-buffering effect and might have benefitted TotalFill hydration by the water content of the bacterial suspension, which may account for the better results found for the antimicrobial action of TotalFill (Zhang *et al.* 2009b, Candeiro *et al.* 2016).

For Peer Review

MTA Fillapex is chemically based on mineral trioxide aggregate and salicylate resin, which results in the release of lower levels of calcium hydroxide (Viapiana *et al.* 2014). The mildly alkaline pH (Silva *et al.* 2013, Viapiana *et al.* 2014) correlated to its ability to release Ca^{2+} ions (Viapiana *et al.* 2014) is below the killing threshold for *E. faecalis*. Its antimicrobial action might be related to its main component - salicylate resin, together with diluting resin, and silica that strongly affected cell viability (Bin *et al.* 2012, Scelza *et al.* 2012).

AH Plus exhibited low antimicrobial activity in agreement with previous reports (Zhang *et al.* 2009a, Bailon-Sanchez *et al.* 2014, Arias-Moliz *et al.* 2015). When limited by the presence of a smear layer, the antimicrobial agents released by AH Plus did not reach lethal levels against

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

E. faecalis within the dentinal tubules. The use of EDTA was important for AH Plus as it improved the penetration of sealer tags into dentinal tubules and therefore increased the range of its antimicrobial action and mechanical bond to dentine. The collagen exposed by EDTA chemically bonds to the epoxy rings of AH Plus to initiate it setting reaction (Johns 1982), but it is not impaired by the proteolytic action of NaOCl on Protocol A, as collagen ratios were the same after the NaOCl flush (Neelakantan *et al.* 2011).

It is well established that intra-radicular microorganisms either remaining or recolonizing the filled canal system are the main aetiological agents of apical periodontitis (Kakehashi *et al.* 1965). The essential basis for the choice of a sealer must thus be to optimize root canal disinfection by eradicating the root canal microbes or substantially reducing the microbial load and to prevent re-infection by an effective dentin-sealer seal (Nair *et al.* 2005). Although none of the sealers tested eliminated the bacteria present in total, BioRoot reduced the bacterial load even when EDTA was not used in the irrigation protocol. Furthermore, when EDTA irrigation was employed, BioRoot restored the calcium levels of the dentine.

For Peer Review

Conclusions

A smear-layer-free dentine surface increased the elimination of bacteria inside dentinal tubules for all sealers. The use of EDTA on dentine did not negatively affect the HCSC sealer antimicrobial action and was important for tubule penetration of AH Plus. Bacterial susceptibility to BioRoot was not impaired by the presence of the smear layer. None of the sealers were able to eliminate live bacteria cells completely. The interaction of HCSC sealers with dentine was influenced by the irrigation protocol but the sealers also affected the dentine chemistry further enforcing the mineral exchange at the interface.

Statement

The authors declare no conflict of interest

For Peer Review

References

Aggarwal V, Jain A, Kabi D (2011) In vitro evaluation of effect of various endodontic solutions on selected physical properties of white mineral trioxide aggregate. *Australian Endodontic Journal* **37**, 61-4.

Andrade FBd, Arias MPC, Maliza AGA *et al.* (2015) A new improved protocol for in vitro intratubular dentinal bacterial contamination for antimicrobial endodontic tests: standardization and validation by confocal laser scanning microscopy. *Journal of Applied Oral Science* **23**, 591-8.

Arias-Moliz MT, Camilleri J (2016) The effect of the final irrigant on the antimicrobial activity of root canal sealers. *Journal of Dentistry* **52**, 30-6.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Arias-Moliz MT, Ruiz-Linares M, Cassar G *et al.* (2015) The effect of benzalkonium chloride additions to AH Plus sealer. Antimicrobial, physical and chemical properties. *Journal of Dentistry* **43**, 846-54.

Atmeh AR, Chong EZ, Richard G, Festy F, Watson TF (2012) Dentin-cement interfacial interaction: calcium silicates and polyalkenoates. *Journal of Dental Research* **91**, 454-9.

Bailon-Sanchez ME, Baca P, Ruiz-Linares M, Ferrer-Luque CM (2014) Antibacterial and anti-biofilm activity of AH plus with chlorhexidine and cetrimide. *Journal of Endodontics* **40**, 977-81.

Bin CV, Valera MC, Camargo SEA *et al.* (2012) Cytotoxicity and Genotoxicity of Root Canal Sealers Based on Mineral Trioxide Aggregate. *Journal of Endodontics* **38**, 495-500.

