



Optimised clinical protocol for root canal obturation using single cone and hydraulic cement sealer

Sweta Surana Bhandari^a, William M. Palin^a, Sarah A. Kuehne^b, Josette Camilleri^{a,*}

^a Dentistry, College of Medicine and Health, University of Birmingham, Birmingham, United Kingdom

^b Nottingham Trent University, School of Science and Technology, Nottingham, United Kingdom

ARTICLE INFO

Keywords:

Root canal obturation
Hydraulic cements
Irrigation protocol
Continuous chelation
Dentine characterisation
Antimicrobial characteristics
Split tooth model

ABSTRACT

Introduction: This study aimed to establish an optimised clinical protocol based on matching the root canal preparation, irrigation and obturation by choosing an adequate that prepared the dentin and reduced the microbial load followed by a root canal obturation that maintained the seal.

Methods: A split tooth model was used to assess the irrigation and obturation quality. Three irrigation sequences including sodium hypochlorite, EDTA and HEDP with water used as a control. Four commercial sealers (AH Plus, BioRoot RCS, BioRoot Flow and TotalFill) were used to obturate the root canal using single cone obturation technique. The ultrastructure and chemical properties of the dentin and sealers and the dentin-sealer interface were assessed before and after obturation and contact with the irrigation sequences using scanning electron microscopy, energy dispersive spectroscopy, and Fourier transform infrared spectroscopy. Microbiological analysis by direct contact test and minimum inhibitory concentration was undertaken with and without dentin contact. All results were analysed using a two-way ANOVA and Tukey post hoc tests ($p < 0.05$).

Results: Irrigation with HEDP resulted in less erosion of dentin and a larger mineral concentration zone at the interface of hydraulic cements with dentin. Furthermore, continuous chelation with HEDP improved the antimicrobial properties of the hydraulic cement sealers. All sealers exhibited a reduction in their antimicrobial effectiveness when in contact with irrigated dentin.

Conclusions: The irrigation techniques affected the interfacial characteristics and antimicrobial properties of root canal sealers, indicating that a selection of appropriate irrigation protocols for clinical practice is required to optimize treatment outcomes.

1. Introduction

Root canal treatment involves the removal of the diseased dental pulp followed by chemo-mechanical preparation and filling of the root canal space undertaken to preserve the function of natural teeth. The debridement and shaping of the canal space facilitates the placement of the root canal filling. To predictably remove all microbial aetiology from the root canal system and dentinal tubules, mechanical debridement should be accompanied with chemical disinfection through copious antimicrobial irrigation using a variety of solutions [1].

Irrigating solutions are required for elimination of microbial load and dentin preparation during root canal treatment. It is suggested that these irrigating solutions may cause structural and chemical alterations of root dentin [2] affecting the adhesion of sealers to dentin as well as

their antimicrobial properties [3]. Consideration should be given to appropriate selection of irrigation protocols for dentin preparation and matching to sealer type [4,5].

The most reliable irrigating solution is sodium hypochlorite (NaOCl) as it reduces the microbial load but unfortunately, it also denatures the collagen in dentin [6,7]. Mechanical instrumentation creates a smear layer, blocking the dentinal tubules which can be cleared by the chelating agents such as ethylene diamine tetracetic acid (EDTA). When utilising epoxy-resin-based sealers like AH Plus (Dentsply, DeTrey GmbH, Konstanz, Germany), calcium chelation of the dentin by EDTA is required for the sealer to attach to the exposed collagen with the help of resin tags and penetrate the dentinal tubules [8,9,10], improving the seal and minimising the chances of apical leakage [11,12].

Contrary to that, when utilising hydraulic calcium silicate (HCS)

* Correspondence to: Birmingham Dental Hospital and School of Dentistry, University of Birmingham, 5, Mill Pool Way Edgbaston, Birmingham B5 7EG, United Kingdom.

E-mail address: J.Camilleri@bham.ac.uk (J. Camilleri).

<https://doi.org/10.1016/j.dental.2026.02.011>

Received 14 August 2025; Received in revised form 10 February 2026; Accepted 11 February 2026

Available online 16 February 2026

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Table 1a
Sealers and irrigating solutions used in the study.

Sealer/Irrigant	Manufacturer	Batch number
AH Plus Jet - AH	Dentsply DeTrey GmbH, Konstanz, Germany	1904000728
BioRoot RCS - BR	Septodont, Saint-Maur-des-Fossés, France	B27015
BioRoot Flow - BRF	Septodont, Saint-Maur-des-Fossés, France	B24447BB
TotalFill BC - TF	FKG Dentaire, La Chaux-de-Fonds, Switzerland	21001SP
5.25 % NaOCl	Chloraxid; Cerkamed, Stalowa Wola, Poland	
17 % EDTA	Endo Solution, Cerkamed, Stalowa Wola, Poland)	
9 % HEDP	Dual Rinse HEDP, MedCem gmbH, Weinfelden, Switzerland	
Distilled water	/	

sealers, the interface with the dentin is different. The release of calcium hydroxide in contact with the dentin results in changes to the dentin with movement of mineral from material to tooth across the interface [13]. The chelation and opening of the dentinal tubules also allows the formation of sealer tags as well. Due to the reactivity of the HCS sealers, the final irrigating solution is important as it changes the dentin

characteristics [3] and also reacts with the sealer modifying the reaction mechanics with dentin [13,14]. The antimicrobial characteristics of hydraulic calcium silicate sealers are modified by contact with irrigating solutions [3,15].

Several studies have addressed the effect of irrigation solutions on sealer properties and the effect of disinfection on dentin [5,14,16,17,18,19,20]. However, there is limited information regarding the effect of the irrigation and smear layer removal protocols on the chemical and antimicrobial properties of sealers and the best irrigation sequence that prepares the dentin substrate for interaction with obturating materials. Consequently, the aim of this research is to assess the changes to the microstructure and chemical properties of dentin and root canal sealers with different chemistries and delivery after using various irrigation sequences. Furthermore, the effect of the irrigation and obturation on the antimicrobial properties of the dentin and obturating materials will also be investigated. The null hypothesis is that continuous chelation by irrigation does not optimise the root dentin and improve the antimicrobial properties of HCS sealers, leading to improved interfacial integrity. The data will be used to propose an optimised clinical protocol for root canal therapy. The matched irrigation to obturation strategy is an innovative clinical approach where the root canal is prepared to accommodate specific sealer chemistry. This ensures the microenvironment in the root canal is conducive to a better seal through

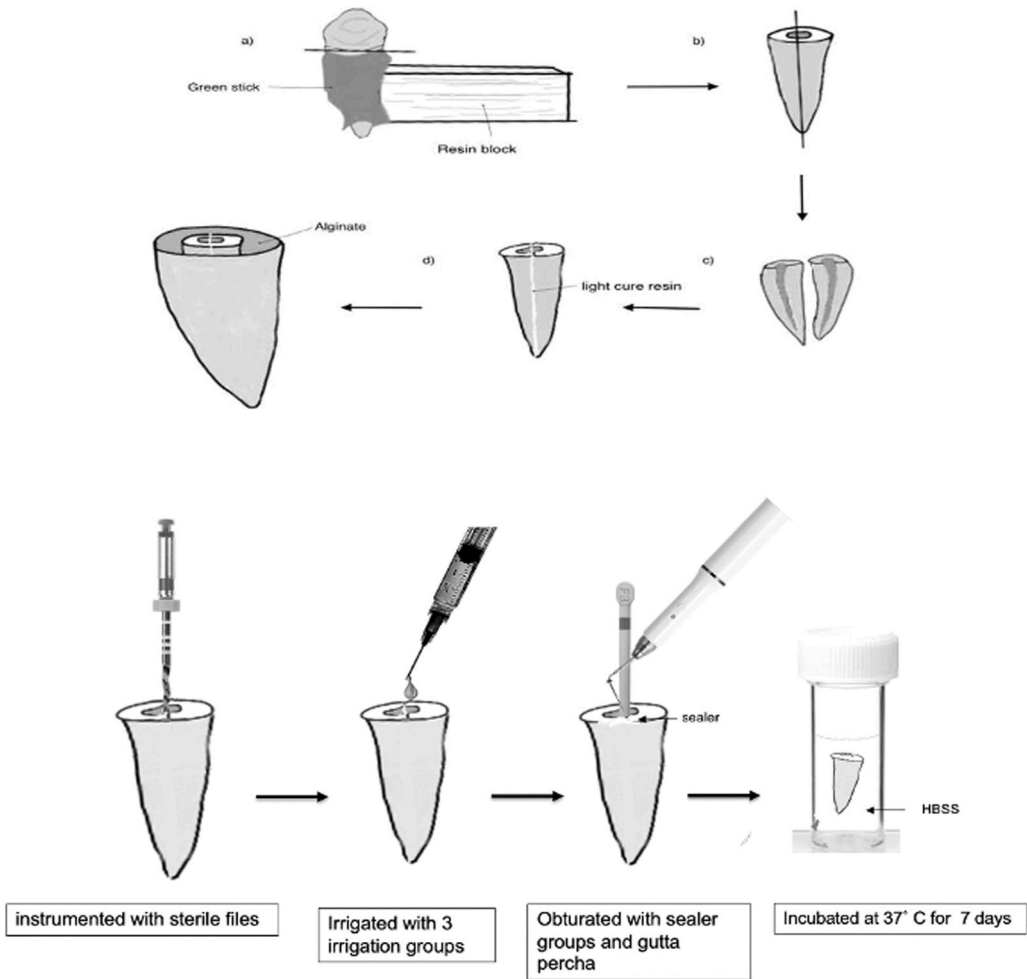


Fig. 1. Schematic representation of the split tooth model used to prepare the teeth enabling the undertaking of endodontic procedures followed by atraumatic root splitting and retrieval of the endodontic materials for analyses. The preparation involves (a) fixing the tooth to an acrylic block with green stick compound. The tooth was decoronated and root length standardised to 14 mm using a hard tissue microtome (b). The roots were then split longitudinally (c) to obtain 2 root halves; (d) The root halves were reassembled and held in place with light cured unfilled resin; (e) the assembly was placed in alginate to simulate a closed apical system. It is then followed by instrumentation of the root canal space with sterile files, irrigation with different groups, obturation and coronal seal with GIC followed by immersion in HBSS and incubation for 7 days at 37C.

