

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

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

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3 **The presence of smear-layer affects the antimicrobial action of root canal**
4 **sealers**
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8

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17 **Key words:** antimicrobial activity, AH Plus, BioRoot, MTA Fillapex, smear layer,
18 TotalFill BC Sealer.
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23 **Running title:** Smear-layer on dentine affects sealer properties
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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 Ca²⁺ 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

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

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

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3 HCSC with their surroundings resulting in the improvement of the biological properties of the
4 materials (Yildirim *et al.* 2008, 2010). EDTA's ability to chelate calcium ions has been shown
5 to disrupt HCSC hydration (Lee *et al.* 2007) compromising their hardness, flexural and bond
6 strength (Yan *et al.* 2006, Yildirim *et al.* 2010, Aggarwal *et al.* 2011). The interaction of the
7 HCSC with the dentine and their enhanced antimicrobial characteristics following hydration
8 has led to a paradigm shift in the use of chelators prior to root canal filling.
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16 HCSCs are affected by variations in pH, temperature and humidity (Prati & Gandolfi 2015,
17 Khalil *et al.* 2016), therefore experimental models should be chosen with care. Agar diffusion
18 tests (ADTs), direct contact tests (DCTs) and dentine block models are commonly employed
19 to assess the antimicrobial activity of endodontic sealers (Zhang *et al.* 2009b, Andrade *et al.*
20 2015, Arias-Moliz & Camilleri 2016, Candeiro *et al.* 2016). The buffering capacity of the agar
21 plate and its possible interaction with the disinfecting agents invalidate ADTs (Editorial-Board
22 2007). Despite being a widely accepted technique, it's substitute DCTs (Editorial-Board 2007)
23 may affect the chemistry and hydration of HCSCs by bacteriological media. The use of dentine
24 block models provides a reliable method to assess the complex interactions between dentine,
25 biofilm and sealer (Wang *et al.* 2014).
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38 To date the effect of the irrigation protocol used prior to root filling has not been investigated
39 in depth. The antimicrobial activity of HCSC sealers is well established and several studies
40 have evaluated the effect of the final irrigant on properties of the sealer (Lee *et al.* 2007, Arias
41 Moliz & Camilleri 2016). However, no studies have evaluated the effect of the disinfection and
42 smear layer removal protocols on the antimicrobial properties of HCSC sealers and the best
43 irrigation protocol that enhances the sealer properties and also how sealers with a different
44 chemistry interact with dentine after placement.
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53 The null hypotheses tested are that: 1. irrigated dentine does not influence the antimicrobial
54 activity of ~~the~~ HCSC and epoxy resin sealers against *Enterococcus faecalis* (*E. faecalis*)
55 intratubular infection in dentine, taking into consideration the presence of smear layer for the
56 non-chelator protocol; 2. sealers with a different chemistry interact in the same way with
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3 dentine; 3. dentine modified by the irrigation protocols interacts with sealers in the same way
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5 as unmodified dentine.
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8 **Material and Methods**

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10 The following irrigation protocols were used:

- 13 - Protocol A: 2% NaOCl (Cerkamed, Stalowa Wola, Poland) - 5 mL/5 minutes;
- 14 - Protocol B: 2% NaOCl - 5 mL/5 minutes - followed by 17% EDTA (Sigma, Stoinhem,
15
16 Germany) – 5 mL/3 minutes.
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20 *Microstructural and chemical analysis of the dentine*