Camilleri J (2007) Hydration mechanisms of mineral trioxide aggregate. *International Endodontic Journal* **40**, 462-70.

Camilleri J, Sorrentino F, Damidot D (2013) Investigation of the hydration and bioactivity of radiopacified tricalcium silicate cement, Biodentine and MTA Angelus. *Dental Materials* **29**, 580-93.

Candeiro GTM, Moura-Netto C, D'Almeida-Couto RS *et al.* (2016) Cytotoxicity, genotoxicity and antibacterial effectiveness of a bioceramic endodontic sealer. *International Endodontic Journal* **49**, 858-64.

Colombo M, Poggio C, Dagna A *et al.* (2018) Biological and physico-chemical properties of new root canal sealers. *Journal of Clinical and Experimental Dentistry* **10**, e120-e6.

Editorial-Board (2007) Wanted: A Base of Evidence. *Journal of Endodontics* **33**, 1401-2.

Estrela C, Pecora JD, Souza-Neto MD, Estrela CR, Bammann LL (1999) Effect of vehicle on antimicrobial properties of calcium hydroxide pastes. *Brazilian Dental Journal* **10**, 63-72.

Ghorbanzadeh A, Aminsobhani M, Sohrabi K *et al.* (2016) Penetration Depth of Sodium Hypochlorite in Dentinal Tubules after Conventional Irrigation, Passive Ultrasonic Agitation and Nd:YAG Laser Activated Irrigation. *Journal of Lasers in Medical Sciences* **7**, 105-11.

Gutiérrez JH, Jofré A, Villena F (1990) Scanning electron microscope study on the action of endodontic irrigants on bacteria invading the dentinal tubules. *Oral Surg Oral Med Oral Pathol* **69**, 491-501.

Haapasalo M, Orstavik D (1987) In vitro infection and disinfection of dentinal tubules. *Journal of Dental Research* **66**, 1375-9.

Hill PK (1959) Endodontics. *The Journal of Prosthetic Dentistry* **9**, 142-8.

Johns RB (1982) Endodontics – Science and Practice: A Textbook for Student and Practitioner. *Journal of the Royal Society of Medicine* **75**, 574-5.

Koutroulis A, Kuehne SA, Cooper PR, Camilleri J (2019) The role of calcium ion release on biocompatibility and antimicrobial properties of hydraulic cements. *Scientific Reports* **13**;9(1):19019.

Kakehashi S, Stanley HR, Fitzgerald RJ (1965) THE EFFECTS OF SURGICAL EXPOSURES OF DENTAL PULPS IN GERM-FREE AND CONVENTIONAL LABORATORY RATS. *Oral surgery, oral medicine, and oral pathology* **20**, 340-9.

Khalil I, Naaman A, Camilleri J (2016) Properties of Tricalcium Silicate Sealers. *Journal of Endodontics* **42**, 1529-35.

Lee YL, Lin FH, Wang WH, Ritchie HH, Lan WH, Lin CP (2007) Effects of EDTA on the hydration mechanism of mineral trioxide aggregate. *Journa of Dental Research* **86**, 534-8.

Loushine BA, Bryan TE, Looney SW *et al.* (2011) Setting Properties and Cytotoxicity Evaluation of a Premixed Bioceramic Root Canal Sealer. **37**, 673-7.

For Peer Review

Ma J, Wang Z, Shen Y, Haapasalo M (2011) A New Noninvasive Model to Study the Effectiveness of Dentin Disinfection by Using Confocal Laser Scanning Microscopy. *Journal of Endodontics* **37**, 1380-5.

Marciano MA, Duarte MA, Camilleri J (2015) Dental discoloration caused by bismuth oxide in MTA in the presence of sodium hypochlorite. *Clinical Oral Investigations* **19**, 2201-9.

Marciano MA, Duarte MAH, Camilleri J (2016) Calcium silicate-based sealers: Assessment of physicochemical properties, porosity and hydration. *Dental Materials* **32**, e30-e40.

Mayer BE, Peters OA, Barbakow F (2002) Effects of rotary instruments and ultrasonic irrigation on debris and smear layer scores: a scanning electron microscopic study. *International Endodontic Journal* **35**, 582-9.

Molander A, Reit C, Dahlén G, Kvist T (1998) Microbiological status of root-filled teeth with apical periodontitis. *International Endodontic Journal* **31**, 1-7.

Nair PNR, 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.

Neelakantan P, Sharma S, Shemesh H, Wesselink PR (2015) Influence of Irrigation Sequence on the Adhesion of Root Canal Sealers to Dentin: A Fourier Transform Infrared Spectroscopy and Push-out Bond Strength Analysis. *Journal of Endodontics* **41**, 1108-11.