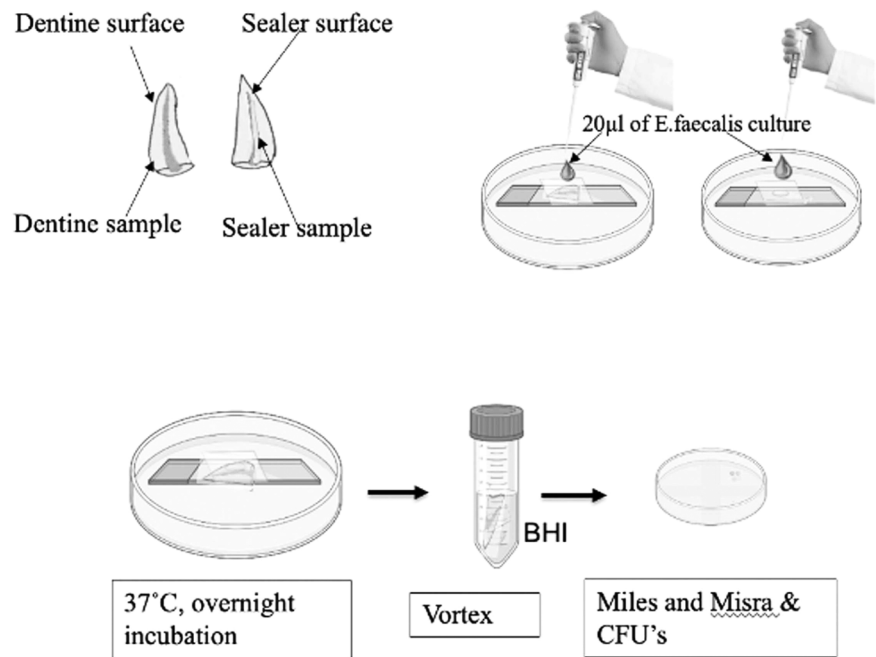


Fig. 1. (continued).

Table 1b
Irrigating solutions tested in different sequences with the time employed per solution. NaOCl (sodium hypochlorite), EDTA (ethylenediaminetetraacetic acid), HEDP (1-hydroxyethylidene-1,1-diphosphonic acid) (mins= minutes).

Groups	Main Irrigants (During instrumentation)	Time mins	Final Irrigants (After instrumentation)	Time mins
Group A	Distilled water (control)	5	Distilled water (control)	5
Group B	NaOCl 5.25 %	5	NaOCl 5.25 %	5
Group C	EDTA 17 %	5	EDTA 17 %	5
Group D	NaOCl 5.25 %	5	EDTA 17 %	5
Group E	HEDP 9 % + NaOCl 5.25 %	5	HEDP 9 % + NaOCl 5.25 %	5
Group F	HEDP 9 % + Distilled water	5	HEDP 9 % + Distilled water	5

interaction with the materials and antimicrobial characteristics. A split tooth model that mimicked the clinical set up will be used.

2. Materials and methods

The current study is an *in vitro* assessment of the changes to the dentin using different irrigation sequences after which the chemical and antimicrobial properties of different root canal sealers will be assessed. The interaction of material to substrate at the tooth to material interface will also be analysed.

2.1. Materials and reagents

The irrigating solutions and the sealer types used in the current study are shown in Table 1a. The 5.25 % NaOCl and 17 % EDTA were used as supplied by the manufacturer. The 9 % HEDP was freshly prepared by mixing 0.9 g of Dual Rinse® HEDP powder (mean content per capsule) as recommended by the manufacturer with 10 ml of 5.25 % NaOCl before each experiment. Another group was created by mixing with distilled water.

The sealers were dispensed directly for AH Plus using the jet system and BioRoot Flow and Totalfill BC sealers using the nozzles provided by

the manufacturer. The BioRoot RCS was mixed as instructed by the manufacturer and was placed in the root canals using a hand file. Material and dentin characterisation was done initially followed by assessment of tooth to material interface along with microbiology.

2.2. Sealer characterisation

Sealer specimens (n = 3) were prepared in 10 mm diameter and 1 mm thick rubber moulds in triplicates. All sealers were allowed to be set at 37 °C, 100 % humidity in the incubator (Memmert GmbH, Schwabach, Germany). The sealers were then characterised using various methods, immediately after setting and after immersion in Hank's balanced salt solution (HBSS, Sigma Aldrich, H6648) for 7 and 90 days. The characterisation was undertaken by scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) (EVO MA10, Carl Zeiss Ltd., Cambridge, UK) after hardening and retrieval from immersing solution, embedding in resin, polishing and gold coating. In terms of elemental content and elemental distribution was assessed under 1 K magnification with the following parameters, EHT = 20 kV, I probe = 1000 PA, and WD = 8.5 mm for a 35°take-off (elevation angle). Furthermore, phase analysis by X-ray diffraction (XRD; Bruker D8 Advance, Bruker Corp., Billerica, MA, USA) was conducted with a search-match software using the COD database (Crystallography Open Database). FT-IR spectra were collected and analysed in the infrared spectrometer (Thermo Nicolet Nexus 4700 FT-IR Spectrometer) using transmitted infrared spectroscopy. All spectra were collected within the spectral range of 4000–400 cm⁻¹. The leachate collected after 7 and 90 days and every 15 days for a period of 90 days from the solutions was assessed for calcium release by inductively coupled plasma mass spectroscopy (ICP-OES; Optima 8000 ICP-OES, Perkin Elmer, Waltham, MA, USA).

2.3. Dentin characterisation

A split tooth model [21] which has been used and validated in previous research was used to provide a clinically relevant assessment of the dentin characterisation and microbial investigation. The preparation of the specimens (n = 3) with single sample per root section, is shown in Fig. 1. Extracted human premolars with single straight canals with fully

formed roots were obtained from the Birmingham Dental School Tissue Bank, School of Dentistry, University of Birmingham, Birmingham, UK (ethical approval number: BCHC Dent 005.2024). Root canals were prepared with Wave One Gold rotary instruments (Dentsply Sirona, North Carolina, USA) to an apical size of 35 and 0.06 taper. Irrigation using groups A, B, C, D, E, and F (Table 1b) was performed with a disposable 5 ml plastic syringe coupled to a 27-G monoject endodontic irrigation needle (Covidien LLC, Mansfield, MA, USA) placed passively to about 2 mm from the apical foramen without binding. Apical patency was maintained with the #10 K-file. The canals were dried with paper points and separated into their two halves along the cleavage created earlier.

Characterisation of dentin was undertaken by scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and Fourier infrared (FT-IR) spectroscopy after the application of different irrigation protocols. The dentin ultrastructure of one-half of each tooth and the irrigation protocol from apical, middle, and coronal points were assessed by SEM at 2000 x magnification in backscatter mode. For FT-IR, the mean of two separate acquisitions of spectra data was obtained for each sample using a Nicolet 6700 FT-IR machine (Thermo Scientific Instruments Corp., Madison, WI, USA) and Omnic 8 software suite (Thermo Scientific Instruments Corp.) within the mid-IR spectrum (range: 2000–700 cm^{-1}) at a resolution of 0.482 cm^{-1} and 32 scans.

2.4. Assessment of the tooth to material interface

Root segments of 4 mm length were obtained by sectioning the decoronated tooth segment horizontally, 1 mm below the cemento-enamel junction, using a microtome (Accuton 50, Struers, Ballerup, Denmark). These root blocks were further sectioned longitudinally to get two halves. The outer cementum of each half was then removed and polished on all sides with 220–800-grit silicon carbide paper to obtain a flat 4x4x2 mm specimen. These samples were then washed with distilled water. A mould was created by silicon putty (Affinis Putty Soft, Coltene) by embedding a tooth sample (which was removed later). Teeth were irrigated with Groups A, D and E, dried and different sealers was placed on the specimen mentioned in Table 1b. The assembly was incubated overnight. After 24 h, the dentin sealer assembly was embedded in cold cure resin (Epofix, Struers, Ballerup, Denmark). Once the resin was set, the block was sectioned longitudinally into two halves with a microtome (Accuton 50, Struers, Ballerup, Denmark). Samples were then incubated in 5 ml Hank's balanced salt solution (HBSS) in a bijoux tube to age for 7 days. After aging the dentin sealer interface of the samples was analysed by SEM, EDS and FT-IR.