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23 Extracted human maxillary incisors were obtained from the Birmingham Dental School Tissue
24 Bank – School of Dentistry, University of Birmingham, Birmingham, U.K. (ethical approval
25 number: 14/EM/2811- BCHCDent397), embedded in auto polymerizing epoxy resin (Epoxyfix;
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27 Struers GmbH, Ballerup, Denmark) and sectioned through their root long axis using a hard
28 tissue microtome (Isomet, Buhler, Lake Buff, IL, USA). The resulting halves were then ground
29 using progressively finer diamond discs (Stuers ApS, Ballerup, Denmark) and diamond-
30 impregnated solutions (Stuers ApS) on an automatic polishing machine (Buehler Phoenix Beta
31 Grinder/Polisher, Dusseldorf, Germany), finishing with a silicon suspension of 1 µm.
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41 The dentine specimens were treated using the chemical irrigation Protocols A or B, after which
42 they were dried in a vacuum desiccator, attached to aluminum stubs and sputter coated with
43 a conductive layer of gold using a TK8842 Gold Target (Emitech Limited, Ashford, U.K.). The
44 smear layer removal on the dentine ultrastructure after each stage of the irrigation protocol
45 was assessed by scanning electron microscopy (SEM; Zeiss MERLIN Field Emission SEM;
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47 Carl Zeiss NTS GmbH, Oberkochen, Germany) at 2K× magnification ($n = 5$). For Energy
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49 Dispersive Spectroscopy (EDS) analyses, the following parameters were used, EHT= 20 kV,
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51 Iprobe = 1000 pA and WD= 8.5 mm for a 35° take off (elevation angle). Alterations to the
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53 dentine surfaces by the different irrigation protocols were measured by monitoring changes in
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55 the calcium, phosphorus and chlorine.
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3 Fourier transform infrared spectroscopy (FT-IR) was used to determine compositional
4 changes of human dentine ($n = 5$) after each irrigation protocol. The mean of three separate
5 acquisitions of spectra data was obtained for each sample using a Nicolet 6700 FTIR machine
6 (Thermo Scientific Instruments Corp., Madison, WI, USA) and Omnic 8 software suite (Thermo
7 Scientific Instruments Corp.) within the mid-IR spectrum (range: $1600\text{--}750\text{ cm}^{-1}$) at a
8 resolution of 0.482 cm^{-1} and 32 scans. After scanning, the baseline tracing was performed,
9 and the areas under the infrared bands amide III ($1298\text{--}1216\text{ cm}^{-1}$), phosphate (1170--
10 780 cm^{-1}) and carbonate ($888\text{--}816\text{ cm}^{-1}$) were calculated using Microsoft Excel (Seattle, WA,
11 USA). Subsequently, the ratio of the amide III/phosphate was determined indicating the
12 organic components of dentine. The inorganic components were calculated by the
13 carbonate/phosphate band area ratios. As the interpositions of the bands of carbonate and
14 phosphate, the latter was subtracted from the former to obtain the real value of the phosphate
15 band.

31 *Sealer characterization*

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33 The following sealers were assessed:

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

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

53 *Antimicrobial activity test*

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3 Caries-free maxillary human incisors obtained from the Birmingham Dental School Tissue
4 Bank – School of Dentistry, University of Birmingham, Birmingham, U.K. (ethical approval
5 number: 14/EM/2811- BCHCDent397), were prepared following previously described
6 protocols (Ma *et al.* 2011) with some modifications. The teeth were sectioned horizontally
7 using a hard tissue microtome (Isomet; Buhler Ltd.) at 1 and 5 mm below the cemento-enamel
8 junction, thereby obtaining cylindrical root segments standardized to a length of 4 mm.
9 Subsequently, the root canal was enlarged with a size 6 Gates-Glidden bur (1.5 in diameter;
10 Tulsa Dentsply, Tulsa, OK, USA) under water cooling. Each root segment was sectioned into
11 two semi cylindrical halves using a hard tissue microtome (Isomet; Buhler Ltd.). The outer
12 cementum was ground with 600-grit silicon paper (Carbide; Buehler Ltd.) and the specimen
13 size was adjusted to 4 × 4 × 2 mm to fit the inner wall of a 2 mL filter tube (Pall Corporation,
14 Ann Arbor, MI, USA). The samples were then autoclave sterilized at 121°C for 20 min. After
15 this the specimens were treated with the irrigating solutions as per Protocol A or Protocol B.
16 The antimicrobial activity tests were conducted under aseptic conditions in a laminar flow
17 chamber (Guardian MSC1200; Monmouth Scientific, Bridgewater, U.K.). A standard strain of
18 *E. faecalis* (American Type Culture Collection [ATCC 29212]) was used for intratubular
19 infection. After confirmation of the strain purity by Gram staining and colony morphology, cells
20 were grown in sterile brain-heart-infusion (BHI; Oxoid, Basingstoke, UK) broth and incubated
21 at 37°C overnight. Subsequently, a spectrophotometer (Jenway 7315, Stone, UK) was used
22 to adjust the bacterial density to 10⁷ cells/mL in BHI broth, at an optical density of 1 at 600 nm
23 according to the 0.5 MacFarland standard.
24