Neelakantan P, Subbarao C, Subbarao CV, De-Deus G, Zehnder M (2011) The impact of root dentine conditioning on sealing ability and push-out bond strength of an epoxy resin root canal sealer. *International Endodontic Journal* **44**, 491-8.

Oguntebi BR (1994) Dentine tubule infection and endodontic therapy implications. *International Endodontic Journal* **27**, 218-22.

Perez-Heredia M, Ferrer-Luque CM, Gonzalez-Rodriguez MP, Martin-Peinado FJ, Gonzalez-Lopez S (2008) Decalcifying effect of 15% EDTA, 15% citric acid, 5% phosphoric acid and 2.5% sodium hypochlorite on root canal dentine. *International endodontic journal* **41**, 418-23.

Prati C, Gandolfi MG (2015) Calcium silicate bioactive cements: Biological perspectives and clinical applications. *Dental Materials* **31**, 351-70.

Reyes-Carmona JF, Felippe MS, Felippe WT (2009) Biomineralization Ability and Interaction of Mineral Trioxide Aggregate and White Portland Cement With Dentin in a Phosphate-containing Fluid. *Journal of Endodontics* **35**, 731-6.

Rödig T, Döllmann S, Konietschke F, Drebenstedt S, Hülsmann M (2010) Effectiveness of Different Irrigant Agitation Techniques on Debris and Smear Layer Removal in Curved Root Canals: A Scanning Electron Microscopy Study. *Journal of Endodontics* **36**, 1983-7.

Saleh IM (2002) The effects of dentine pretreatment on the adhesion of root-canal sealers. *International Endodontic Journal* **35**, 859-66.

Scelza MZ, Linhares AB, da Silva LE, Granjeiro JM, Alves GG (2012) A multiparametric assay to compare the cytotoxicity of endodontic sealers with primary human osteoblasts. *International Endodontic Journal* **45**, 12-8.

Schembri-Wismayer P, Camilleri J (2017) Why Biphasic? Assessment of the Effect on Cell Proliferation and Expression. *Journal of Endodontics* **43**, 751-9.

Silva EJNL, Rosa TP, Herrera DR, Jacinto RC, Gomes BPFA, Zaia AA (2013) Evaluation of Cytotoxicity and Physicochemical Properties of Calcium Silicate-based Endodontic Sealer MTA Fillapex. *Journal of Endodontics* **39**, 274-7.

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.

Stuart CH, Schwartz SA, 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.

Tartari T, Wichnieski C, Bachmann L *et al.* (2018) Effect of the combination of several irrigants on dentine surface properties, adsorption of chlorhexidine and adhesion of microorganisms to dentine. *International Endodontic Journal* **51**, 1420-33.

Tay FR, Loushine RJ, Lambrechts P, Weller RN, Pashley DH (2005) Geometric factors affecting dentin bonding in root canals: a theoretical modeling approach. *Journal of Endodontics* **31**, 584-9.

Tay FR, Pashley DH, Rueggeberg FA, Loushine RJ, Weller RN (2007) Calcium Phosphate Phase Transformation Produced by the Interaction of the Portland Cement Component of White Mineral Trioxide Aggregate with a Phosphate-containing Fluid. *Journal of Endodontics* **33**, 1347-51.

Teixeira CS, Felippe MC, Felippe WT (2005) The effect of application time of EDTA and NaOCl on intracanal smear layer removal: an SEM analysis. *International Endodontic Journal* **38**, 285-90.

Vahdaty A, Ford TRP, Wilson RF (1993) Efficacy of chlorhexidine in disinfecting dentinal tubules in vitro. *Endodontic Dental Traumatology* **9**, 243-8.

Versiani MA, De-Deus G, Vera J *et al.* (2015) 3D mapping of the irrigated areas of the root canal space using micro-computed tomography. *Clinical Oral Investigations* **19**, 859-66.

Viapiana R, Guerreiro-Tanomaru JM, Hungaro-Duarte MA, Tanomaru-Filho M, Camilleri J (2014) Chemical characterization and bioactivity of epoxy resin and Portland cement-based sealers with niobium and zirconium oxide radiopacifiers. *Dental Materials* **30**, 1005-20.

Viapiana R, Moinzadeh AT, Camilleri L, Wesselink PR, Tanomaru Filho M, Camilleri J (2016) Porosity and sealing ability of root fillings with gutta-percha and BioRoot RCS or AH Plus sealers. Evaluation by three ex vivo methods. *International Endodontic Journal* **49**, 774-82.