For EDS four different point scans for silicon (Si), calcium (Ca), and zirconium (Zr) were taken on material, interface, and dentin. The ratios of Ca/Si and Ca/Zr were thus calculated. For the FT-IR spectra, seven equidistant points were selected in a vertical direction, three in material, three in dentin, and one on the interface as undertaken in the EDS analyses.

2.5. Microbiological analysis

The antimicrobial properties of the sealers were assessed on freshly set sealers and after contact with irrigated dentin.

2.5.1. Bacteria and media

The antimicrobial activity tests were conducted under aseptic conditions in a laminar flow chamber against *Enterococcus faecalis* planktonic bacteria. *E. faecalis*, American type Cell Culture Collection number (ATCC) 29212, was grown on Brain Heart Infusion Agar (BHI agar) plates overnight in an incubator at 37°C, (Thermo Scientific, Langensfeld, Germany) from frozen stock cultures stored at –80°C; subsequently, three to four colonies from the agar plate were inoculated in 5 ml Brain Heart Infusion broth (BHI) and incubated in a shaking incubator (N-Biotek, INC, 110 rpm) at 37°C. The overnight bacterial

culture was suspended again in BHI and adjusted spectrophotometrically (Jenway, 7315, Staffordshire, UK) to an optical density at 600 nanometres (OD600) to 1.6, corresponding to approximately 2×10^6 Colony Forming Units (CFUs)/ml.

2.5.2. Antimicrobial assessment of set sealers

All sealer samples ($n = 3$) for microbiological assays were prepared in the laminar flow cabinet (Guardian MSC T1200, Monmouth Scientific, Bridgwater, UK) under aseptic conditions with autoclaved (121°C, 15 psi for 15 mins) instruments (tweezer, mixing spatula) and materials (glass slab, rubber moulds). Sealer discs of dimension 10 × 1 mm were prepared in rubber moulds in triplicates for four sealer groups. 1 set of sealer discs was prepared in unsterile conditions in open at the lab bench to be used as control. The sealers were mixed and dispensed according to manufacturers' instructions. They were allowed to at 37°C and 100 % relative humidity. After setting, the specimens were aged in 5 ml of sterile HBSS in sterile 7 ml polystyrene bijoux containers in an incubator at 37°C for 7 days and 90 days. After aging, the samples were rinsed with sterile water, dried on sterile filter paper, and subjected to antimicrobial testing using attachment assay/ direct contact test (22) and minimum inhibitory concentration (MIC) assay (23–31).

2.5.2.1. Attachment assay. The procedure used for the attachment assay has been previously published [45]. The specimens ($n = 3$) were placed on top of a microscopic slide which was then inoculated with 20 μl of the diluted *E. faecalis* culture. A 22 mm diameter sterile glass coverslip (Chance Propper Ltd., Warley, England) was gently placed on the droplet to spread out the solution evenly and for better control of the contact between the specimen and solution. The petri dish was then closed and sealed with cling film to decrease evaporation during the long incubation times. All the petri dishes were incubated at 37 °C in the dark without shaking. Bacterial suspension placed on the sterile glass coverslip, covered with another sterile glass cover slip on top, with no sealer disc, was used as a control for set sealer. Bacterial suspension in contact with AH plus was the positive control. Testing was carried out for 0, 7, and 90 days of incubation in sterile HBSS.

To assess the survival of bacteria, the complete specimen with the cover slip assembly was immersed in 5 ml of BHI in a falcon tube and vortexed for 30 s to allow for dispersion of the viable and non-viable bacteria from the surface of the specimen. The Miles and Misra method was used to enumerate the microbial counts [46]. Briefly, 200 μl of suspension was placed in the top row of a 96-well plate and 180 μl of BHI broth was placed in all remaining rows underneath. 20 μl of suspension from the top row was removed and mixed it into the well just below containing only BHI and 10 -fold serial dilution was performed. This was repeated for all remaining rows. Finally, 20 μL aliquots were plated in triplicate onto BHI agar plates and incubated for 24 h at 37°C. After the incubation period, the number of colonies was counted, and the Colony Forming Units/ml (CFU/ml) were calculated. The number of colonies formed on the agar represents the number of viable bacteria that survived the contact with the sealer surface enabling us to know the anti-bacterial efficacy. All experiments were performed in triplicate.

2.5.2.2. Minimum inhibitory concentration. For minimum inhibitory concentration test (MIC), the leachates retrieved from soaking material discs for 7 and 90 days were microbiologically assessed by minimum inhibitory concentration. Experiments were performed in 96-well plates. One hundred microliters of the final bacterial inoculum were added to each well and an equal amount of the test leachate was then transferred. The test leachates were analysed either undiluted or following 4 sets of two-fold serial dilutions in sterile water (1/2, 1/4, 1/8, 1/16). Sterile water served as a negative control in the experiments; independent negative controls were carried out for each group, containing sterile broth and the respective test leachate.

Plates were incubated overnight for 24 h at 37°C; subsequently, the

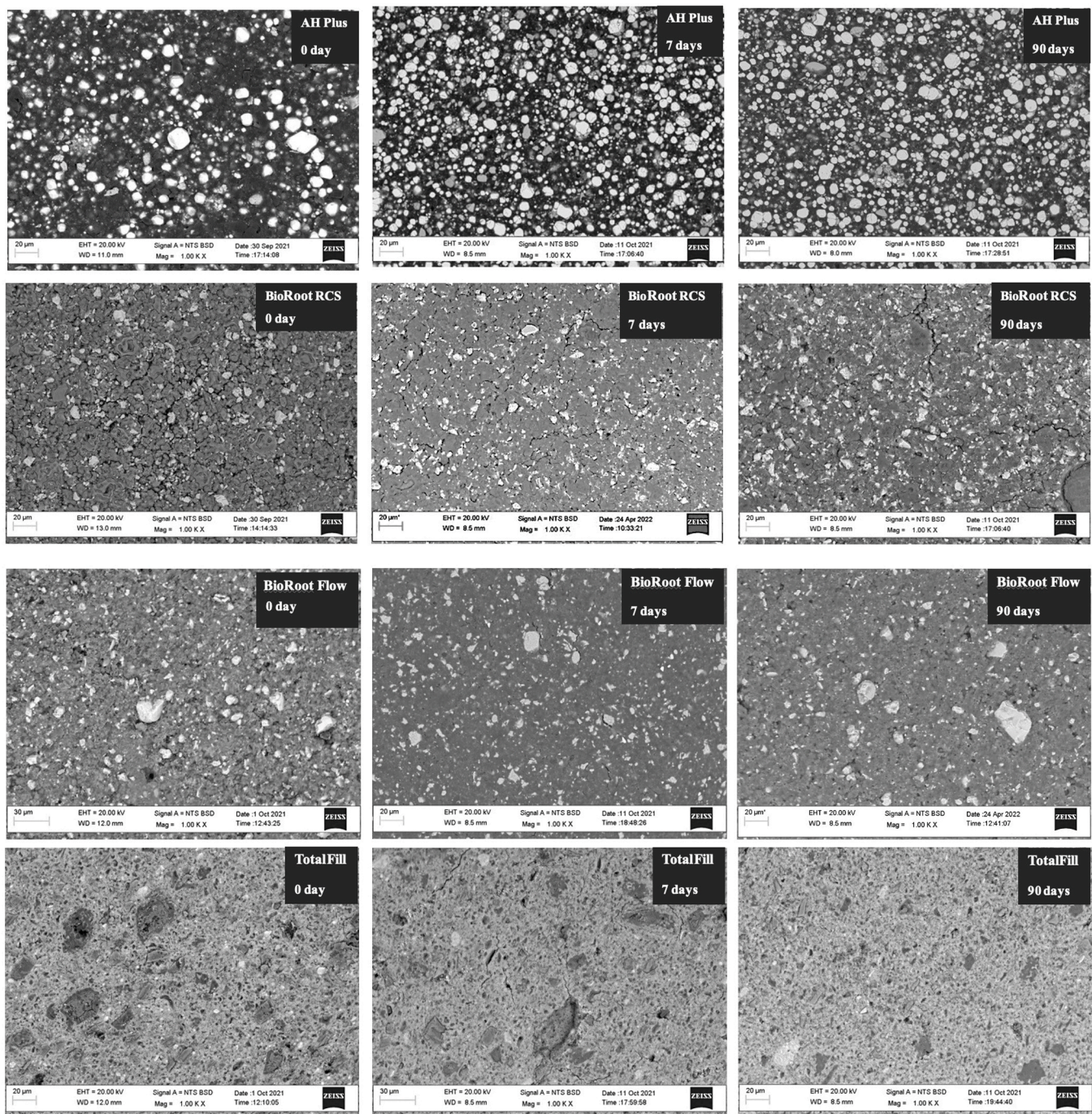


Fig. 2. Backscatter Scanning Electron Microscopy (SEM) (1000X magnification) showing microstructural components of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill sealer after immersion in HBSS for 0 day, 7 days and 90 days.