25 Dentine specimens were placed inside the filter tubes with the internal root canal surface
26 facing outwards, followed by addition of 500 µL of the adjusted bacterial culture. The tubes
27 were centrifuged at 1400g, 2000g, 3600g, and 5000g sequentially, twice for each sample, for
28 5 minutes. After each centrifugation, the supernatant was replaced with fresh 500 µL culture.
29 The samples were then incubated at 37°C in BHI broth and in air. After every 48 hours the
30 procedure was repeated. The incubation lasted for 5 days.
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3 The dentine specimens were removed from each tube, followed by rinsing in sterile water for
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5 1 minute and air drying. The specimens were randomly divided into four experimental groups
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7 according to the sealer applied and a control group that received no treatment. Another group
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9 was prepared and was not infiltrated with bacteria. The root canal walls were dried with sterile
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11 absorbent paper points (Dentsply) and the sealers were applied into it surface using a cavity
12
13 liner applicator achieving an approximate film thickness of 0.5 mm measured with a digital
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15 pachymeter (Barasti, Amsterdam, Netherlands). The samples were set in an oven at 37°C in
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17 100% relative humidity for 7 days.
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21 For Confocal Laser Scanning Microscopy (CLSM) analysis, bacterial viability was analyzed
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23 using the SYTO 9/propidium iodide technique (Live/Dead BacLight Viability Kit; Molecular
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25 Probes, Eugene, OR, USA). Dentine discs were washed with 1000 µL phosphate-buffered
26
27 saline (PBS) to eliminate sealer residues and samples were subsequently stained with 30 µL
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29 of the dye, in a light free environment, for 10 minutes. The samples were then placed in a 35
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31 mm petri dish, covered with water and examined with an inverted Leica TCS-SP8 confocal
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33 system equipped with an DM6 upright microscope and a 40x/0.80 HCX water immersion lens
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35 (Leica Microsystems GmbH, Mannheim, Baden-Württemberg, Germany). A blank internal
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37 control comprising of a root filled tooth without bacterial contamination was used to set the
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39 threshold for the CLSM. Four confocal “stacks” of random areas were obtained for each
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41 sample at 0.5-µm step size. In total, there were 5 samples per group, thus a total of 20
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43 operative fields per group. For quantification Image J software (<https://imagej.net/Fiji>) was
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45 used to calculate the percentage of green (live cells) and red (dead cells) found after the sealer
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47 application.
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50 51 *Microstructural and chemical analysis after sealer placement*

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55 Extracted human single rooted teeth were obtained from the Birmingham Dental School
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57 Tissue Bank – School of Dentistry, University of Birmingham, Birmingham, U.K. (ethical
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59 approval number: 14/EM/2811- BCHCDent397), The teeth were stored in water. After coronal
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3 access, the working length (1 mm short of the apical foramen) was established by inserting a
4 size 10 K-file (Dentsply) until its tip was observed in the foramen through a stereomicroscope
5 (Stemi 2000C; Carl Zeiss, Jena, Germany). The root canals were chemo-mechanically
6 prepared up to the working length with ProTaper Gold system (Dentsply) in the following
7 sequence of files: SX, S1, S2, F1, F2 and F3. After the use of each instrument, each root canal
8 was irrigated with either 5 mL NaOCl (Protocol A) in a 1mL/10 seconds flow rate. In Protocol
9 B, NaOCl was followed by 5 mL of 17% EDTA for 3 minutes. The irrigants were delivered into
10 the root canal through a plastic syringe and capillary tip cannula (Ultradent, Southlake, TX,
11 USA) using an in-and-out pecking motion. The canals were then dried with paper points and
12 root filled using single cone technique with a matched gutta-percha cone and a test sealer.
13 The teeth were stored in an oven at 37°C for 7 days to completely set. Samples were then
14 embedded in auto polymerizing epoxy resin (Epoxyfix; Struers GmbH), sectioned
15 longitudinally using a hard tissue microtome and polished using an automatic polishing
16 machine. The sealer to dentine interface was imaged at 500 X magnification ($n = 5$). EDS
17 analysis of the dentine was performed for calcium, phosphorus and chlorine at a distance of
18 100 μm from the interface. This was compared to control dentine that had been irrigated using
19 either Protocol A or B but not obturated.

20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 **Statistical analysis**

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

48 49 50 51 52 53 54 55 56 57 **Results**

58 59 60 *Microstructural and chemical analysis of the dentine*

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3 The scanning electron micrographs of the dentine irrigated with Protocols A or B with untreated
4 dentine used as control are shown in Figure 1a and the elemental analysis for calcium,
5 phosphorus and chlorine is shown in Table 1a. Smear layer was still present after 2% NaOCl
6 irrigation. When NaOCl was followed by EDTA widening of the dentinal tubules was observed
7 (Figure 1). EDS analysis revealed deposition of chlorine in the dentine for both irrigation
8 protocols, with higher values ($p < 0.05$) following 2% NaOCl final flush (Table 1). The lowest
9 values of calcium and phosphate resulted after EDTA rinse.
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18 The results of dentine treatment with Protocols A and B on the amide III/phosphate and
19 carbonate/phosphate ratio are presented in Table 2. Dentine surfaces before (initial) and after
20 immersion in the irrigation solutions were not significant in terms of organic and inorganic
21 components.
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27 *Sealer characterization*