Virdee SS, Farnell DJJ, Silva MA, Camilleri J, Cooper PR, Tomson PL (2020) The influence of irrigant activation, concentration and contact time on sodium hypochlorite penetration into root dentine: an ex vivo experiment. *International Endodontic Journal*, 986-97.

Wang X, Bank RA, TeKoppele JM, Agrawal CM (2001) The role of collagen in determining bone mechanical properties. *Journal of Orthopaedic Research* **19**, 1021-6.

Wang Z, Shen Y, Haapasalo M (2014) Dentin Extends the Antibacterial Effect of Endodontic Sealers against Enterococcus faecalis Biofilms. *Journal of Endodontics* **40**, 505-8.

White RR, Goldman M, Lin PS (1984) The influence of the smeared layer upon dentinal tubule penetration by plastic filling materials. *Journal of Endodontics* **10**, 558-62.

Wong DT, Cheung GS (2014) Extension of bactericidal effect of sodium hypochlorite into dentinal tubules. *Journal of Endodontics* **40**, 825-9.

Xuereb M, Vella P, Damidot D, Sammut CV, Camilleri J (2015) In Situ Assessment of the Setting of Tricalcium Silicate–based Sealers Using a Dentin Pressure Model. *Journal of Endodontics* **41**, 111-24.

Yan P, Peng B, Fan B, Fan M, Bian Z (2006) The Effects of Sodium Hypochlorite (5.25%), Chlorhexidine (2%), and Glyde File Prep on the Bond Strength of MTA-Dentin. *Journal of Endodontics* **32**, 58-60.

Yildirim T, Er K, Taşdemir T, Tahan E, Buruk K, Serper A (2010) Effect of smear layer and root-end cavity thickness on apical sealing ability of MTA as a root-end filling material: A bacterial leakage study. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **109**, e67-e72.

Yildirim T, Oruçoğlu H, Çobankara FK (2008) Long-term Evaluation of the Influence of Smear Layer on the Apical Sealing Ability of MTA. *Journal of Endodontics* **34**, 1537-40.

For Peer Review

Zancan RF, Vivan RR, Milanda Lopes MR *et al.* (2016) Antimicrobial Activity and Physicochemical Properties of Calcium Hydroxide Pastes Used as Intracanal Medication. **42**, 1822-8.

Zancan RF, Hadis MA, Burgess D, Zhang ZJ, DiMaio A, Tomson P, Duarte MAH, Camilleri J. (2021) A matched irrigation and obturation strategy for root canal therapy. *Scientific Reports* ***11,*** *4666 (2021). https://doi.org/10.1038/s41598-021-83849-y*

Zehnder M (2006) Root canal irrigants. *Journal of Endodontics* **32**(5), 389-98

Zhang H, Pappen FG, Haapasalo M (2009a) Dentin enhances the antibacterial effect of mineral trioxide aggregate and bioaggregate. *Journal of Endodontics* **35**, 221-4.

Zhang H, Shen Y, Ruse ND, Haapasalo M (2009b) Antibacterial Activity of Endodontic Sealers by Modified Direct Contact Test Against Enterococcus faecalis. *Journal of Endodontics* **35**, 1051-5.

Zou L, Shen Y, Li W, Haapasalo M (2010) Penetration of Sodium Hypochlorite into Dentin. *Journal of Endodontics* **36**, 793-6.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

Tables

Table 1. Mean (X) and standard deviation (SD) values for the energy dispersive spectroscopy (EDS) chemical analysis of dentine surface for calcium (Ca), phosphorus (P) and chlorine (Cl) after immersion in the irrigant solutions using Protocol A: 2% NaOCl or Protocol B: 2% NaOCl + 17% EDTA.

	Control	**2% NaOCl**	**2% NaOCl + 17% EDTA**
	X ± SD	X ± SD	X ± SD
Calcium	24.76 ± 1.15^{A}	23.94 ± 2.64^{A}	18.68 ± 2.72^{B}
Phosphorus	13.29 ± 0.45^{A}	13.01 ± 1.23^{A}	10.33 ± 0.94^{B}
Chlorine	0.0 ± 0.0^{B}	1.20 ± 0.67^{A}	0.66 ± 0.23^{AB}

Different capital letters in rows indicate statistically significant intergroup differences (One -way Anova and Holm-Sidak's multiple comparisons test p -value < 0.05).