OD of each well was determined using a microplate reader (ELx800, Bio Tek Instruments, Swindon, UK) at 600 nm. Experiments were performed in triplicate with three independent wells for each group, 3×3 (3 technical repeats from 3 biological repeats). Results were normalised by subtracting the values of the negative control (leachate + BHI) from each group, to eliminate false positive results from increased OD values obtained due to potential sedimentation effects from the leachates. The MIC is equivalent to the lowest concentration of the eluent where no growth was seen.

2.5.3. Antimicrobial assessment of sealers in contact with irrigated dentin

The split tooth model (Fig. 1) was used to prepare the teeth. The

exterior surface of the tooth ($n = 9$) was wiped with 70 % ethanol in the microbiology laminar flow hood and the root sample was rolled on BHI agar plates and incubated at 37°C overnight. The next day, the agar plate was checked for any contamination of the exterior surface of the root sample. After wrapping with parafilm as mentioned above, the root canals were chemo-mechanically prepared using WaveOne Gold and the irrigation protocols A, D and E (Table 1b). The root canals were filled using single cone gutta-percha and a sealer from the ones described in Table 1a. The roots were coronally sealed with glass ionomer cement (Ionofil Plus, Voco, Cuxhaven, Germany). Specimens were then aged in 5 ml of sterile HBSS in sealed sterile 7 ml polystyrene bijoux containers in an incubator at 37°C for 7 days.

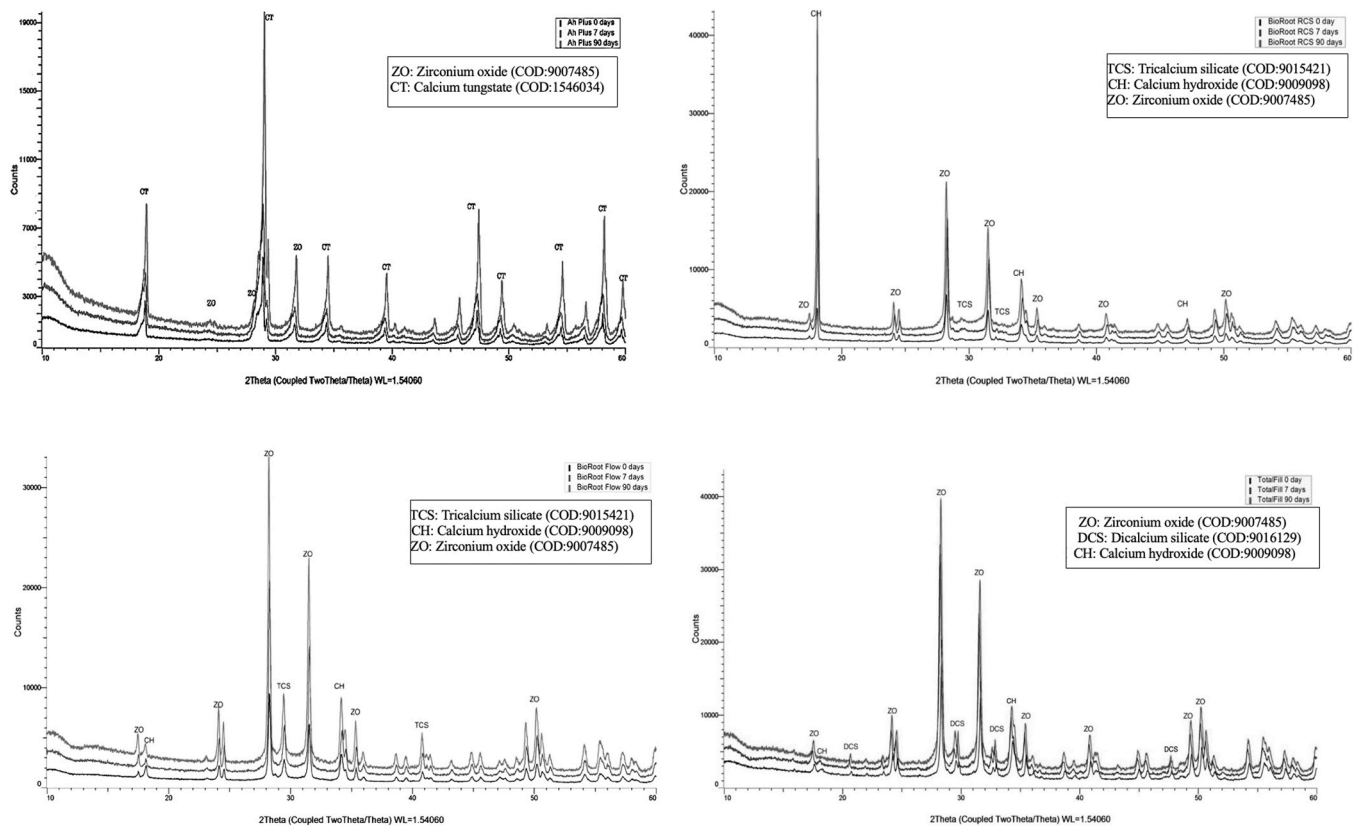


Fig. 3. Xray diffraction plots of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill at 0,7 and 90 days showing the crystalline phases present after immersion in Hank's balanced salt solution.

Table 1c

Cumulative data of leachate collected after total of 7 days and 90 days: Calcium (Ca) ion concentration in leachate with standard deviation of four sealers: AH Plus, BioRoot RCS, BioRoot Flow and TotalFill. BDL: Below Detection Limit.

Sealer	Ca- 7 days	Ca- 90 days
AH Plus	0.03 ± 0.01	BDL
BioRoot RCS	278.33 ± 33.29 *	300.73 ± 54.15 *
BioRoot Flow	142.29 ± 52.01	84.08 ± 8.73
TotalFill	123.50 ± 11.68	247.25 ± 37.09

* represents mean values with significant difference $p \leq 0.05$.

Table 1d

Data of leachate collected every 15 days for a total of 90 days duration: Mean calcium ion concentration in leachate media of four sealers: AH Plus, BioRoot RCS, BioRoot Flow and TotalFill with standard deviation.

Duration	AH Plus	BioRoot RCS	BioRoot Flow	TotalFill
15 days	0.06 ± 0.10	20.97 ± 1.29	17.34 ± 1.17	17.34 ± 1.17
30 days	0.01 ± 0.01	16.08 ± 0.93	19.36 ± 0.43	19.36 ± 0.43
45 days	0.03 ± 0.03	13.34 ± 0.50	12.62 ± 0.69	12.62 ± 0.69
60 days	0.01 ± 0.01	9.31 ± 0.41	9.27 ± 0.8	9.27 ± 0.8
75 days	0.01 ± 0.01	3.91 ± 0.91	5.72 ± 1.06	5.72 ± 1.06
90 days	0.03 ± 0.03	2.81 ± 1.51 *	3.59 ± 0.63 *	3.59 ± 0.63 *

* represents mean values after 90 days with significant difference $p \leq 0.05$.

After the incubation period, each root was unwrapped from the parafilm, and the root segments were gently detached, one side dentin sample was cleared of sealer remnants, and the sealer was detached from the other half and the sealer sample facing the irrigated dentin was identified.

For the attachment assay, same method was followed as mentioned

in the previous section. A root sample with distilled water irrigation, without any sealer, was used as a dentin control and a sealer sample without contact with dentin was used as sealer control for dentin/sealer samples. Testing was carried out after 7 days of incubation in sterile HBSS. All experiments were performed in triplicate.

2.6. Statistical analyses

Graph Pad Prism software version 9.3.1 (GraphPad, San Diego, CA, USA) was used for data handling, statistical analyses, and the expression of results in graphical formats. All data were normally distributed according to Shapiro -Wilk test ($p > 0.05$). Mean values and standard deviations were expressed. The significance level of $p < 0.05$ was used for all analyses. For the microbiological analyses, all results were analysed using a one-way ANOVA and Tukey post-hoc tests for multiple comparisons. Additionally, the Spearman correlation coefficient (r) was calculated to evaluate the relationship between the mean values of calcium ion release and the respective antimicrobial performance of the eluates.