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29 The scanning electron micrographs (Figure 2a) revealed a microstructure with particles of
30 various sizes and opacity for all the test sealers. The shiny particles (marked as red arrows)
31 were rounded for AH Plus, and were evenly distributed across the sample. BioRoot, MTA
32 Fillapex and TotalFill had non-uniformly distributed particles, with no shape standardization.
33 The latter exhibited particles with lower levels of brightness occurring in smaller quantities.
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41 EDS scans are shown in Figure 2b. AH Plus and MTA Fillapex exhibited shiny particles which
42 were rich in calcium (Ca) and tungsten (W) and the former also had particles composed of
43 zirconium (Zr) and oxygen (O). BioRoot and TotalFill also contained zirconium whilst presence
44 of chlorine (Cl) was found in BioRoot. MTA Fillapex, BioRoot and TotalFill all included calcium
45 (Ca), silicon (Si) and oxygen (O) while the MTA Fillapex included also aluminium and traces
46 of titanium.
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54 *Antimicrobial activity test*

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3 Table 3 shows the percentage of live cells in the dentinal tubules after contact with different
4 sealers after the dentine was irrigated using either Protocol A or B. Representative confocal
5 laser scanning microscope images of the groups described in Table 3 are shown in Figure 3.
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10 After root canal dentine was irrigated with 2% sodium hypochlorite only (Protocol A), BioRoot
11 eliminated more bacteria than the other sealers ($p < 0.05$). AH Plus and TotalFill were not
12 statistically significant ($p > 0.05$) and were similar to the control group (unirrigated and unfilled
13 teeth) thus ineffective against *E. faecalis*. When EDTA was used in conjunction with NaOCl
14 as in Protocol B, BioRoot and AH Plus both showed a reduction in viable bacteria in the
15 dentinal tubules ($p < 0.05$).
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18 *Microstructural analysis after sealer placement*

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21 The scanning electron micrographs of the dentine to sealer interface for all sealers using both
22 irrigation protocols are shown in Figure 4. Protocol A was associated with interfaces with poor
23 sealer adaption to dentine for AH Plus and MTA Fillapex (marked as red arrows). For AH Plus
24 the removal of smear layer allowed the penetration of the sealer in the dentinal tubules when
25 using irrigation Protocol B. MTA Fillapex also exhibited tubular penetration but this was not
26 as marked as the AH Plus (marked as white arrows). The BioRoot and TotalFill sealers
27 showed adequate had sealer to dentine interfaces without gaps using both protocols.
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32 The EDS analyses for calcium, phosphorus and chlorine performed on the dentine 100 μm
33 away from the sealer-dentine interface after root canal filling are shown in Table 4. After sealer
34 placement the levels of calcium in dentine increased for HCSC groups for both irrigation
35 protocols with the highest values for BioRoot ($p < 0.05$). When the dentine was irrigated with
36 both sodium hypochlorite and EDTA (Protocol B), TotalFill exhibited lower values of
37 phosphorus when compared with the other sealers ($p < 0.05$), while in Protocol A, no
38 differences occurred between them ($p > 0.05$). The control (unfilled root canal) had higher
39 values of chlorine than dentine obtained from root-filled teeth.
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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

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2
3 dentine-sealer hermetic seal is not easy to achieve (Tay *et al.* 2005) the release of
4 antimicrobial compounds by sealers ~~would~~ may prevent bacterial ingress from the root canals
5
6 to the periapical tissues.
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10 Although endodontic infections are caused by multispecies biofilms, the survival and virulence
11 factors possessed by *E. faecalis* make it the preferred single microorganism used for
12 laboratory research to mimic persistent endodontic infections (Stuart *et al.* 2006).
13
14 Furthermore, this microorganism withstands centrifuging which is an essential step of the
15 intratubular infection process. This method provides a robust model to investigate the
16 antimicrobial properties of materials since it takes into consideration the effect that the dentine
17 has on material characteristics. This is particularly important when investigating materials such
18 as HCSCs which interact chemically and physically with dentine. The use of NaOCl followed
19 by a calcium chelator has been recommended and used in a number of studies (Ma *et al.*
20 2011, Andrade *et al.* 2015, Arias-Moliz & Camilleri 2016) to facilitate bacterial penetration into
21 open dentinal tubules (Haapasalo & Orstavik 1987). By modifying a previously described and
22 validated experimental procedure the present study verifies that bacteria are present in
23 dentinal tubules regardless of the presence of the smear layer as shown in Figure 3.
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38 The antimicrobial activity of HCSCs occurs after cement hydration, where Ca^{2+} ions from the
39 calcium silicate particles of the cement reacting with the water added or present in the clinical
40 environment are released (Camilleri 2007). Elemental migration of the experimental sealers,
41 indicated by enhanced levels of Ca^{2+} on smear-layer-rich dentine interface, suggests the
42 material interacts with dentine (Atmeh *et al.* 2012) which was not displayed by AH Plus. EDTA
43 has been shown to interfere with cement hydration due its chelating ability in the absence of
44 dentine (Lee *et al.* 2007), whereas the only source of Ca^{2+} ions would be those released by
45 HCSCs. To mimic the clinical situation, in this study, the effect of NaOCl (Protocol A) and
46 NaOCl + EDTA (Protocol B) was investigated on dentine, relative to the antimicrobial action
47 of HCSCs. A 3 minutes flush with EDTA to reduce debris on the dentine in the apical third of
48 the root was employed (Teixeira *et al.* 2005), resulting in no improvements on smear layer
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3 scores following irrigant agitation in straight root canals (Mayer *et al.* 2002, Rödig *et al.* 2010).
4
5 In the current study with the use of NaOCl only, BioRoot had the highest percentage of dead
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7 intra-tubular bacteria. TotalFill which is also a HCSC sealer had a similar response to AH Plus
8
9 and the control group.
10