Table 2. Medians (Med) and minimum and maximum (Min–Max) values for the ratio of amide III/phosphate and phosphate/carbonate on dentine surface before (initial) and after immersion in the irrigant solutions using Protocol: A. 2% NaOCl or Protocol B: 2% NaOCl + 17% EDTA.

	Initial	**2% NaOCl**	**2% NaOCl + 17% EDTA**
	Med (Min-Max)	Med (Min-Max)	Med (Min-Max)
Amide/Phosphate	32.99^{A} (13.1 – 131.8)	17.27^{A} (9.28 – 64.82)	49.23^{A} (27.92 – 277.4)
Carbonate/Phosphate	20.58^{A} (17.58 - 21.95)	21.45^{A} (15.67 - 29.08)	14.49^{A} (11.67 - 28.96)

Median (med) and minimum and maximum (min–max) values are multiplied by 10^{-3}; Kruskal-Wallis with a Dunn post hoc P value <.05. Different capital letters in rows indicate statistically significant intergroup differences.

For Peer Review

Table 3. Median (Med) and minimum and maximum (Min–Max) values of the *Enterococcus faecalis* percentage of live cells after 7 days inside dentinal tubules after irrigating with either Protocols: A. 2% NaOCl or Protocol B: 2% NaOCl + 17% EDTA and sealer placement

	AH Plus	**BioRoot BCS**	**MTA Fillapex**	**Total Fill**	**Control**
	Med (Min-Max)	Med (Min-Max)	Med (Min-Max)	Med (Min-Max)	Med (Min-Max)
Protocol A	81.63^{Aa}	70.09^{Ba}	77.43^{ABa}	88.06^{Aa}	83.46^{Aa}
	(63.99 – 96.42)	(59.23 – 82.53)	(50.74 – 98.46)	(54.23 – 96.96)	(68.01 – 99.10)
Protocol B	76.18^{ABb}	53.06^{Cb}	67.00^{BCa}	76.92^{ABa}	94.49^{Aa}
	(50.02 – 98.26)	(15.82 – 73.00)	(50.93 – 92.21)	(61.54 – 94.89)	(60.57 – 99.65)

Protocol A: Bartlett's test for equal variances P value <05. Protocol B: Kruskal-Wallis with a Dunn post hoc P value <.05. Different capital letters in rows indicate statistically significant intergroup differences; F test to compare variances was used to compare protocols, different lowercase letters in columns indicate statistically significant differences for the same sealer.

For Peer Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

Table 4. Mean (X) and standard deviation (SD) values for the Energy Dispersive Spectroscopy (EDS) chemical analysis of dentine surface irrigated either using Protocol A: 2% NaOCl or Protocol B: 2% NaOCl + 17% EDTA. Values for calcium (Ca), phosphorus (P) and chlorine (Cl) were obtained before and after the root canal filling 100 µm from the dentin-sealer interface.

	AH Plus	**BioRoot**	**MTA Fillapex**	**TotalFill**	**Control**
	X ± SD	X ± SD	X ± SD	X ± SD	X ± SD
Protocol A- Ca	24.67 ± 1.32^{Ba}	31.08 ± 1.36^{Aa}	25.89 ± 5.10^{ABa}	27.98 ± 1.04^{ABa}	23.94 ± 2.64^{Ba}
Protocol B- Ca	25.95 ± 0.76^{Ba}	34.90 ± 3.78^{Aa}	27.31 ± 3.60^{Ba}	25.94 ± 0.42^{Bb}	18.68 ± 2.72^{Cb}
Protocol A- P	14.35 ± 0.76^{Aa}	15.30 ± 0.59^{Aa}	13.54 ± 2.45^{Aa}	13.39 ± 0.66^{Aa}	13.01 ± 1.23^{Aa}
Protocol B- P	13.19 ± 2.76^{ABa}	16.62 ± 1.20^{Aa}	13.33 ± 3.37^{ABa}	12.30 ± 0.36^{Bb}	10.33 ± 0.94^{Bb}
Protocol A- Cl	0.11 ± 0.01^{Bb}	0.68 ± 0.22^{ABa}	0.43 ± 0.35^{Ba}	0.17 ± 0.01^{Bb}	1.20 ± 0.67^{Aa}
Protocol B- Cl	0.28 ± 0.09^{Ba}	0.35 ± 0.10^{Bb}	0.25 ± 0.01^{Ba}	0.22 ± 0.03^{Ba}	0.66 ± 0.23^{Aa}

Different capital letters in rows indicate statistically significant intergroup differences (One -way Anova and Holm-Sidak's multiple comparisons test p -value < 0.05); different lowercase letters in columns indicate indicate statistically significant differences for the same sealer and ion comparing protocols (One -way repeated measures Anova and Tukey post -hoc p -value < 0.05).