3. Results

3.1. Sealer characterisation

The backscatter SEM at 1,000 x magnification are shown in Fig. 2. The AH Plus showed no changes in morphology over the 90-day testing period, while the hydraulic cement sealers exhibited aging with development of reaction rims composed of a cement and radiopacifier phase. The material matrix was dense, and the phases were uniformly distributed, the radiopacifier appeared more radio dense. The EDS spectra taken at random sites for AH Plus showed the presence of calcium, zirconium, carbon, oxygen, and tungsten while that of TotalFill, BioRoot

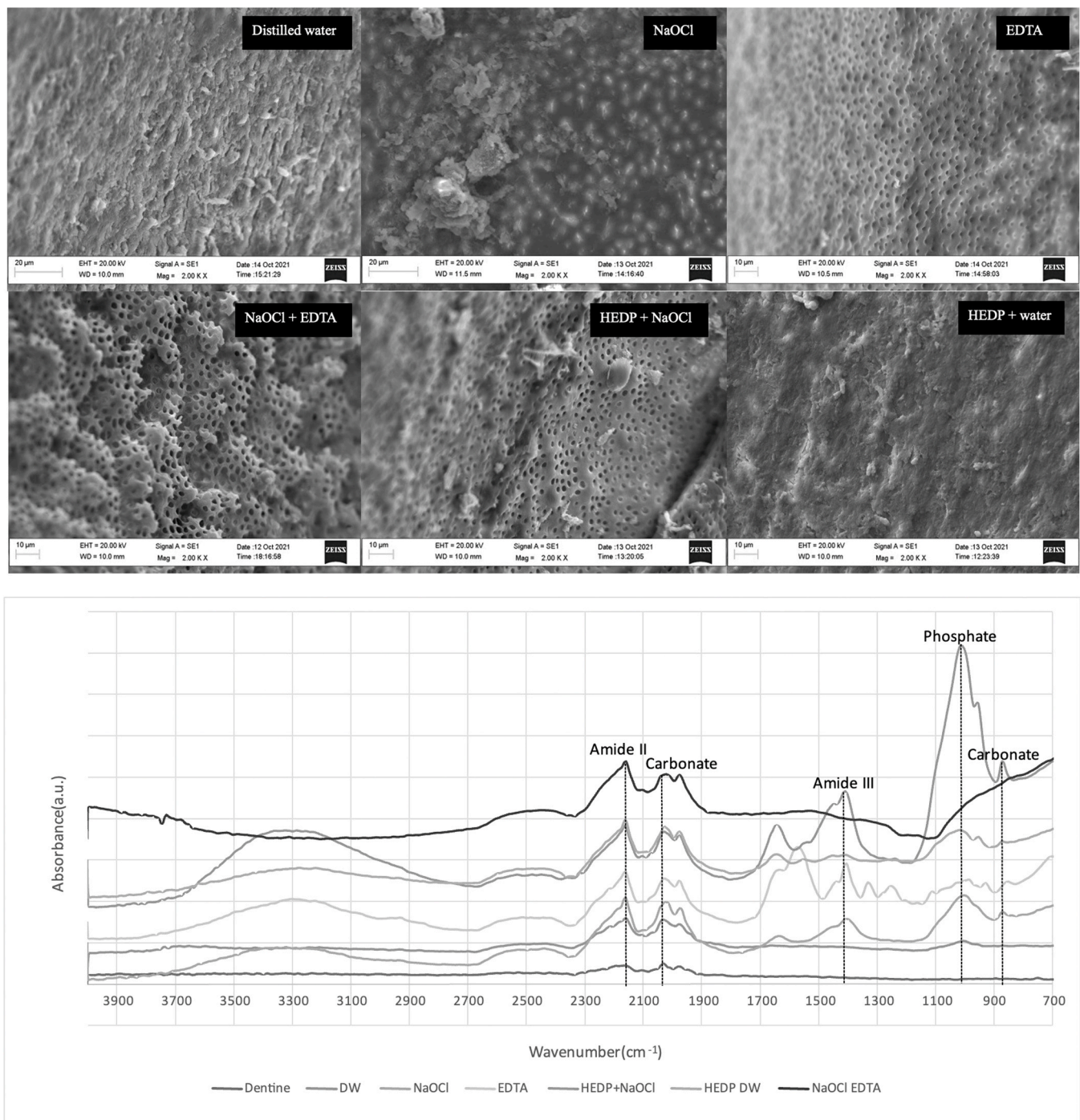


Fig. 4. a: Representative secondary electron photomicrographs (2000 X magnification) showing microstructural components of human root dentin after irrigation with distilled water - Group A, sodium hypochlorite - Group B, ethylene diamine tetracetic acid - Group C, sodium hypochlorite followed by EDTA - Group D, etidronic acid mixed with sodium hypochlorite - Group E and etidronic acid mixed with distilled water - Group F. **Fig. 4b:** Fourier transform infrared spectroscopy (FT-IR) of dentin plain and dentin exposed to different irrigation protocols: distilled water (DW) - Group A, sodium hypochlorite (NaOCl)-Group B, EDTA - Group C, sodium hypochlorite followed by EDTA (NaOCl-EDTA) - Group D, etidronic acid mixed with hypochlorite (HEDP+NaOCl) - Group E and etidronic acid mixed with distilled water (HEDP+DW) - Group F. **Fig. 4c:** Fourier transform infrared spectroscopy point scans of (A) all sealers in contact with all solutions and (B) BioRoot RCS in contact with Dentin irrigated with sodium hypochlorite and HEDP. The interface is marked with a dotted line, the points within dentin in black and points within material in grey. The points within the sealer and the dentin were taken at 300 μ m, 200 μ m and 100 μ m from interface. The phosphate peak is marked on the graph.

RCS, and BioRoot Flow showed peaks of calcium, zirconium, oxygen, and silicon.

XRD scans shown in Fig. 3, show that AH Plus exhibited peaks for zirconium oxide (Crystallography Open Database number COD: 9005833) and calcium tungstate (COD: 1546034) with no changes over

the 90-day period. The XRD plots of BioRoot RCS, BioRoot Flow, and TotalFill showed peaks of zirconium oxide (COD: 9007485), (COD: 9005843) (COD: 9007485) and tricalcium silicate (COD: 9015421) (COD: 9015421) respectively. The BioRoot RCS exhibited a peak of calcium hydroxide (COD: 9006832) at $18^\circ 2\theta$ which increased in

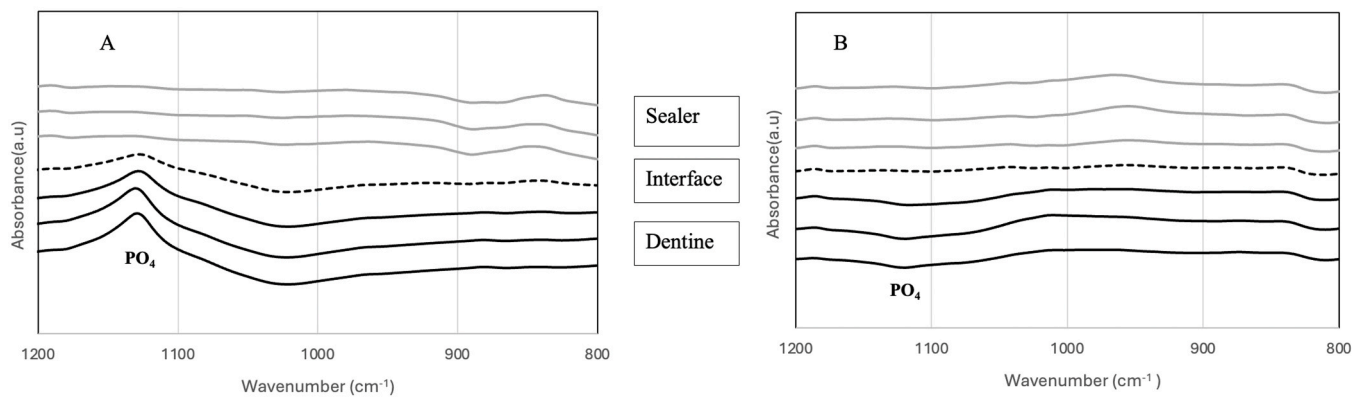


Fig. 4. (continued).

intensity with aging reaching maximum intensity by 90 days. The TotalFill and BioRoot Flow exhibited a peak for calcium hydroxide which was stable throughout the testing phase but of lower intensity than that of BioRoot RCS.

The leachate analysis for calcium release tested after 7 and 90 days is shown in Table 1c. AH Plus leachate released minimal amounts of calcium after 7 days which were not present after 90 days. BioRoot RCS exhibited the highest calcium release with similar levels for the 2 time periods ($p = 0.023$). The 7-day calcium release of BioRoot Flow were similar to TotalFill ($p = 0.148$) with a decline in BioRoot Flow after 90-days ($p < 0.0001$). TotalFill increased the calcium released over the 90 days with similar levels to BioRoot RCS ($p = 0.1495$).

Results for calcium leaching in media changed after every 15 days for a total of 90 days duration is shown in Table 1d. BioRoot Flow and TotalFill showed a similar constant decrease in calcium ion release ($p = 0.7242$) for all the changes until 90 days. BioRoot RCS exhibited a comparable decline in the leaching of calcium for the first three changes up till 45 days compared to the other TCS sealers ($p = 0.356$) but at 60 days there was significant difference in calcium ion leaching ($p = 0.034$) with the same reducing pattern further till 90 days. AH Plus being a resin revealed insignificant calcium ion leaching overall.

3.2. Dentin characterisation

Scanning electronic microscope images of dentin after irrigation with the different irrigation sequences indicated in Table 1b are shown in Fig. 4a. When the NaOCl was used as the only irrigant after instrumentation, smear layer was still observed on the dentin surface which was though eliminated when the HEDP or EDTA were added to the irrigation sequence. The use of EDTA on its own as irrigating solution and hypochlorite followed by EDTA as in Group C and D resulted in the widening of the dentinal tubules but some smear layer and erosion were still observed. The HEDP was only effective in smear layer removal when used in conjunction with the sodium hypochlorite.