11
12 The use of NaOCl followed by EDTA resulted in fewer live bacteria for all sealers. The reason
13
14 for the enhanced sealer activity could be multifactorial where the chelation preferentially
15
16 affected dentine rather than the sealer (Hill 1959), and the Ca^{2+} released through the sealer
17
18 surpassed the levels of Ca^{2+} extracted by EDTA. The smear-layer-free dentine and its greater
19
20 wettability favoured the diffusion of hydraulic cements and fluids through dentinal tubules,
21
22 increasing the ion exchange and hydration of HCSC (Tartari *et al.* 2018).
23

24
25 The hydration patterns of various HCSCs have been shown to be different (Camilleri *et al.*
26
27 2013, Marciano *et al.* 2016, Schembri-Wismayer & Camilleri 2017). BioRoot is mixed with
28
29 water and thus the hydration is always guaranteed regardless of the environmental presence
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31 of fluids. This leads to a lower bacterial viability (Arias-Moliz & Camilleri 2016, Colombo *et al.*
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33 2018) which has also been shown in the current study. It killed even bacteria inside the dentinal
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35 tubules without the removal of the smear layer while the TotalFill was ineffective. The hydration
36
37 of HCSC significantly influences the release of calcium hydroxide. Therefore, as a water-
38
39 based sealer, BioRoot RCS does not rely on OH^- ions derived from tissue fluids to form calcium
40
41 hydroxide, as does MTA Fillapex which is a 2-paste system (Marciano *et al.* 2015) or TotalFill,
42
43 a premixed ready-to-use injectable sealer (Loushine *et al.* 2011). Since BioRoot RCS
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45 has higher Ca^{2+} ion leaching regardless of the dentine pre-treatment (Table 4) and thus,
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47 calcium hydroxide peaks (Xuereb *et al.* 2015), the present results show an expected higher
48
49 percentage reduction of *E. faecalis* viability for both irrigation protocols. This is in agreement
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51 with previous research that reported the superiority of BioRoot BCS against intratubular
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53 infection and in the direct contact test (Arias-Moliz & Camilleri 2016, Colombo *et al.* 2018).
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55 The vehicles used in the TotalFill and the MTA Fillapex have an influence on rates of ionic
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57 dissociation and diffusion of calcium hydroxide (Estrela *et al.* 1999, Zancan *et al.* 2016).
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3 The lesser antimicrobial effect of TotalFill against *E. faecalis* might have been influenced by
4 insufficient levels of calcium hydroxide released. The hydration of TotalFill cannot be assumed
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6
7 *in vivo* and conditions of tissue fluids cannot be monitored or reproduced in laboratory settings.

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9 The TotalFill specimen discs exposed to 100% relative humidity required 10 days to achieve
10 their final setting (Loushine *et al.* 2011), raising doubts about their complete hydration in a
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14 period of 7 days enclosed within the root canal, as demonstrated in this study. The hydration
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17 of TotalFill might be affected by the addition of calcium phosphate to tricalcium silicate,
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20 resulting in a reduction of crystalline calcium hydroxide (Loushine *et al.* 2011, Schembri-
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23 Wismayer & Camilleri 2017); its entire dependence on an aqueous environment to hydrate
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26 and set, and its presentation as a water-free paste (Xuereb *et al.* 2015). The higher
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29 susceptibility of *E. faecalis* to TotalFill achieved in previous report (Wang *et al.* 2014) might be
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32 explained by the wider dentinal tubule openings obtained by means of previous irrigation with
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35 a stronger chelating agent than the one used in the present study. This allowed better sealer
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38 penetration and enhanced antimicrobial action, while maintaining the order of susceptibility,
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41 since no differences were found between TotalFill and AH Plus in both studies. The direct
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44 contact test disregarded the dentine-buffering effect and might have benefitted TotalFill
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47 hydration by the water content of the bacterial suspension, which may account for the better
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50 results found for the antimicrobial action of TotalFill (Zhang *et al.* 2009b, Candeiro *et al.* 2016).