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

Figure Legends

Figure 1. Secondary electron scanning electron micrographs at 2Kx magnification of dentine irrigated with protocols (A) 2% sodium hypochlorite and (B) 2% NaOCl + EDTA and saline as a control showing the ability of the EDTA to remove the smear layer.

Figure 2a. Backscatter scanning electron micrographs at 2Kx magnification of polished sections of test sealers showing microstructural components (A) AH Plus showing the presence of rounded shiny particles of various sizes evenly distributed across the section; (B) BioRoot and (C) MTA Fillapex exhibiting the presence of shiny particles of various shapes and sizes non-uniformly distributed, and (D) TotalFill exhibited particles with lower levels of brightness occurring in smaller quantities.

Figure 2b. Corresponding energy dispersive spectroscopic scans of the test sealers shown in Figure 1b displaying high peaks of calcium, carbon and oxygen. Silicon was detected only for HCSC. The shiny particles on SEM (marked as red arrows in Figure 1b) are mainly composed of (A) AH Plus, calcium, tungsten, zirconium and oxygen; (B) BioRoot zirconium and oxygen; (C) MTA Fillapex calcium and tungsten; and (D) TotalFill zirconium and oxygen.

For Peer Review

Figure 3. Representative confocal laser scanning microscope images of *E. faecalis* infected dentinal tubules obturated with the test sealers or not (control) after irrigation with Protocols A: 2% NaOCl and B. 2% NaOCl + 17% EDTA. The viability staining indicated live cells in green, and dead cells in red. Each picture represents an area of 275 x 275 µm.

Figure 4. Secondary electron scanning electron micrographs of the interfacial interactions between sealers (left side of each image) and dentine (right side) previously irrigated with 2% NaOCl (Protocol A) or 2% NaOCl + 17% EDTA (Protocol B). Interfaces with poor adaptation are marked as red arrows and sealer penetration in the dentinal tubules as white arrows.

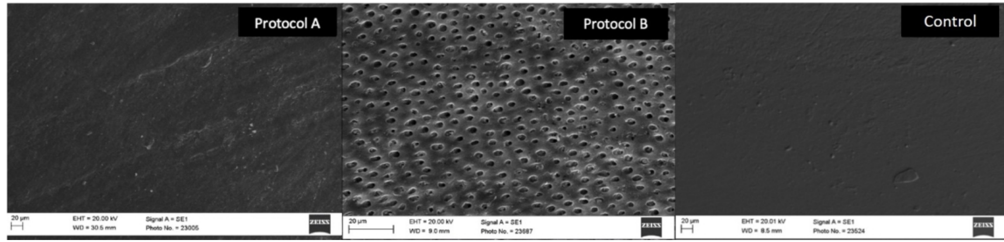


Figure 1. Secondary electron scanning electron micrographs at 2Kx magnification of dentine irrigated with protocols (A) 2% sodium hypochlorite and (B) 2% NaOCl + EDTA and saline as a control showing the ability of the EDTA to remove the smear layer.