The FT-IR spectra of dentin (Fig. 4b) showed peaks at 1650, 2148, and 1398 cm^{-1} , characteristic of the amide I, amide II, and III bands respectively. Bands were also observed at 3350 and 3307 cm^{-1} , representing the stretching of (OH). 860 and 2009 cm^{-1} bands represented carbonate peaks while 980–1000 peaks were of phosphate. The samples of the EDTA group demonstrated a prominent amide peak as compared to phosphate, eliciting demineralisation of dentin in comparison to the HEDP+NaOCl group where amide peaks exhibited weakening and superior phosphate peak indicating denaturation of collagen. The NaOCl-EDTA group displayed a reduction in all phosphate, amide, and carbonate peaks implying both denaturation and demineralisation of dentin.

3.3. Assessment of the tooth to material interface

The scanning electron micrographs in the backscatter mode showing the tooth to material interface are presented in Fig. 5. The specimens revealed cracks, debonding and shrinking from dentin at the interface due to vacuum drying and desiccation during sample preparation.

EDS analyses of the mean atomic ratios (with standard deviation) of calcium (Ca) with silicon (Si) and calcium (Ca) with zirconium (Zr), at four different points, are shown in Table 1e (1–3). Since AH Plus is an inert material, the data could not be calculated. EDS analysis of BioRoot RCS on the dentin surface (Table 1e) exhibits higher concentrations of Si and Zr detected towards the dentin sealer interface in the NaOCl+HEDP irrigant group as compared to the NaOCl-EDTA group and least in DW. At the interface, the irrigated dentin (Inter-D) showed a high Ca/Si and Ca/Zr in contact with BioRoot when EDTA was used. This was different in BioRoot Flow where both EDTA and HEDP showed high Ca/Si but only HEDP exhibited high Ca/Zr. For TotalFill, the HEDP exhibited different dentin characteristics compared to distilled water and EDTA.

On the sealer aspect, at the interface (Inter-M), irrigation with EDTA resulted in an increase in Ca/Si ratio with BioRoot RCS, BioRoot Flow while irrigation with HEDP increased the Ca/Si ratio for TotalFill obturation.

The intensity of the dentin characteristic phosphate peak at 1100 cm^{-1} was used to quantify the degree of mineralization across the sealer-dentin interface. Representative FT-IR spectra of the dentin sealer interface are shown in Fig. 4c. NaOCl-EDTA group displayed a reduction in all phosphate peaks implying demineralisation of dentin (Fig. 4c-A).

None of the sealers showed any changes in the PO_4 peak across the dentin (Fig. 4c - A) for all irrigation groups. The irrigation with sodium hypochlorite and HEDP and obturation with BioRoot RCS resulted in the elimination of the PO_4 peak within dentin to a distance of 300 μm from the interface and intensified carbonate peak was visible representing high mineral infiltration zone (Fig. 4c - B).

3.4. Microbiological analysis

3.4.1. Antimicrobial assessment of set sealers

A preliminary experiment was conducted to verify the association between optical density and CFU/ml, as shown in Table 1f. The results of the Direct Contact Test for the antimicrobial activity of all four sealers against *E. faecalis* are shown in Fig. 6a. Groups of BioRoot RCS, BioRoot Flow, and TotalFill specimens exhibited nondetectable viable counts at all time points suggesting inhibition of the microbial activity. The limit of detection was marked at 50 CFU/ml. The bacterial concentration that was recovered from the AH Plus control group at 0 days and 7 days was similar with 1×10^4 CFU ml^{-1} . After ninety days of aging, Colony Forming Unit values for the AH Plus sealer disc increased up to 1×10^6 CFU/ml.

Results from the MIC assay testing 7-day eluates on *E. faecalis* are

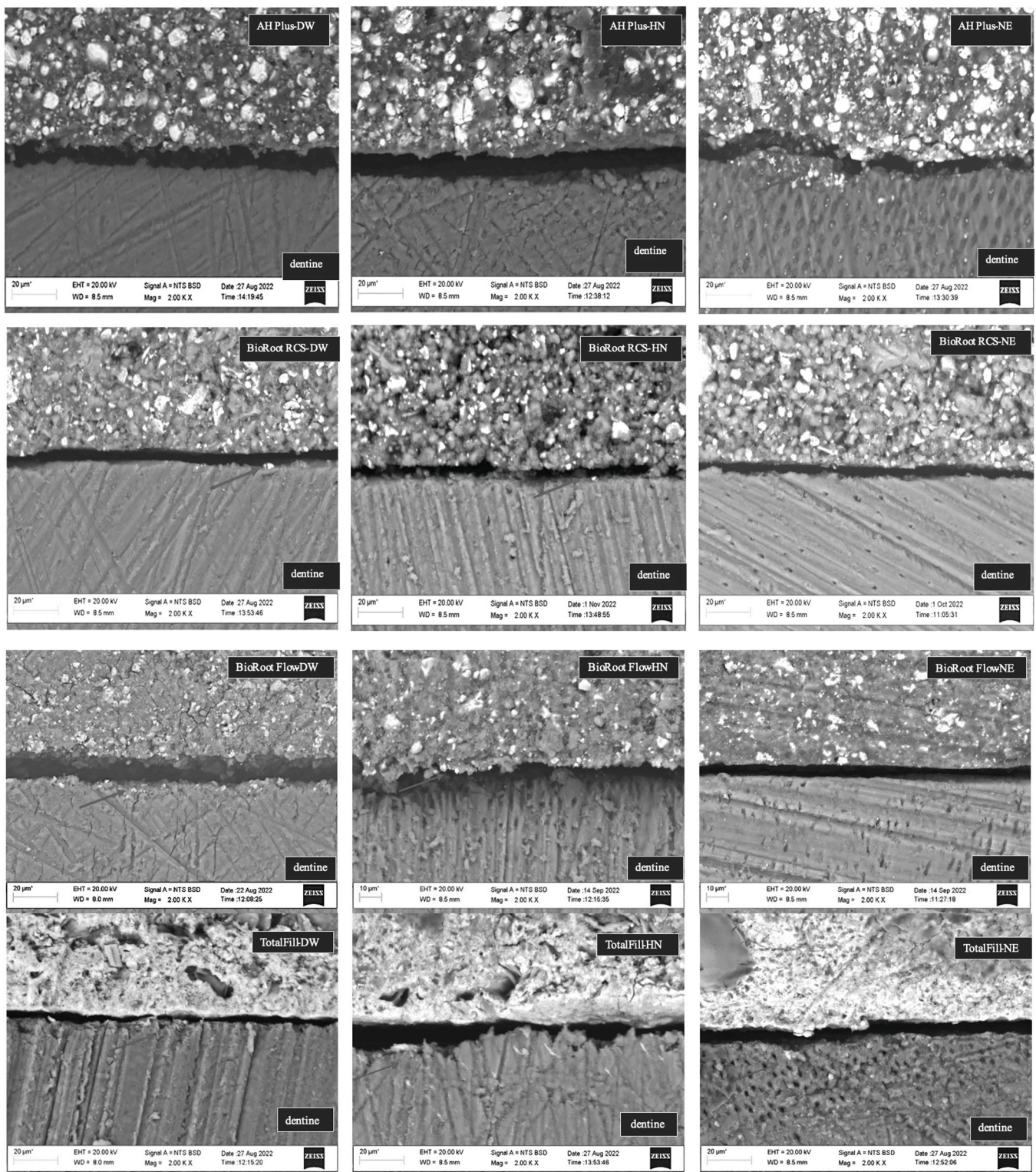


Fig. 5. Back-scatter scanning electron micrographs of dentin sealer interface of AH Plus, BioRoot RCS, BioRoot Flow and TotalFill sealers (2000 × magnification) showing microstructural components irrigated with three irrigant groups; Distilled water-(DW), HEDP mixed with hypochlorite (NaOCl)-(HN) and Hypochlorite (NaOCl) followed by EDTA-(NE). Red arrow depicts the detached and cracked interface of sealer to dentin.

presented in Fig. 6b. The leachates of BioRoot RCS ($OD_{600} = 0.323$ CFU/ml), BioRoot Flow ($OD_{600} = 0.313$ mg/ml), and Total Fill ($OD_{600} = 0.390$ mg/ml) resulted in a significant growth reduction of *E. faecalis* ($p < 0.0001$) when compared with the bacterial control (0.410 mg/ml), however, no MIC could be obtained. The leachate of BioRoot Flow and BioRoot RCS was more antibacterial compared with other test leachates.

However, AH Plus leachate demonstrated the least inhibition of microbial growth ($OD_{600} = 0.399$ mg/ml).

The 90-day testing on the inhibition of *E. faecalis* showed that BioRoot RCS ($OD_{600} = 0.298$ mg/ml), exhibited a greater inhibitory effect ($p < 0.001$) on bacterial growth as compared to its 7-day eluate and all other test sealers. The 90-day leachate of BioRoot Flow ($OD_{600} =$

Table 1e

EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium (Ca/Zr) after irrigation with three groups and using sealer (1) BioRoot RCS, (2) BioRoot Flow and (3) TotalFill at three distinct locations, material, interface, and dentin at 4 different points. One point in material, two points at interface and one point in dentin, with standard deviation. (NaOCl+HEDP(NH) = sodium hypochlorite mixed with etidronic acid, NaOCl-EDTA(NE) = sodium hypochlorite followed by EDTA, DW = distilled water), BDL: below detection limit; Inter-M: interface towards material; Inter-D: interface towards dentin).