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53 MTA Fillapex is chemically based on mineral trioxide aggregate and salicylate resin, which
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56 results in the release of lower levels of calcium hydroxide (Viapiana *et al.* 2014). The mildly
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59 alkaline pH (Silva *et al.* 2013, Viapiana *et al.* 2014) correlated to its ability to release Ca^{2+} ions
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(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

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3 *E. faecalis* within the dentinal tubules. The use of EDTA was important for AH Plus as it
4 improved the penetration of sealer tags into dentinal tubules and therefore increased the range
5 of its antimicrobial action and mechanical bond to dentine. The collagen exposed by EDTA
6 chemically bonds to the epoxy rings of AH Plus to initiate its setting reaction (Johns 1982), but
7 it is not impaired by the proteolytic action of NaOCl on Protocol A, as collagen ratios were the
8 same after the NaOCl flush (Neelakantan *et al.* 2011).
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16 It is well established that intra-radicular microorganisms either remaining or recolonizing the
17 filled canal system are the main aetiological agents of apical periodontitis (Kakehashi *et al.*
18 1965). The essential basis for the choice of a sealer must thus be to optimize root canal
19 disinfection by eradicating the root canal microbes or substantially reducing the microbial load
20 and to prevent re-infection by an effective dentin-sealer seal (Nair *et al.* 2005). Although none
21 of the sealers tested eliminated the bacteria present in total, BioRoot reduced the bacterial
22 load even when EDTA was not used in the irrigation protocol. Furthermore, when EDTA
23 irrigation was employed, BioRoot restored the calcium levels of the dentine.
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36 **Conclusions**

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

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59 The authors declare no conflict of interest
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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
<i>Calcium</i>	24.76 ± 1.15 ^A	23.94 ± 2.64 ^A	18.68 ± 2.72 ^B
<i>Phosphorus</i>	13.29 ± 0.45 ^A	13.01 ± 1.23 ^A	10.33 ± 0.94 ^B
<i>Chlorine</i>	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)
<i>Amide/Phosphate</i>	32.99 ^A (13.1 – 131.8)	17.27 ^A (9.28 – 64.82)	49.23 ^A (27.92 – 277.4)
<i>Carbonate/Phosphate</i>	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.

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)
<i>Protocol A</i>	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)
<i>Protocol B</i>	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.

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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
<i>Protocol A- Ca</i>	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}
<i>Protocol B- Ca</i>	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}
<i>Protocol A- P</i>	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}
<i>Protocol B- P</i>	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}
<i>Protocol A- Cl</i>	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}
<i>Protocol B- Cl</i>	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 statistically significant differences for the same sealer and ion comparing protocols (One -way repeated measures Anova and Tukey post -hoc p -value < 0.05).

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.

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.

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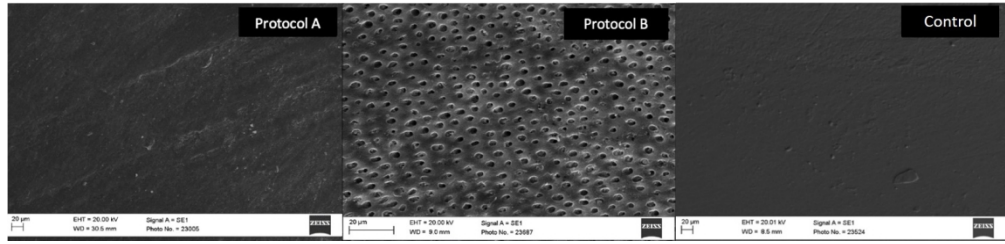


Figure 1. Secondary electron scanning electron micrographs at 2Kx magnification of dentine irrigated with protocols (A) 2% sodium hypochlorite and (B) 2% NaOCl + EDTA and saline as a control showing the ability of the EDTA to remove the smear layer.