281x67mm (150 x 150 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

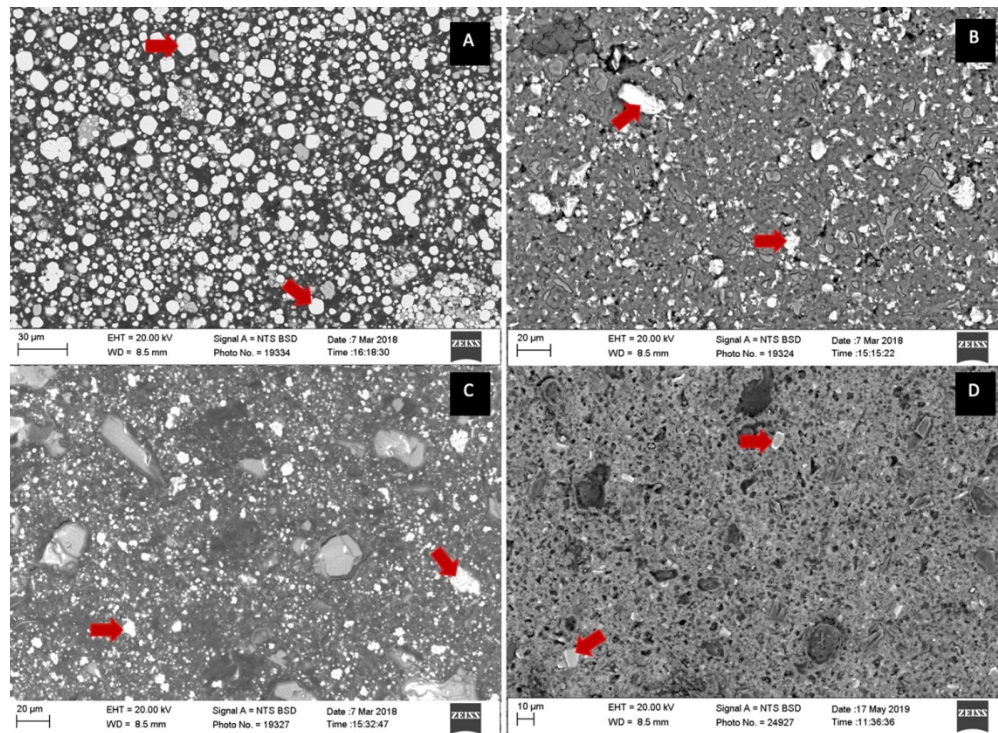


Figure 2a. Backscatter scanning electron micrographs at 2Kx magnification of polished sections of test sealers showing microstructural components (A) AH Plus showing the presence of rounded shiny particles of various sizes evenly distributed across the section; (B) BioRoot and (C) MTA Fillapex exhibiting the presence of shiny particles of various shapes and sizes non-uniformly distributed, and (D) TotalFill exhibited particles with lower levels of brightness occurring in smaller quantities.

261x190mm (150 x 150 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

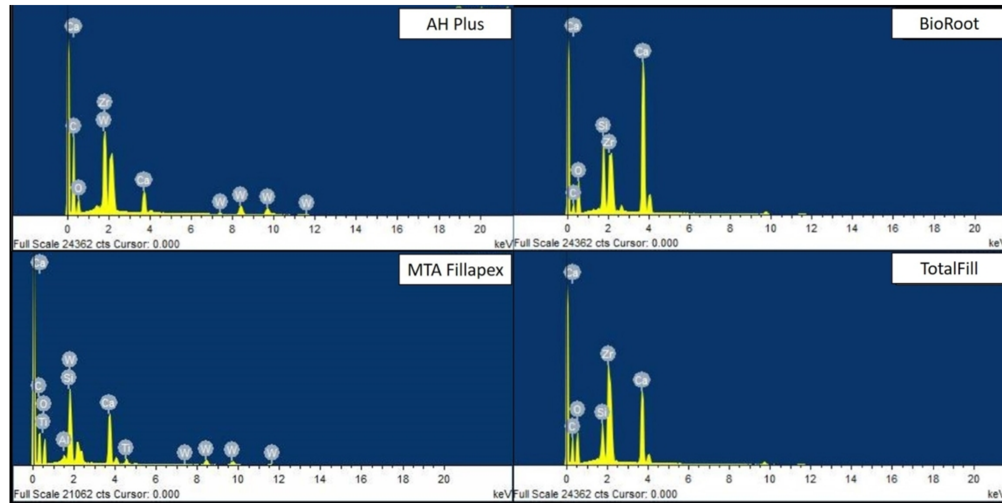
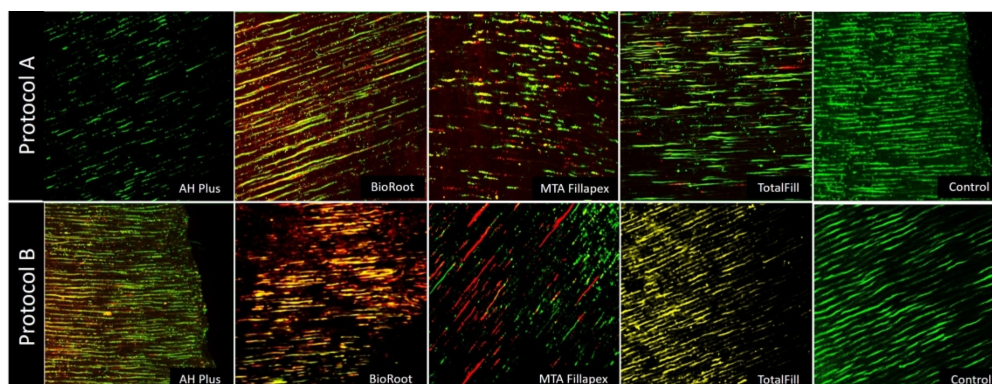


Figure 2b. Corresponding energy dispersive spectroscopic scans of the test sealers shown in Figure 1b displaying high peaks of calcium, carbon and oxygen. Silicon was detected only for HCSC. The shiny particles on SEM (marked as red arrows in Figure 1b) are mainly composed of (A) AH Plus, calcium, tungsten, zirconium and oxygen; (B) BioRoot zirconium and oxygen; (C) MTA Fillapex calcium and tungsten; and (D) TotalFill zirconium and oxygen.