Irrigant group	BioRoot RCS (Mean ratio)		Inter - M (Mean ratio)		Inter - D (Mean ratio)		Dentin (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
Distilled water DW	4.87 ± 0.84	1.21 ± 0.59	4.36 ± 2.03	4.54 ± 5.52	37.12 ± 46.96	2.09 ± 3.63	Si BDL	Zr BDL
NaOCl-EDTA (NE)	3.69 ± 0.72	13.62 ± 15.11	7.21 ± 3.24	4.57 ± 3.06	105.44 ± 61.79	11.35 ± 5.49	28.13 ± 36.24	7.29 ± 2.46
NaOCl+HEDP (NH)	5.89 ± 1.94	25.14 ± 21.57	4.11 ± 0.59	3.69 ± 4.05	41.72 ± 28.26	5.92 ± 3.35	51.46 ± 18.37	Zr BDL

Irrigant group	BioRoot Flow (Mean ratio)		Inter-M (Mean ratio)		Inter -D (Mean ratio)		Dentin (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
DW	3.73 ± 1.08	2.49 ± 1.8	5.07 ± 2.75	9.33 ± 10.11	Si BDL	Zr BDL	Si BDL	Zr BDL
NaOCl-EDTA (NE)	3.68 ± 0.44	2.38 ± 1.97	27.59 ± 6.95	5.64 ± 5.96	28.03 ± 48.5	Zr BDL	Si BDL	Zr BDL
NaOCl+HEDP (NH)	2.73 ± 0.46	0.88 ± 0.47	3.57 ± 0.71	2.04 ± 0.53	27.30 ± 4.34	13.97 ± 4.35	Si BDL	Zr BDL

Irrigants group	TotalFill (Mean ratio)		Inter-M (Mean ratio)		Inter -D (Mean ratio)		Dentin (Mean ratio)	
	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr	Ca/Si	Ca/Zr
Distilled water DW	4.23 ± 1.46	0.56 ± 0.1	5.07 ± 1.45	0.35 ± 0.06	31.88 ± 6.72	2.36 ± 0.89	0.63 ± 0.89	7.66 ± 8.13
NaOCl-EDTA (NE)	3.73 ± 0.31	0.29 ± 0.16	4.99 ± 1.53	0.41 ± 0.06	45.14 ± 58.67	4.64 ± 2.99	Si BDL	9.56 ± 0.63
NaOCl+HEDP (NH)	3.61 ± 0.45	0.58 ± 0.44	24.67 ± 19.67	1.80 ± 1.85	Si BDL	Zr BDL	Si BDL	3.81 ± 1.81

Table 1f

Association between optical density and CFU/ml conducted in a preliminary experiment.

Optical density(nm)	CFU/ml
0.272	1 × 10 ⁵
1.531	1.2 × 10 ⁴
1.855	1.6 × 10 ⁶
2.111	6.3 × 10 ⁶
3.793	18.6 × 10 ⁶

0.353 mg/ml), demonstrated a rise in microbial viability as opposed to the 7-day result. However, the eluent of TotalFill (OD₆₀₀ = 0.314 mg/ml) exhibited the opposite effect. AH Plus revealed comparable results for both time frames (OD₆₀₀ = 0.397 mg/ml).

On comparison, the difference in bacterial inhibition was highly significant between tricalcium silicate sealers and the bacterial control suggesting high antibacterial activity. Whilst results were non-significant for AH plus, making it the least antimicrobial.

3.4.2. Antimicrobial assessment of sealers in contact with irrigated dentin

The results of the DCT after 7 days of aging and 24 hrs of direct contact are shown in Fig. 7a and b. This test was used to evaluate changes in the antimicrobial effect of each sealer in contact with dentin and vice versa at the dentin sealer interface. In Fig. 7b all the sealers exhibited a reduction in their antimicrobial effect when in contact with dentin as compared to sealers with no contact (control). AH Plus showed the least antibacterial activity of 2×10^4 CFU/ml ($p < 0.05$) with all the irrigants. However, BioRoot RCS sealer showed the highest bactericidal effect as compared to all other sealers. Overall, the sealers with NaOCl-EDTA irrigation exhibited maximum bactericidal effect as opposed to other irrigation groups at 1×10^5 CFU/ml ($p < 0.05$).

4. Discussion

The current research investigated three hydraulic calcium silicate (HCS) cement sealers and an epoxy resin-based sealer to evaluate how effectively these materials interacted with dentin that had undergone irrigation with different protocols as well as how well they could alter the properties of the dentin after application at the dentin sealer interface. The null hypothesis was rejected.

The ‘split tooth’ model [21], was used in this study as it mimics the clinical procedure of root canal therapy closely and enables the specimen retrieval from contact with the root canal dentin for further testing. This model which has been validated in previous research [21] was an effective *ex vivo* method to conduct root canal therapy and retrieve the materials for testing with minimal disruption to the specimen. Various techniques were employed to assess the changes to the e and sealer and to assess the antimicrobial characteristics of the sealer without and in contact with the irrigated dentin. Scanning electron microscopy was very useful to determine the most effective way of smear layer removal after dentin preparation but was less effective in imaging the dentin-sealer interface due to shrinkage and distortion caused by specimen preparation especially when working with hydraulic cements. The model enabled the retrieval of sealers in contact with dentin for antimicrobial analyses. The single species biofilm model, although accepted, is not representative of the complex polymicrobial biofilm found within infected root canals. *E. faecalis* survives the material processing thus is the best microbe to be used to test a new method as was done in previous research [21]. Additional studies focusing on polymicrobial biofilm are warranted.

EDS point scans of mean ratio of calcium with silicon (Ca/Si) and calcium with zirconium showed higher concentrations of Si and Zr elements towards the dentin sealer interface signifying superior sealer penetration for BioRoot RCS with the NaOCl+HEDP group as compared to the other group which indicated that it was the best way to prepare the dentin. The irrigation with NaOCl+HEDP followed by obturation with BioRoot also led to the elimination of the PO₄ band in the dentin

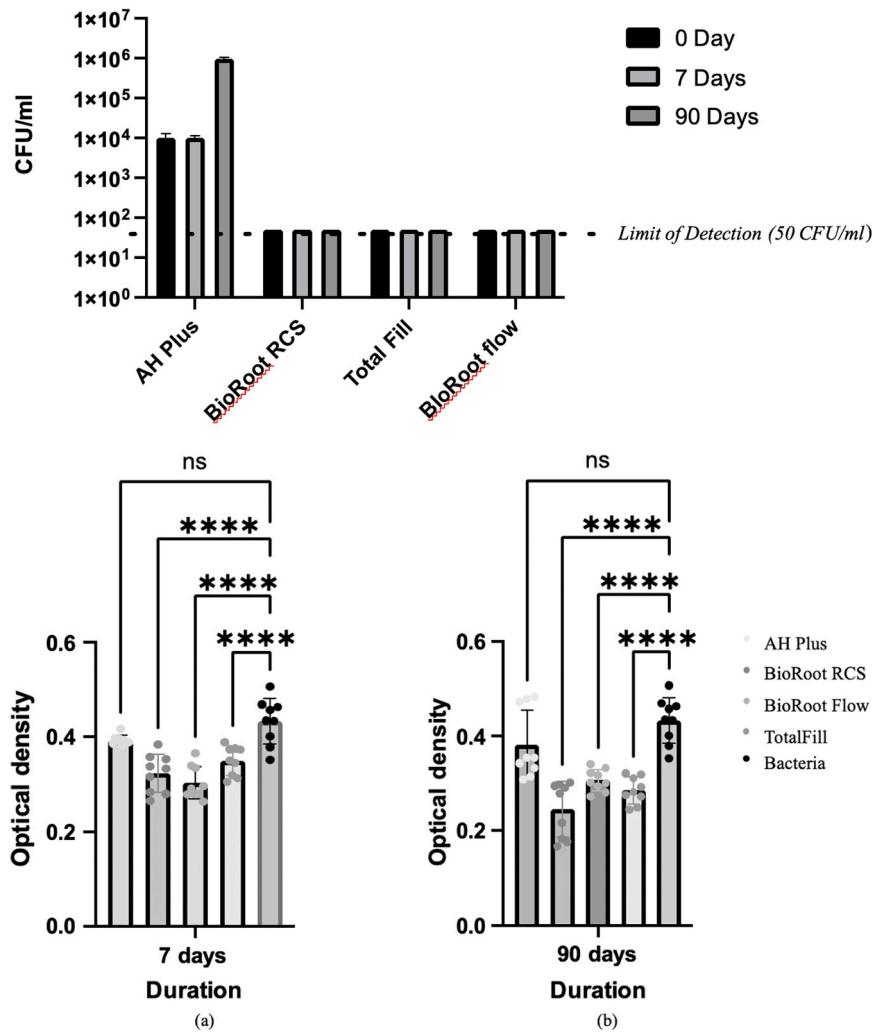


Fig. 6. a: Antimicrobial activity in CFU/ml for four different sealers (AH Plus, BioRoot RCS, BioRoot Flow and TotalFill) at 0-day, 7 days and 90 days with the Direct Contact Test. AH plus was the control. Results of two-way ANOVA test with $p < 0.0001$: statistically significant difference. The limit of detection was at 50 CFU/ml. ANOVA: analysis of variance; ($n = 3$). **Fig. 6b:** Bacterial viability (measured as optical density at 600 nm) when incubated with various sealer eluents 3×3 (3 technical repeats from 3 biological repeats) for a time frame of (a) 7 days and (b) 90 days. Bacteria only are the control. Statistical significance was determined using a one-way ANOVA / Dunnett's multiple comparisons test. The bar graphs represent the mean and the error bars the standard deviation (mean $\pm$ SD). Differences between means are indicated. ****, $p < 0.0001$. ns, not significant ($n = 9$).