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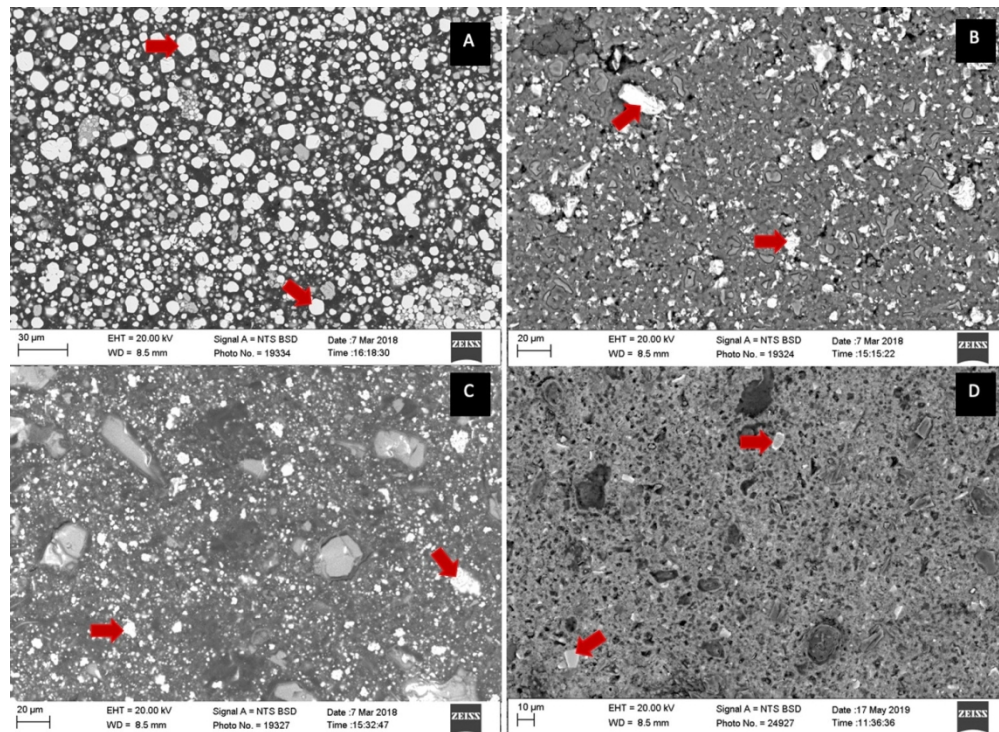


Figure 2a. Backscatter scanning electron micrographs at 2Kx magnification of polished sections of test sealers showing microstructural components (A) AH Plus showing the presence of rounded shiny particles of various sizes evenly distributed across the section; (B) BioRoot and (C) MTA Fillapex exhibiting 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.

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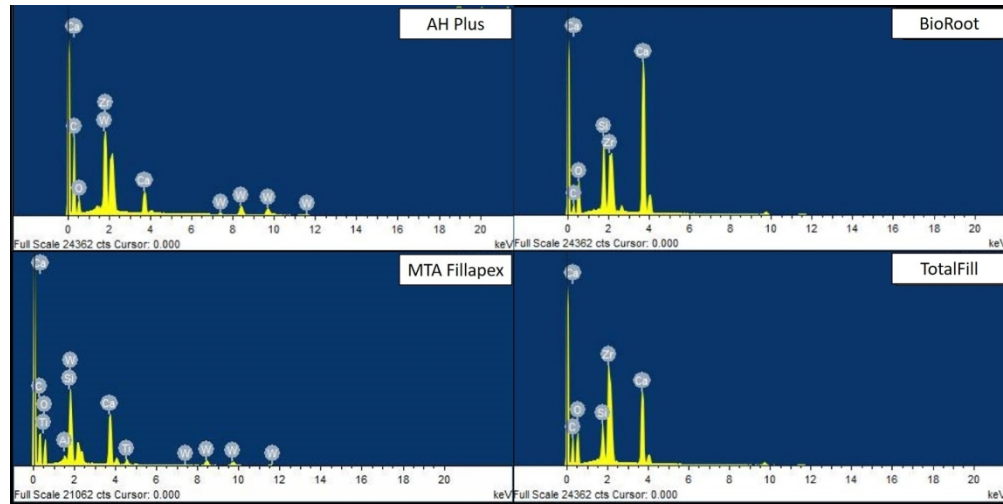


Figure 2b. Corresponding energy dispersive spectroscopic scans of the test sealers shown in Figure 1b displaying high peaks of calcium, carbon and oxygen. Silicon was detected only for HCSC. The shiny particles on SEM (marked as red arrows in Figure 1b) are mainly composed of (A) AH Plus, calcium, tungsten,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