338x168mm (150 x 150 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18



19
20
21
22
Figure 3. Representative confocal laser scanning microscope images of E. faecalis infected dentinal tubules obturated with the test sealers or not (control) after irrigation with Protocols A: 2% NaOCl and B. 2% NaOCl + 17% EDTA. The viability staining indicated live cells in green, and dead cells in red. Each picture represents an area of 275 x 275 μm.

23
24
419x161mm (150 x 150 DPI)

25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

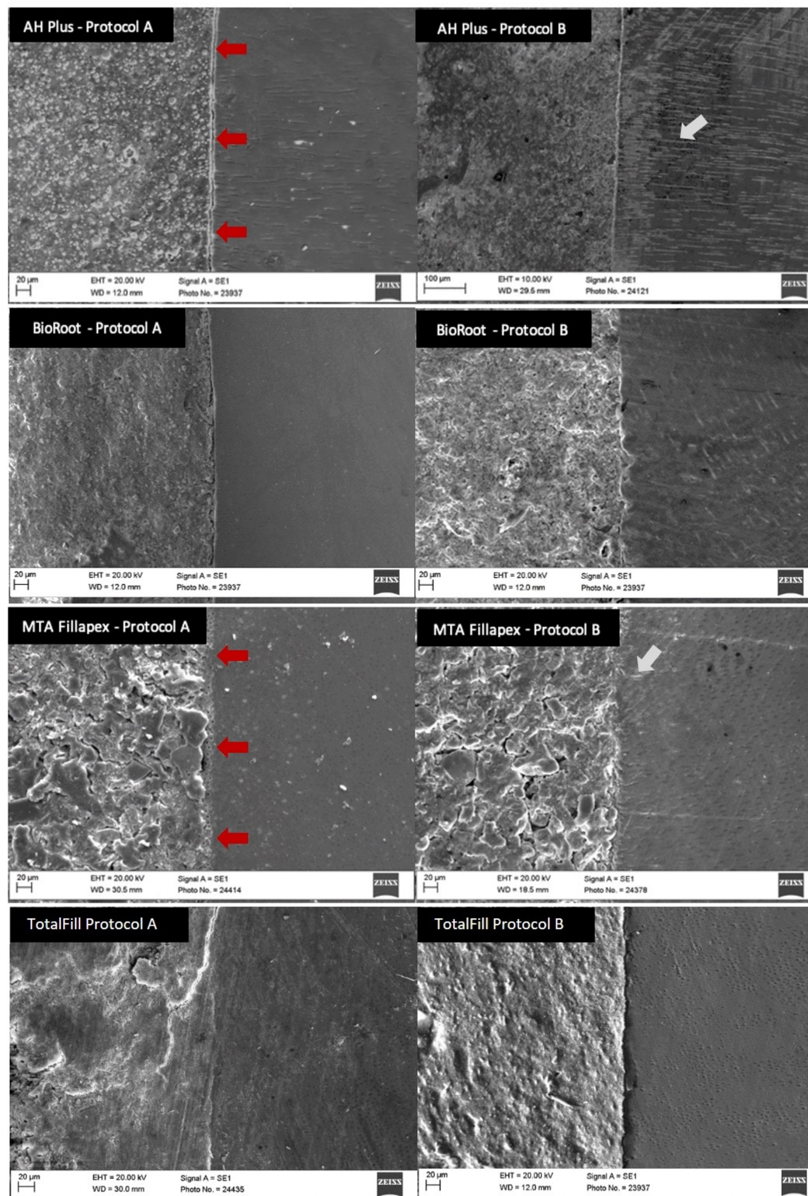


Figure 4. Secondary electron scanning electron micrographs of the interfacial interactions between sealers (left side of each image) and dentine (right side) previously irrigated with 2% NaOCl (Protocol A) or 2% NaOCl + 17% EDTA (Protocol B). Interfaces with poor adaptation are marked as red arrows and sealer penetration in the dentinal tubules as white arrows.

179x261mm (150 x 150 DPI)