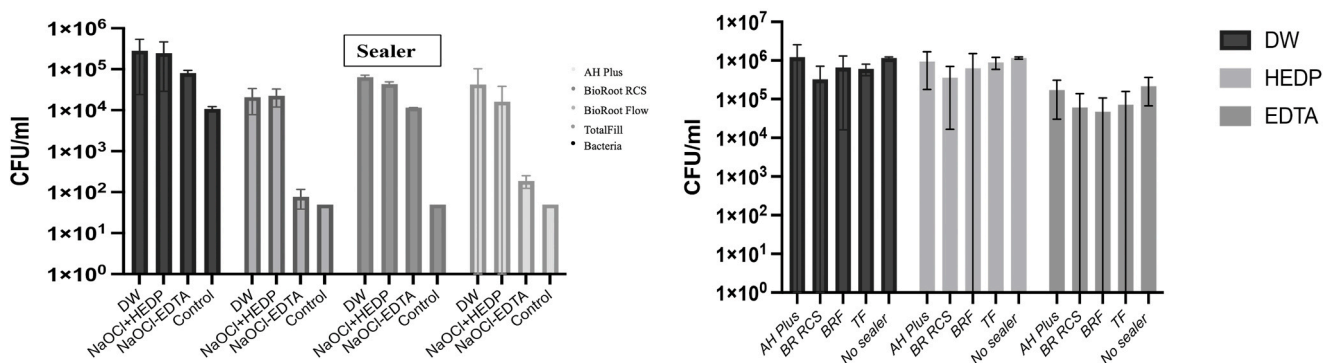


Fig. 7. a: Number of CFU (Colony Forming Unit) of *E. faecalis* in the presence of AH Plus, BioRoot RCS, BioRoot Flow, TotalFill sealer in contact with dentin at the dentin sealer interface after 3 irrigation protocols (DW= distilled water; NaOCl-EDTA= Hypochlorite followed by EDTA; NaOCl+HEDP= hypochlorite mixed with etidronic acid), aged for 7 days and 24 h of direct contact: ($p < 0.05$). Sealer with no contact with demtin was used as a control. Results of two-way ANOVA and error bar (mean $\pm$ SD). ANOVA: analysis of variance; ($n = 3$). **Fig. 7b:** Number of CFU (Colony Forming Unit) of *E. faecalis* in Dentin in contact with (AH Plus, BioRoot RCS, BioRoot Flow, Total Fill) sealer at the dentin sealer interface, after 3 irrigation protocols (DW= distilled water; NaOCl-EDTA= Hypochlorite followed by EDTA; NaOCl+HEDP= hypochlorite mixed with etidronic acid), aged for 7 days and 24 h of direct contact: ($p < 0.05$). Dentin in no contact with sealer was used as control. Results of two-way ANOVA and error bar (mean $\pm$ SD). ANOVA: analysis of variance; ($n = 3$).

adjacent to BioRoot RCS. The BioRoot RCS matured over the 90-day period and resulted in the formation of calcium hydroxide as shown in the XRD scans. The TotalFill and BioRoot Flow did not change their chemical characteristics over the 90-day study phase exhibiting sharp peaks of unreacted tricalcium silicate.

Strong chelating agents like EDTA decreases the apatite/collagen ratio due to demineralization of the hydroxyapatite components of dentin, reducing the root dentin microhardness and can be one of the contributing factors to post treatment root fracture [16,22]. On the other hand, HEDP, a weak chelating agent used alongside NaOCl, prevents the formation of a resistant smear layer during instrumentation without excessively degrading its organic components thus causing less deterioration of dentin [23]. Further research is necessary to show the effectiveness of the use of HEDP to prevent root fractures.

The interaction of hydraulic cement sealers with dentin was influenced by the irrigation protocol. The sealers also affected the dentin chemistry further enforcing the mineral exchange at the interface along with the changes to the dentin structure/chemistry after the sealer placement [24]. Hence, it becomes necessary to verify changes in the dentin microstructure and its properties following exposure to irrigating solutions. It also becomes important to match the best irrigation protocol to the sealer for a better adaptation at dentin sealer interface.

In the current study, the direct contact test was modified and instead of testing the root with the sealer together as one unit, sealer was separated from the root dentin and studied individually. The sealer was aged for 7 days inside root canal with 24 h of direct contact with the bacteria, followed by CFU counts. This enabled to represent the material's true antimicrobial activity without being impacted by dentin buffering action, which was a drawback of DCT in previous research studies [25,26]. However, the investigated material's adhesion to dentin may result in a carryover effect [27] or impede the removal of all sealer from the dentin and impact the dentin's antibacterial activity.

All sealers showed a decrease in antibacterial efficacy in contact with dentin in comparison to sealers without contact. No inhibitory effect against *E. faecalis* was observed after 7 days of aging for AH Plus which is likely a consequence of the setting chemistry, which results in a neutral pH and reduced solubility [28,29,30,31]. BioRoot RCS exerted the highest antibacterial efficacy in comparison with other test sealers in agreement with previous literature [15,28,32]. BioRoot RCS exhibited the highest release of calcium hydroxide (from XRD; Fig. 3). BioRoot RCS is mixed with water, ensuring hydration, which results in dissolution and higher calcium and hydroxyl ion release resulting in elevated alkalinity, and therefore greater antimicrobial properties than the pre-mixed sealers [33]. Sealers delivered by single syringes, and that rely on moisture from the oral environment to set such as BioRoot Flow and TotalFill exhibit delayed calcium ion release [34]. All hydraulic cement sealers showed considerable antibacterial action after 7 days in contact with irrigated dentin as indicated by the direct contact test with planktonic bacteria. The testing using a multispecies biofilm is recommended.

BioRoot RCS had the greatest bactericidal effect with different type of irrigant groups. Irrigation with NaOCl and EDTA led to the greatest antimicrobial activity for both dentin and BioRoot sealer groups. This finding confirms the results obtained by previous studies [35,36,37].

Hydraulic cement sealers in contact with dentin exhibited reduced antimicrobial properties. One of the reasons could be the ability of bacteria to penetrate dentinal tubules, build biofilms contributing to their survival and thus may be shielded from the direct antibacterial action of sealers [38]. Further, the interaction of the hydraulic cement with the surroundings [39,40] may result in the formation of calcium carbonate by consuming the available calcium hydroxide [41]. This lowers pH, which has a detrimental effect on antibacterial action [42].

BioRoot RCS showed decreased microbial growth with distilled water or the HEDP irrigant group. However, dentin in contact with the premixed sealers showed better antimicrobial response when irrigated with EDTA. The reason for this effect could be that the smear layer must

be removed for single-syringe sealers to function since they need the dentin fluid to hydrate [33,43]. Formulations that include powder and liquid, like BioRoot RCS, chemically interact with dentin to produce an antibacterial activity that is not dependent on the smear layer [33,44].

Findings of this study have several clinical implications, primarily concerning the relationship between irrigation protocol and selection of obturating material. The final irrigation strategy must be a deliberate choice matched to the specific chemistry of the chosen sealer to optimize the root canal seal and enhance clinical outcomes.

The continuous chelation protocol using NaOCl and HEDP presents a clinically relevant final irrigating solution especially when using HCS sealers as being a weak chelating agent HEDP maintains a mineralized dentin surface, which is ideal for the chemical interaction and interfacial layer formation characteristic of HCSC sealers.

The antimicrobial properties of the HCS sealers deteriorate when in contact with the dentin. Thus, the disinfection protocol and the modification of the dentin surface to establish the best interface with the sealer of choice is a concept that should be encouraged.

The current study has used verified models to attempt to mimic the clinical scenario. Regardless of this, the findings still need to be established clinically. The recent S3 clinical guidelines [47] suggests the use of sodium hypochlorite and EDTA as a chelator with a hypochlorite final irrigation [48]. The current research suggests the need to change this recommendation. Furthermore, no difference in outcome of root canal therapy when using different materials and techniques was shown in clinical trials [49] while currently, we show differences in the behaviour of different sealers which one hopes to improve the outcome of root canal therapy. Further research is necessary using initially *in vivo* and *ex vivo* models followed by animal and clinical trials to establish the validity of the matched irrigation to obturation strategy for root canal therapy.

5. Conclusions

Irrigation with mixtures of NaOCl and HEDP resulted in higher mineral concentration zone for hydraulic cements at the dentin sealer interface as well as minimal effects on dentin being a weak chelating agent as compared to EDTA. BioRoot RCS interacts chemically with dentin to create antibacterial action that is independent on the smear layer and has shown lower microbial growth with HEDP irrigant group compared to other groups. The antimicrobial effectiveness of tricalcium silicate-based sealers can be affected by contact with irrigated dentin.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Septodont for providing BioRoot Flow

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