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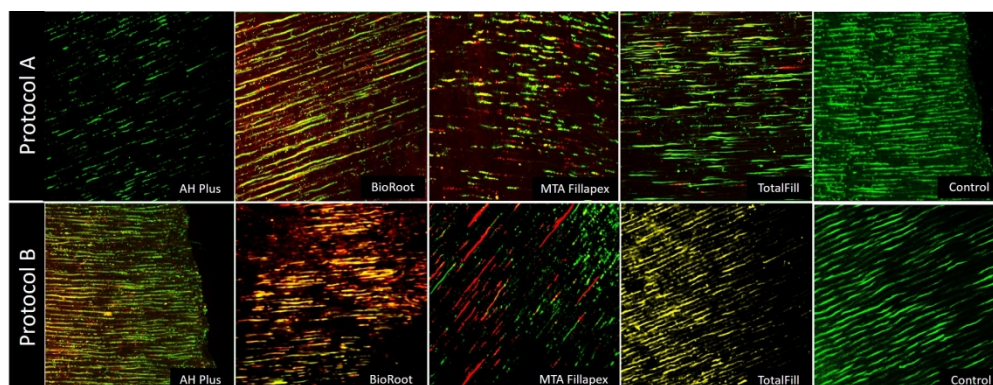


Figure 3. Representative confocal laser scanning microscope images of *E. faecalis* infected dentinal tubules obturated with the test sealers or not (control) after irrigation with Protocol A: 2% NaOCl and B. 2% NaOCl + 17% EDTA. The viability staining indicated live cells in green, and dead cells in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

419x161mm (150 x 150 DPI)

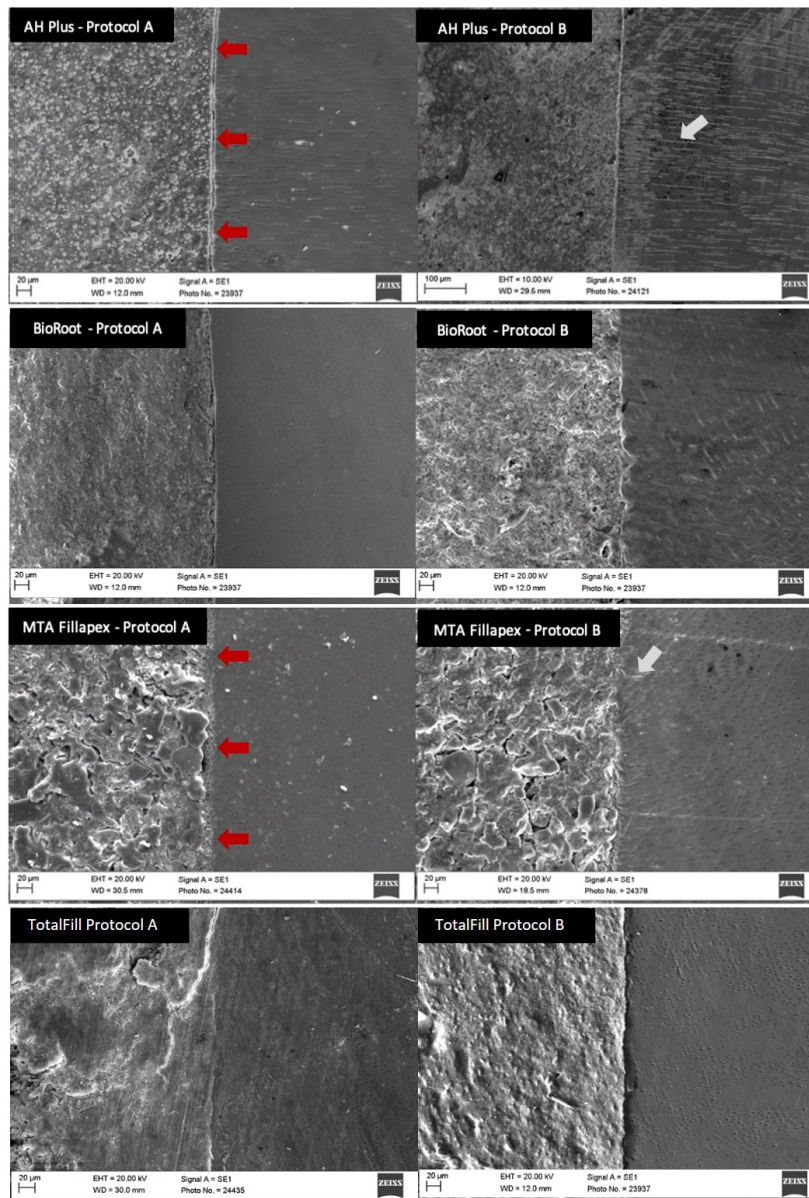


Figure 4. Secondary electron scanning electron micrographs of the interfacial interactions between sealers (left side of each image) and dentine (right side) previously irrigated with 2% NaOCl (Protocol A) or 2% NaOCl + 17% EDTA (Protocol B). Interfaces with poor adaptation are marked as red arrows and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

179x261mm (150 x 150 DPI)